ROLE OF TWO SECRETED PROTEINS FROM *Trichoderma virens* IN MYCOPARASITISM AND INDUCTION OF PLANT RESISTANCE

A Dissertation

by

SLAVICA DJONOVIC

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Charles M. Kenerley
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ABSTRACT

Role of Two Secreted Proteins from *Trichoderma virens* in Mycoparasitism and Induction of Plant Resistance. (December 2005)

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The soil-borne filamentous fungus *Trichoderma virens* is a biocontrol agent with a well known ability to produce antibiotics, parasitize pathogenic fungi and induce systemic resistance in plants. Here we report the identification, purification and characterization of an elicitor secreted by *T. virens*; a small protein designated Sm1 (small protein 1). Confrontation and disk assays demonstrated that Sm1 lacks toxic activity against plants and microbes. Native, purified Sm1 triggers production of reactive oxygen species in rice (*Oryza sativa*) and cotton (*Gossypium hirsutum*), and induces the expression of defense related genes both locally and systemically in cotton. Gene expression analysis revealed that *SM1* is expressed throughout fungal development and is transcriptionally regulated by nutrient conditions and the presence of a host plant. When *T. virens* was co-cultured with cotton in an axenic hydroponic system, *SM1* expression and secretion of the protein was significantly higher than when the fungus was grown alone. These results indicate that Sm1 is involved in plant-*Trichoderma* recognition and the induction of resistance by activation of plant defense mechanisms.

Following the cloning of *SM1*, strains disrupted in or over-expressing *SM1* were generated. Targeted gene disruption revealed that *SM1* was not involved in fungal development. Expression of defense related genes in cotton and maize (*Zea mays*) was induced locally and systemically following colonization by *T. virens* in the hydroponic system. Low levels of expression of cotton or maize defense genes were found when seedlings were grown with a *T. virens* strain disrupted in *SM1*, ssupporting the Sm1-elicitor hypothesis. Additionally, unique proteins in *T.virens*-cotton/maize interaction were identified. Thus, the induction of defense responses in two agriculturally important crops appears to be microbially mediated.

Functional analysis of a cell wall degrading enzyme, β-1,6-glucananse (Tv-bgn3) from *T. virens*, demonstrated involvement of this enzyme indirectly in mycoparasitic activity of *T. virens*. Protein extracts from the strain disrupted in *TV-BGN3* displayed reduced capability to inhibit growth of *Pythium ultimum* as compared to the wild-type. Additionally, protein extracts from the strains co-expressed with *TV-BGN2* (β-1,3-glucananse) from *T. virens* showed a significantly increased capability to inhibit growth of *P. ultimum* and *Rhizoctonia solani* hyphae.

DEDICATION

To my parents, Zarija and Srbislava, and to my husband Sava,

for their love and belief in me

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CHAPTER I

INTRODUCTION

Plant pathogens continue to reduce the availability of food resources on a global scale as well as diminish the economic potential of greenhouse and nursery industries (Pinstrup-Andersen, 2000). As fungi are one of the major causal agents of plant diseases, modern agriculture still highly depends on the use of fungicides to control plant diseases (Zadoks and Waibel, 2000). Combined with the continued public concern of the safety of pesticides and the effect these compounds have on the environment, a tremendous opportunity exists to develop biocontrol agents as direct substitutes for chemicals or as key components in integrated management systems that are more biointensive or ecologically based (Ristaino and Thomas, 1997; Whipps and Lumsden, 2001; Gerhardson, 2002; Noling, 2002).

Trichoderma virens (J.H. Miller, Giddens and A.A. Foster) Arx is a saprophytic deuteromycete (Domsch et al., 1980) ubiquitous in the soil environment through out the world with well demonstrated biological activity (Lumsden and Locke, 1989; Papavizas, 1992; Lumsden et al., 1996). Isolates of *Trichoderma* are characterized by rapid growth, abundant conidial formation and a high degree of ecological adaptability (Domsch et al., 1980; Papavizas, 1985; Bissett, 1991a).

This dissertation follows the style of Plant Cell.

Based on taxonomic studies, including genetic information from 28S rRNA, an internal transcribed spacer region 1 (ITS1) of rDNA, 1-α-TEF (protein-coding gene translation elongation factor 1-α) and fingerprinting analysis, *Gliocladium virens* has been reclassified as *Trichoderma virens* (Bulat et al., 1998; Dodd et al., 2000; Kindermann et al., 1998; Rehner and Samuels, 1994; Bissett et al., 2003; Chaverri et al., 2003; Lu et al., 2004). Morphologically, *T. virens* is distinguishable from *Gliocladium* spp. by a slime that clumps the conidiophores (Bissett, 1991b; Samuels, 1996). Even though *Trichoderma* species are usually considered free-living saprophytes in soil, they are now known to be opportunistic, avirulent plant symbionts as well as being parasites of other phytopathogenic fungi (Harman et al., 2004b). Some *Trichoderma* spp. are very good cellulase producers, and therefore are important for the biotechnological industry (Reczey et al., 1996; Juhasz et al., 2003). However, some isolates of *T. harzianum* have been shown to cause green mold disease of the commercial mushroom *Agaricus bisporus* (Mumpuni, et al., 1998; Mamoum et al., 2000). Very rarely they can be human pathogens, and then only in immunocompromised patients (Samuels, 1996; Kredics et al., 2003).

Species of the genus *Trichoderma* have been used effectively world-wide as biocontrol agents for a wide range of economically important plant pathogens with more than 50 registered *Trichoderma*-based bioproducts available on the market (Papavizas, 1985; Lumsden et al, 1996; Chet, 1987; Harman and Kubicek, 1998; Fravel, 2005). Biocontrol activity by *Trichoderma* spp. has been demonstrated against a wide range of soilborne pathogens including *Rhizoctonia solani*, *Pythium ultimum*, *Sclerotium rolfsii*, *Botrytis*

cinerea, *Fusarium oxysporum*. Strains of *T. virens* has been shown to decrease disease caused by *R. solani*, *P. ultimum*, *B. cinerea*, and *S. sclerotiorum* (Howell, 1987; Howell, 1991; Lumsden and Locke, 1989; Baek et al., 1999) not only by parasitizing the fungal hyphae, but also destroying some of the fungal resting structures, and reducing the pathogen inoculum in soil (Howell, 1982; Tu, 1980; Zhang et al., 1996; Chernin and Chet, 2002). Understanding the biology and mechanisms *Trichoderma* spp. employ to control plant pathogens is essential to achieve the most effective and reliable pest management systems.

The mechanisms that have been described to account for biocontrol of plant disease by *Trichoderma* spp. include mycoparasitism (induction and secretion of cell wall degrading enzymes and antibiotics), induced resistance in the plant host, and competition for nutrients and potential plant infection sites. All these mechanisms have been shown to be employed efficiently by *T. virens*. This biocontrol agent has been recognized as an aggressive mycoparasite capable of competing ecologically when colonizing potential sites of infection (Park et al., 1992; Baek et al., 1999; Howell, 1982; Howell, 2002). In addition, *T. virens* is unique among other *Trichoderma* spp. as it produces an array of antimicrobial compounds (e.g. gliotoxin, glioviridin, peptaibols) known to inhibit growth of wide range of pathogens (Howell, 1998; Howell et al., 1993; Wiest et al., 2002). Mycoparasitic activity of *T. virens* due to the activity of hydrolytic enzymes, such as chitinases and proteases, has been demonstrated against several soil-borne pathogens (Baek et al., 1999; Pozo et al., 2004). Different strains have been shown to induce phytoalexin production and host resistance (Howell et al., 2000). Furthermore, the

opportunity for enhancing the biocontrol ability of these strains has greatly increased as our understanding of the molecular elements directing the mechanisms of biocontrol and plant-pathogen interaction has expanded enormously within the last decade. A genome sequencing project of *T. virens* Gv29-8 recently initiated by Dr. Kenerley (personal communication, CMK) will greatly contribute to the goal of developing *T.virens* as a model system for studying mechanisms of biological control by fungal agents. Therefore, *T. virens* represents an excellent choice to demonstrate the development of an effective biocontrol strain through molecular technology and provide a greater understanding of the mechanisms involved in pathogen suppression.

Chet and Chernin (2002) have divided the mechanisms of biocontrol into two broad categories: direct or classical biocontrol, including competition for limited recourses, antibiosis, production of lytic enzymes and mycoparasitism; and, indirect or augmentative biocontrol, encompassing plant growth promotion and induction of plant resistance.

The process of mycoparasitism is complex and involves not only recognition of the host, but also active ingress and nutrient uptake by the parasitizing fungus (Howell, 1991; Herrera-Estrella and Chet, 1998). Lytic enzymes, such as chitinases (endochitinases, chitobiosidase, and N-acetylglucosaminidases), β-1,3- and β-1,6-glucanases, and proteases (Flores et al., 1997; Benitez et al., 1998; Lorito, 1998; Kim et al., 2002; Ramot et al., 2004) have been shown to play a critical role in this process (Kubicek et al., 2001; Chernin and Chet, 2002; Viterbo, 2002). *In vitro* assays with purified enzyme preparations inhibited growth of plant pathogens such as *Pythium*

ultimum, *Sclerotium rolfsii* and *Botrytis cinerea* (de la Cruz et al., 1995; Thrane et al., 2000; El-Katatny et al., 2001; Waite, 2001). In addition, *in vitro* confrontation assays have demonstrated the induction of several chitinases of *Trichoderma* when in contact with its fungal host, strongly implicating these enzymes in the degradation of host cell walls (Dana et al., 2001; Kullnig et al., 2000; Mendoza-Mendoza et al., 2003). Several individual hydrolytic enzymes have been over-expressed in *T. virens* and other *Trichoderma* spp., often resulting in enhanced biocontrol activity. A gene encoding one of three 42 kDa endochitinases from *T. virens* was over-expressed and disrupted by Baek et al. (1999), resulting in enhanced and reduced biocontrol against *Rhizoctonia solani*, respectively. A similar effect was seen when a 29 kDa serine protease from *T.virens* was disrupted or over-expressed (Pozo et al., 2004). The over-expression of a 33 kDa chitinase, a β-1,3- or β-1,6-glucanase all resulted in increased biocontrol activity (Flores et al., 1997; Limon et al., 1999; Rey et al., 2001). Furthermore, the activity of hydrolytic enzymes is enhanced when they act synergistically with antibiotics, such as gliotoxin or peptaibols, produced by *Trichoderma* spp. (Lorito et al., 1994; Lorito et al., 1996b; Schirmbock et al., 1994). Based on these reports, the potential to enhance the ability of strains of *Trichoderma* to protect seedlings against pathogens by manipulating enzyme expression and/or regulation would appear to be significant.

Moreover, the co-expression of more then one gene encoding for hydrolytic enzymes and/or antimicrobial compounds may provide a broader spectrum of activity against pathogens. Indeed, this was demonstrated by Delgado-Jarana et al. (2002) where co-expression of β-1,6-glucanase and an aspartyl protease in *T. harzianum* strain CECT

2413 resulted in 30% increased activity of β-1,6-glucanase activity. The same strategy was successfully used to improve biocontrol activity of mosquito-larvicidal bacteria against Dipteran species transmitting tropical diseases, such as malaria and filariasis (Sun et al., 2001). Co-expression of *cry4Ba* of *Bacillus thuringiensis* subsp. *israelensis* with *B. sphaericus* binary toxin gene partly suppressed more than 10,000-fold resistance of *Culex pipiens* larvae to the binary toxin. It was suggested that production of Cry4Ba protein and binary toxin interacted synergistically, thereby increasing their mosquito-larvicidal toxicity (Sun et al. 2001). Finally, over-expression of hydrolytic enzymes in plants was shown to be effective against several fungal pathogens (Bolar et al., 2000; Bolar et al., 2001; Lorito et al., 2001; Mora and Earle, 2001). Transgenic cotton plants expressing the *Tv-ech1* gene encoding for a 42 kDa chitinase from *T. virens* showed reduced development of seedling diseases (Emani et al., 2003). Expression of the genes encoding an endochitinase, exochitinase, and an exo- β-1,3-glucanase from *T. atroviride* in rice resulted in increased resistance to *R. solani* and *Magnaporthe grisea* (Liu et al., 2004).

In addition to their ability to directly attack or inhibit growth of pathogens, recent reports indicate that *Trichoderma* spp. also induce systemic and localized resistance in plants against a variety of pathogens (Harman et al., 2004b). Plants in their natural settings are surrounded by a whole range of beneficial or deleterious microorganisms. The plants' 'perception' of microorganisms is usually highly coordinated through cellular processes that will determine the final outcome of the relationship, ranging from parasitism to mutualism (Nimchuk et al., 2003; Bais et al., 2004; Pozo et al., 2005). To

respond to pathogen attack plants activate a variety of biochemical and molecular defenses (Bowles, 1990). These responses include production of reactive oxygen species, programmed cell death in the form of a hypersensitive response (HR), synthesis of antimicrobial secondary metabolites (phytoalexins), deposition of lignin in cell walls, and activation of downstream defense genes that encode pathogenesis related (PR) proteins (Dicke and Hilker, 2003). Also, a number of plant species have been shown to develop systemic acquired resistance (SAR) in response to pathogens resulting in increased systemic disease resistance (Durrant and Dong, 2004; Bostock, 2005; Glazebrook, 2005). Induced defense responses are not just initiated by pathogens, but may result from interactions with avirulent microbes. The colonization of the rhizosphere by certain strains of plant growth-promoting rhizobacteria (PGPR) results in induced systemic resistance (ISR) (Van Loon et al., 1998; Pieterse, et al., 2003). Rhizobacteria-mediated ISR can occur in many plant species and was also demonstrated to be effective against broad range of pathogens (Van Loon et al., 1998). Despite their phenotypic similarity, SAR and ISR differ in many aspects. Extensive work in *Arabidopsis thaliana* has demonstrated the dependence of ISR signal transduction pathway on the perception of plant hormone signals such as jasmonic acid (JA) and ethylene (ET) (Ton et al., 2002), whereas SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA) and accumulation of pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999; Metraux, 2001; Glazebrook, 2003). However, recent findings indicate that there is a cross-talk between SA and JA signaling pathways modulated through the novel function of NPR1 (nonexpressor of PR-genes 1)

in the cytosol (Dong, 2001). Additionally, transcript profiling analysis revealed a high number of genes co-induced or co-repressed by SA and JA (Schenk et al., 2000; Glazebrook et al., 2003) indicating that there is a certain degree of overlap between the two pathways. These studies illustrate the complexity of an interaction among plant defense pathways and supports the flexibility of the plant defense response to fine-tune the appropriate mechanisms (Pozo et al., 2005).

Within the last five years, the induction of host resistance following interactions of several plants with *Trichoderma* spp. has been shown to be a key mechanism involved in biological control, complementing the mechanisms of mycoparasitism, antibiosis and competition. De Meyer et al. (1998) demonstrated that foliar or soil applications of conidia of *T. harzianum* T39 spatially separated from the site of inoculation with the pathogen *Botrytis cinerea* reduced disease severity. Cucumber plants, following inoculation and penetration of the epidermal and outer cortical root cells by *T. asperellum* T-203, were found to strengthen their cells beyond the sites of fungal penetration (Woo et al., 1999). Cell wall appositions enriched with callose were observed as were increases of peroxidase and chitinase in the root and leaf tissues (Yedidia et al., 1999). The response of hydroponically grown cucumber plants to the colonization by T-203 further demonstrated the appearance of PR proteins 72 hours post-inoculation. Higher activities of chitinase, β-1,3-glucanase, cellulase and peroxidase were found in inoculated plants compared to uninoculated controls (Yedidia et al., 2000). The levels were similar to those found when the chemical inducer INA (2,6-dichloroisonicotinic acid) was used (Yedidia et al., 2000). These two studies suggested,

in addition to the well-known mycoparasitic activity, that the biological control activity of *T. asperellum* includes the ability to induce a plant's defense response. In the same biological system, the reduction of disease symptoms, following challenge with leaf bacterial pathogen, *Pseudomonas syringae* pv. *lachrymans*, was associated with increased accumulation of transcripts of two defense genes encoding for phenylalanine ammonia lyase (PAL) and hydroxyperoxide lyase (HPL). There was also an increase in the production of phenolic secondary metabolites (phytoalexins) that were found to have *in vitro* antibacterial activity. As T-203 could not be isolated from the shoots or leaves of protected plants, this study provided strong evidence of systemic induced resistance resulting from root colonization by *T. asperellum* (Yedidia et al., 2003). Other *Trichoderma* species appear capable of inducing defense responses in plants (Howell et al., 2000; Harman et al., 2004a; Harman et al., 2004b). Howell et al., (2000) demonstrated that seed treatment of cotton with *T. virens* (G-6, G-11, G6-5) or application of *T. virens* culture filtrates to cotton seedling radicles induced much higher levels of terpenoid synthesis and peroxidase activity than untreated controls. Additionally, biocontrol activity of *T. virens* against *R. solani* was correlated with induction of terpenoid synthesis in cotton roots. Induction of peroxidase and terpenoid synthesis was not detected in cotton hypocotyls, concluding that in this case, *T. virens* strains seem to induce localized, but not systemic resistance (Howell, 2003; Harman et al., 2004b).

A large array of signaling molecules (elicitors) have been characterized that initiate plant defense responses. These elicitors are not grouped by a common chemical structure, but constitute a wide array of compounds that include peptides, glycolipids and oligosaccharides (Terry and Joyce, 2004). Three types of elicitors have been proposed to be produced by *Trichoderma* spp.: avr homologues, oligosaccharides and low-molecular-weight compounds, and proteins with enzymatic or other functions (Harman et al., 2004b). In the classical 'gene-for-gene' model, a pathogen carrying single dominant genes (avirulence genes) is recognized by plant hosts carrying single dominant resistance (*R*) genes, leading to an incompatible interaction or resistance. However, the recently proposed guard model (de Wit, 2002; Marathe and Dinesh-Kumar, 2003) states that, *R* gene products may not directly bind to avirulence gene product, but rather detect alternations in host proteins that are caused by the pathogen gene products (Mackey et al., 2002; Mackey et al., 2003; Axtell and Staskawicz, 2003; Belkhadir et al., 2004; Lim and Kunkel, 2004a; Lim and Kunkel, 2004b). Two homologous of the avirulence genes, Avr4 and Avr9 from *Cladosporium fulvum,* have been identified in *T. harzianum* T-22 (Harman et al., 2004b).

Oligosaccharides and low-molecular-weight compounds that are produced during multiple interactions that occur in nature between *Trichoderma*, fungal pathogens and plant roots have been described (Harman et al., 2004b). Two enzymes (xylanase and cellulase) have been shown to elicit a defense response in the cell lines of several plants. The ethylene-inducing xylanase (EIX) from *T. viride* has been shown to induce ethylene production, accumulation of PR proteins and phytoalexins, and HR in tobacco and

tomato. The enzymatic activity of EIX is unrelated to the elicitation process, but does require binding to a host protein (Ron et al., 2000). Cellulases from *T. viride* have been shown to induce an HR response and the production of resveratrol (phytoalexin) in grape suspension cells (Calderon et al., 1993). The infiltration of a cellulase from *T. longibrachiatum* into melon cotyledons also induced an HR response and activated defense signaling pathways resulting in an increase in cellular peroxidase and chitinase activities (Martinez et al., 2000). Alamethicin, a peptide mixture (peptaibol) from *T. viride,* has also been shown to induce the biosynthesis of volatile compounds in lima bean and *Arabidopsis thaliana* (Chen et al., 2003; Engelberth et al., 2001). In addition to these known elicitors, *T. virens* produces several proteins and peptides (6 to 42 kDa) that stimulate the production of terpenoids and increases peroxidase activity in cotton radicles (Hanson and Howell, 2004). However, there have not been yet reports of purification in a native form and characterization of proteinaceous elicitors, without enzymatic activity, from *Trichoderma* spp.

As *T. virens* exhibits all the mechanisms that have been indicated in biocontrol of plant pathogens by fungal agents and a major sequencing project of its genome is underway, this fungus represents a model system to further define these mechanisms and associated signaling pathways at the molecular level. In addition, information provided on *T. virens* mediated responses in cotton and maize is of great interest as most of the work on this topic has been conducted with the non-agricultural *Arabidopsis* model system. Providing evidence that the induction of host resistance in two agriculturally important crops is

microbially mediated should have a significant impact on the management of plant pathogens globally.

The studies presented in this dissertation demonstrate the role of an elicitor (Sm1) in the development of plant defense responses at the molecular level using biocontrol system of *T. virens* and cotton (*Gossypium hirsutum)* (Chapter II). The work presented in Chapter III investigates the effect of over-expression or deletion of *SM1* will have on plant-microbe communication in two different hosts. The work presented in Chapter IV characterizes the role an hydrolytic enzyme, β-1,6-glucanase, has for mycoparasitic ability of *T. virens*.

CHAPTER II

SM1, A SMALL PROTEIN SECRETED BY THE BIOCONTROL FUNGUS *TRICHODERMA VIRENS* IS AN ELICITOR OF PLANT DEFENSE RESPONSES

INTRODUCTION

Filamentous fungi from the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. These free-living fungi are ubiquitous in the soil environment and are being successfully used and commercialized to combat a broad range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, and *Botrytis cinerea* (Hjeljord et al., 2000; Desai et al., 2002; Fravel, 2005). *Trichoderma* spp. can directly impact other fungi: after sensing a suitable fungal host, *Trichoderma* responds with the production of antibiotic compounds, formation of specialized structures, and degradation of the host's cell wall, followed by the assimilation of its cellular content, a process known as mycoparasitism (Chet and Chernin, 2002; Steyaert et al., 2003; Benitez et al., 2004). The mechanisms of mycoparasitism, antibiosis and competition afforded by *Trichoderma spp.* have been widely studied (Howell, 2003; Harman et al., 2004b). In fact, more than 100 different metabolites with known antimicrobial activities have been described so far, including antifungal cell-wall degrading enzymes, peptaibols and broad-spectrum antibiotics such as gliotoxin (Howell

et al., 1993; Lorito et al., 1996b; Sivasithamparam and Ghisalberti, 1998; Kim et al., 2002; Wiest et al., 2002; Pozo at al., 2004).

In addition to their mycoparasitic capabilities, many *Trichoderma* strains are "rhizosphere competent", that is, they are able to colonize and grow in association with plant roots and significantly increase plant growth and development (Ahmad and Baker, 1987). Cytological studies have demonstrated that *Trichoderma* hyphae are able to penetrate the root tissue and colonize several epidermal layers, but are restricted from spreading further by formation of plant cell wall appositions enriched with callose (Yedidia et al., 1999). Very rarely is colonization by particular strains detrimental to the plant or results in a pathogenic interaction (Harman et al., 2004b). In contrast, it is frequent that root colonization by *Trichoderma* is associated with induction of both local and systemic resistance to pathogen attack (Yedidia et al., 1999, 2000, 2003; Shoresh, et al, 2005). Indeed, induction of systemic resistance against fungal and bacterial pathogens in diverse dicot and monocot plants has been demonstrated (De Meyer, 1998; Elad, 2000; Howell et al., 2000; Yedidia et al., 2003; Harman et al., 2004a; Khan et al., 2004). Even so, the relevance of the induction of plant resistance by *Trichoderma* spp. in the biocontrol process has only recently been envisaged (Harman et al., 2004b).

Extensive communication occurs between plants and microbes during the early stages of their association, in which signaling molecules play an essential role. Microbes are able to detect the plant host and initiate their colonization strategies, and plants are able to recognize microbe-derived molecules and tailor their defense responses according to the type of microorganism encountered. This molecular dialogue will determine the final

outcome of the relationship, ranging from parasitism to mutualism, usually through highly coordinated cellular processes (Nimchuk et al., 2003; Bais et al., 2004; Pozo et al., 2005). Signaling during plant-pathogen associations has been a central topic in phytopathology for many years, while more recent efforts are being made to understand the communication processes involved in plant interactions with non-pathogenic microorganisms, especially those improving plant fitness or inducing systemic resistance (Pieterse et al., 1998; Geurts and Bisseling, 2002; Limpens and Bisseling, 2003; Parniske, 2004, Levy et al., 2004; Harrison, 2005). A large array of signaling molecules (elicitors) of microbial origin that initiate plant defense responses have been characterized (reviewed in Nimchuk et al., 2003). Plant cells exposed to elicitors, whether crude fungal cell wall fragments or defined molecules such as purified proteins and avirulence gene products, respond with a battery of cellular changes related to defense (Hammond-Kosack and Jones, 1996; Yang et al., 1997). These responses include rapid ion fluxes and the generation of reactive oxygen species, accumulation of phytoalexins, and synthesis of pathogenesis-related (PR) proteins such as chitinases and glucanases (Nicholson and Hammershmidt, 1992; Van Loon et al., 1999; Mittler et al., 2004). Small, cysteine-rich proteins are common among microbial molecules involved in early signaling, and include well characterized types such as fungal avirulence gene products, elicitins, and hydrophobins (Templeton et al., 1994). Besides their involvement in pathogenicity (Tucker and Talbot, 2001), some of these small proteins have been shown to play an important role in the specificity, recognition and adhesion of

symbiotic fungi to their host plants (Martin et al., 1995; Temple et al., 1997; Wosten, 2001; Tagu et al., 2002).

During mycoparasitism, the ability of *Trichoderma* to sense a potential fungal host has been demonstrated and regulatory sequences in the promoter region of mycoparasitism related genes and key elements in the signalling transduction pathways involved in regulation of these genes have been identified (Cortes et al., 1998; Rocha-Ramirez et al., 2002; Mendoza-Mendoza et al., 2003; Zeilinger et al., 2005). However, a clear understanding of the *Trichoderma*-plant recognition and communication process is lacking. Only proteins with enzymatic activity, such as cellulase and xylanase, have been described as proteinaceous elicitors in *Trichoderma* spp., as they induce a hypersensitive response, expression of PR proteins and phytoalexins in different plant species (Calderon et al., 1993; Ron et al., 2000; Martinez et al., 2001). Evidence for the production of other metabolites by *Trichoderma* involved in plant defense elicitation has been provided, but not fully characterized (Hanson and Howell, 2004; Harman et al., 2004b).

In an attempt to bring new insights into the mechanisms underlying the processes of plant-*Trichoderma* recognition, defense elicitation and induction of resistance, we have identified, purified and characterized a small, cysteine-rich protein secreted by *Trichoderma virens*, designated Sm1 (small protein 1). Using an axenic hydroponic system we show that the presence of the plant enhances the expression of *SM1*. The protein belongs to a family of phytotoxic proteins, common among fungal pathogens. However, experiments with the native purified protein confirmed that Sm1 lacks toxic

activity against plants and microbes but it is a potent elicitor able to trigger plant defense reactions both locally and systemically.

RESULTS

Identification and Isolation of Sm1 Protein

Previous reports have indicated that *Trichoderma virens* induces defense responses in plants (Howell et al., 2000; Hanson and Howell, 2004). With the aim of discovering elicitor molecules from this fungus, we analyzed the pattern of proteins secreted by the strain Gv29-8. Electrophoretic analysis of protein extracts from *T. virens* culture filtrates revealed a remarkable abundance of a low molecular weight protein (e.g, see later section) in all media tested. The protein was isolated, subjected to N-terminal sequencing, and the 44-residue sequence DTVSYDTGYDNGSRSLNDVSCSDGPNGLETRYHWSTQGQIPRFP was obtained. Similarity searches using MPsrch at the European Bioinformatics Institute (http://www.ebi.ac.uk/MPsrch/) revealed homology to elicitors and allergens from fungal pathogens of plants and humans. Therefore, the protein, designated Sm1 (small protein 1) appeared to be a good candidate as a signaling compound, and deserved further study.

Cloning of the *SM1* Gene and Nucleotide Sequence Analysis

Sequence similarity searches were performed (Blast) with the peptide sequence against the *T. reseei* EST database (http://trichoderma.iq.usp.br/), and a matching EST was found. Specific primers were designed to amplify the corresponding gene. PCR amplification of *T. virens* genomic DNA using the designed SmF-SmR primer pair yielded a 264 bp PCR product which was further sequenced. The deduced amino acid sequence of the PCR product was in agreement with the 44-residue sequence of Sm1 and the deduced amino acid sequence of *T. reseei* EST. The labeled PCR product was used as a probe to isolate the *SM1* gene from the *T. virens* BAC library (Grzegorski, 2000). A 3.5 kb subclone containing *SM1* was sequenced (GeneBank Accession No. DQ121133). Southern analysis of *T. virens* Gv29-8 genomic DNA digested with restriction enzymes *Eco*RI, *Eco*RV, *Xba*I, *Bam*HI, *Apa*I, and *Xho*I revealed that *SM1* is present as a single copy in the *T. virens* genome (data not shown).

The DNA sequence, shown in Figure 2.1, includes 261 bp of 5'flanking region, an open reading frame (ORF) of 480 bp, and 259 bp of 3'flanking region. The *SM1*-ORF was interrupted by a single intron of 66 bp at positions 62-127, as confirmed by comparative sequence analysis of genomic and cDNA. The *SM1*-ORF encoded a polypeptide of 138 amino acids with a predicted molecular mass of 14,426.0 Da, a pI of 5.76 and a signal peptide of 18 amino acids. The 3' region contained four putative polyadenylation signals (AAATA at 631, 653, 663, and TAAATAA at 700) (Figure 2.1). Analysis of the 5' region of *SM1* gene revealed putative TATA boxes at positions -28,

and -171, and putative CAAT boxes at -81 and -104 upstream of the ATG codon. To study the presence of putative regulatory motifs in the promoter region of *SM1* gene, the sequence analysis was expanded to 2 kb upstream of the ATG codon. Six CreA-binding motifs (5' SYGGRG 3') involved in catabolite repression in *A. nidulans* (Sophianopoulou et al., 1993; Kulmburg et al., 1993) were found at positions -1054, -1033, -1003, -948, -480, and -403. Only one HGATAR site, a possible binding consensus for nitrogen regulator such as AreA in *A. nidulans* (Ravagnani et al., 1997), was observed at position -1830. Three GCCARG binding sites for PacC that mediates pH regulation in *Aspergillus* (Tilburn et al., 1995) were found at -1765, -1674, and -816. A stress response element (STREs) as described by Marchler et al. (1993) was located at -1600. Only two of the four mycoparasitism response elements (MYREs) described in the promoter region of the mycoparasitism-related genes *ech42* and *prb1* of *T. atroviride* (Cortes et al., 1998), MYC1 (GCTTCA) and MYC3 (CGGCAC) were found at positions -1925, -1669, -1218, -119 and -1104, -623, respectively.

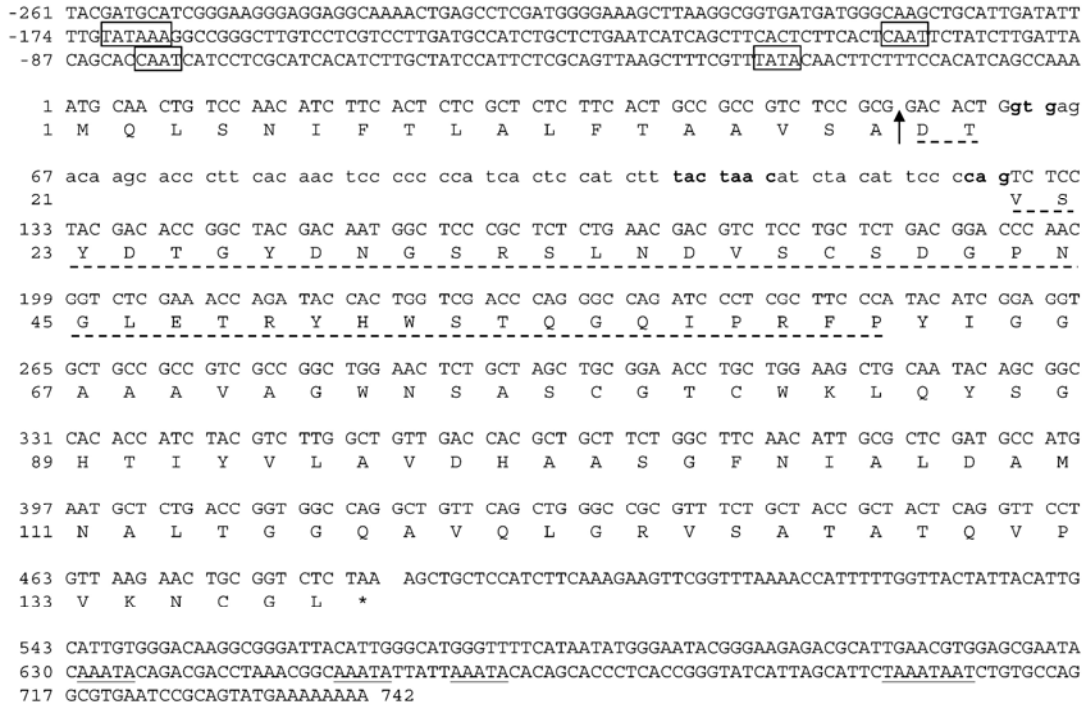

Figure 2.1. Nucleotide and Deduced Amino Acid Sequences of Sm1.

The predicted amino acid sequence of Sm1 is shown below the nucleotide sequence. The dashed underlined peptide sequence corresponds to that obtained by N-terminal sequencing of the protein. The SM1 open reading frame starts at position 1 and ends at 480. The intron sequence (at positions 62-127) is marked in lower case. The consensus sequences for 5' and 3' intron splicing sites and for lariat formation are marked in bold. The arrow indicates the cleavage site of the signal peptide. The putative TATA and CAAT consensus sequences are in clear and shaded boxes, respectively. Solid underlined nucleotide sequences in the 3' region represent putative polyadenylation signal sites.

Analysis of the deduced amino acid sequence of Sm1 (Blastp) showed that Sm1 shares high similarity to proteins of several plant and human fungal pathogens (Figure 2.2). The group includes several genes encoding for proteins known to be related to pathogenesis from phytopathogenic fungi: *snodprot1* gene of *Phaeosphaeria nodorum* (70%) highly expressed during infection of wheat leaves (Hall et al., 1999); *sp1* gene of *Leptosphaeria maculans* (69%) expressed during the infection of *Brassica napus* cotyledons (Wilson et al., 2002); and cerato-platanin (58%), a phytotoxic protein of the plane tree pathogen

Ceratocystis fimbriata f. sp. *platani* (Pazzagli et al., 1999). The highest similarity was to hypothetical proteins FG11205.1 of the cereal pathogen *Gibberella zeae* (77%), 73% to MG05344.4 of *Magnaporthe grisea* (rice blast fungus), and 71% to probable SnodProt1 PRECURSOR from the saprophytic fungus *Neurospora crassa*. Proteins homologous to Sm1 from human fungal pathogens include: an allergen from *Aspergillus fumigatus* (67%) (Hemmann et al., 1997), and an antigen (CS-Ag) with serine proteinase activity from *Coccidioides immitis* (67%) (Pan and Cole, 1995). Interestingly, a 70% similarity was found to an immunomodulatory protein (Aca1) from a medicinal fungus *Antrodia camphorate* (Hsu et al., 2005). Even though the amino acid sequence similarity between Sm1 and its homologues ranged from 58% to 77%, the sequence alignment relies on the pattern of cysteine residues in the sequence (Figure 2.2A). Searches against the Pfam protein families database (Bateman et al., 2004) for conserved domains at the NCBI server (Marchler-Bauer et al., 2004) show that residues 20-138 of the Sm1 protein align 99.2 % (E value= 2e-43) over the length of the cerato-platanin family (pfam07249). This family contains a number of fungal cerato-platanin phytotoxic proteins approximately 150 residues long, containing four cysteine residues that form two disulphide bonds.

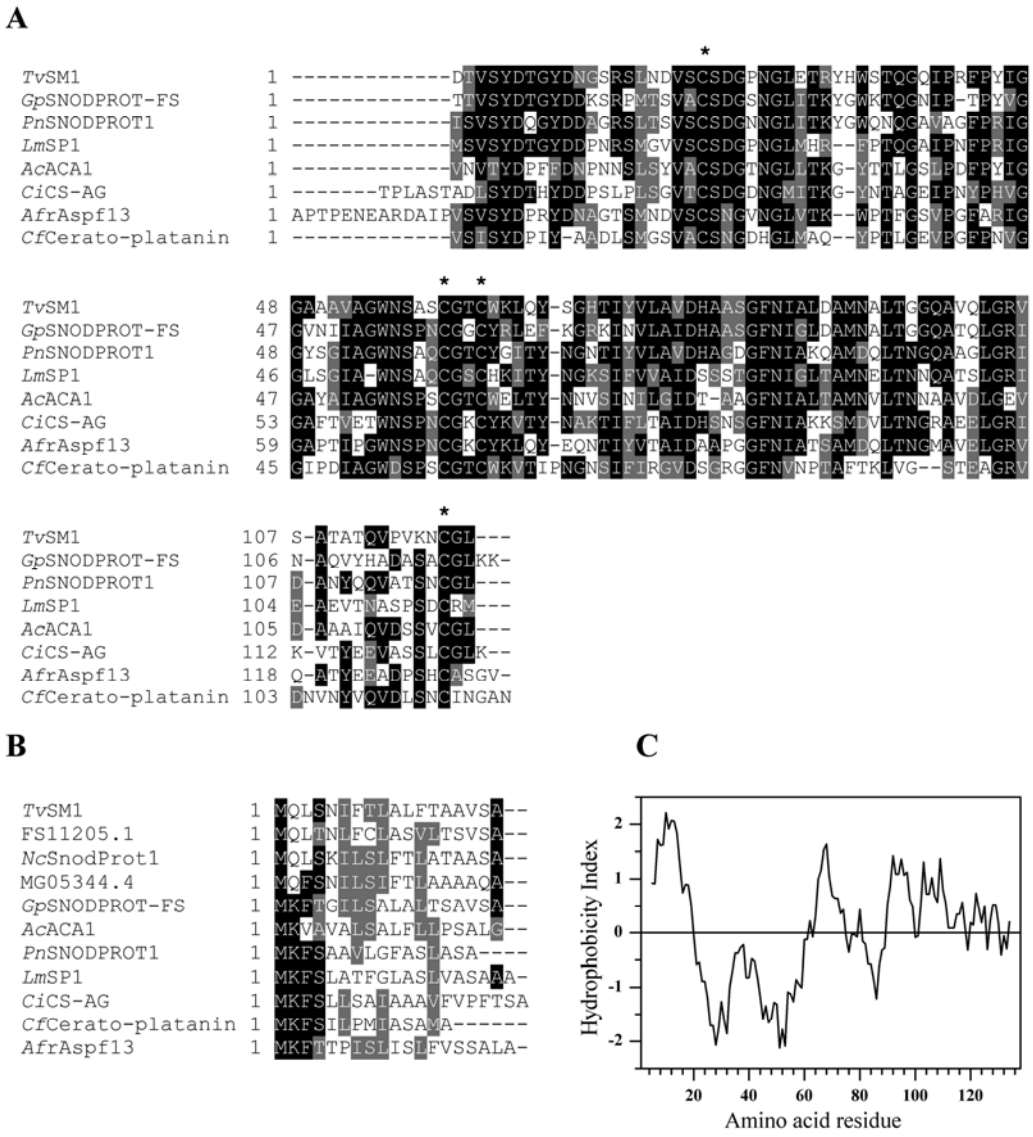

Figure 2.2. Sequence Alignments of Sm1 and Other Members of the Cerato-platanin Family.

(A) Multiple alignment of the amino acid sequences of *T. virens* Sm1 mature protein and its homologues. *Gp*SNODPROT-FS (*G. pulicaris*), *Pn*SNODPROT1 (*P. nodorum*), *Lm*SP1 (*L. maculans*), *Ac*Aca1 (*A. camphorate*), *Ci*CS-AG, (*C. immitis*), *Afr*Aspf13 (*A. fumigatus*), and *Cf*Cerato-platanin (*C. fimbriata f.sp. platani*). The asterisks indicate the conserved cysteine residues. The identical amino acid residues are shaded in black and similar residues are shaded in gray. Alignments were obtained by using ClustalW at the Kyoto University Bioinformatics Center (http://clustalw.genome.jp).
(B) Multiple alignments of the signal peptide sequences of Sm1 and its homologues. Protein names as in (A), with addition of hypothetical proteins: FG11205.1 (*G. zeae* PH-1), MG05344.4 (*M. grisea*) and NCsnodprot1 (*N. crassa*).
(C) Hydrophobicity plot of Sm1. The x-axis represents the amino acid sequences and the y-axis the hydrophobicity (above the base line) or the hydrophilicity (under the base line) of the protein domains. The hydropathic profile was calculated by the method of Kyte and Doolittle (1982) with a window of 9 amino acids.

The amino acid composition of Sm1 reveals a high percentage of hydrophobic residues (40%) including four cysteines and three tryptophans. The hydropathy plot generated from the Sm1 deduced sequence indicates that a putative cleavage site between the signal peptide and the mature, secreted protein is located between residues 18 and 19 (Figure 2.2C). This prediction corresponds to the data obtained by N-terminal sequencing of the secreted protein (indicated by an arrow, Figure 2.1). Interestingly, the similarity of the amino acid sequence of signal peptides of Sm1 and its homologues is as high as 66% (Figure 2.2B). The mature Sm1 protein contains 120 amino acids, predicted molecular mass of 12,545.8 Da and pI of 5.78. Motif analysis (ScanProsite) of the Sm1 deduced aa sequence identified several potential post-translational modifications: tyrosine sulfation site (residue 16), casein kinase II phosphorylation site (residue 25, 33, 38), N-glycosylation site (residue 29), and N-myristoylation site (residue 65).

Sm1 Is Produced and Secreted by Different *Trichoderma* Species

To assess whether Sm1 production is specific to particular *Trichoderma* species or strains, or is common to different species, we screened proteins in culture filtrates from strains of *T. virens, T. harzianum* and *T. atroviride* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analyses. Polyclonal antibody was raised in rabbits against Sm1 protein fraction electro-eluted from SDS-PAGE and confirmed by N-terminal sequencing. Sm1 was produced by all *Trichoderma* species and strains tested, although clear differences in the production were observed.

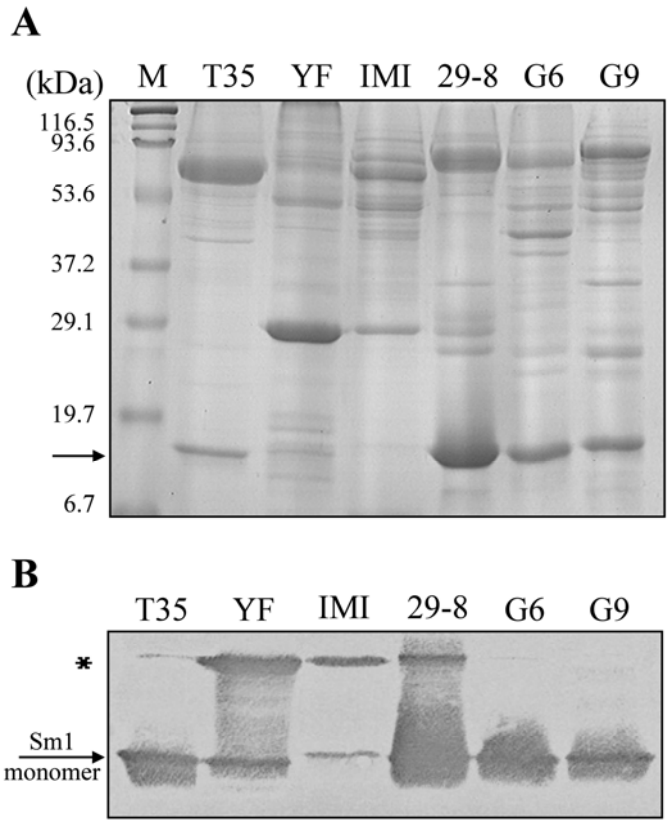

Figure 2.3. Sm1 Production and Distribution in Different *Trichoderma* Species and Strains.

(A) Protein profile of culture filtrates from different *Trichoderma* species. Coomassie stained pattern of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. 6 µg of total protein were loaded per lane. Lanes, from left to right are: molecular weight marker (M), *T. harzianum* strains (T35) and (YF); *T. atroviride* strain (IMI); *T. virens* strains (Gv29-8), (G6) and (G9). Molecular weights, in kiloDaltons, are indicated on the left. The 12.6 kDa-Sm1 is indicated by an arrow.
(B) Immunoblot analysis of protein extracts. SDS-PAGE and lanes as in A, except that 1 µg of total protein was loaded per lane. A polyclonal antibody raised against Sm1 was used. Indicated by an arrow, the 12.6 kDa monomeric form of the protein, present in all strains, and a protein band migrating as the SDS-resistant 25.2 kDa dimeric form (indicated by an asterisk), differentially distributed among the strains.

Coomassie stained SDS-PAGE profile and Western analysis correlated with respect to the amount of Sm1 produced (Figure 2.3). The protein was produced most abundantly in all *T. virens* strains (Gv29-8, G-9 and G-6), with the highest levels in Gv29-8 culture filtrates. *T. atroviride*, strain IMI, showed the lowest level of Sm1 (Figure 2.3A).

Interestingly, Western analysis showed that Sm1 and its homologues were present in two different conformations, monomeric, and a SDS-resistant dimeric form, with the greatest dimerization observed in *T. harzianum* YF strain (Figure 2.3B).

Sm1 Is Expressed in all Developmental Stages of the Fungus and Is Induced in the Presence of the Plant

Northern analysis of *SM1* transcript levels revealed basal levels of expression in all nutritional conditions tested (Figure 2.4A). In the absence of a carbon source (VM), relatively abundant *SM1* transcripts were detected at all time points. However, when Gv29-8 was growing in a medium with glucose as a carbon source (VMG), *SM1* was slightly repressed during the early time points. This early repression was also observed in a medium containing *Rhizoctonia* cell walls as a carbon source (VMR). In contrast to the up-regulation observed in mycoparasitism related genes (Baek et al., 1999; Olmedo-Monfil et al., 2002; Pozo et al., 2004), expression of *SM1* in the presence of cell walls was only observed at later time points. No clear de-repression was found under nitrogen starvation conditions: a similar pattern was found for media with glucose in presence or absence of nitrogen, and only higher levels of expression were found in media containing *Rhizoctonia* cell walls at later time points.

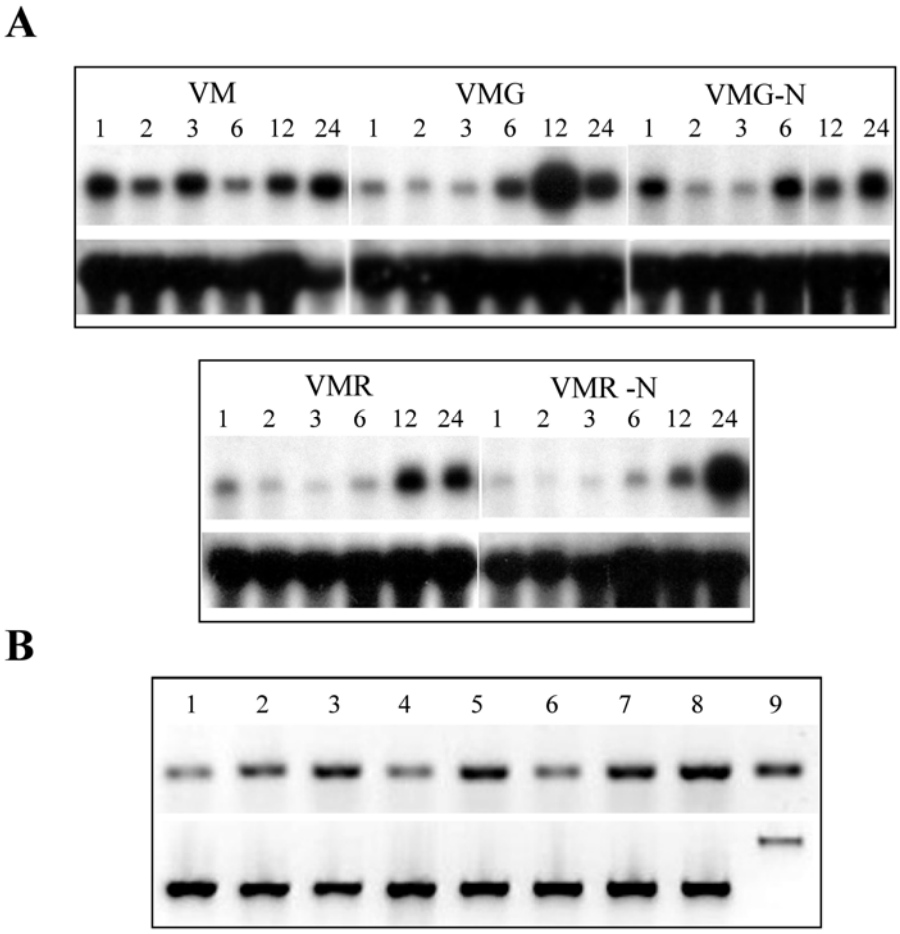

Figure 2.4. Gene Expression Analysis of *T. virens SM1*.

(A) Northern analysis of *SM1* expression during growth of *T. virens* in submerged culture under different nutritional conditions. Media used were: Vogel's minimal medium without carbon source (VM), or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *R. solani* (VMR); and VM lacking nitrogen but supplemented with either 1.5% glucose (VMG-N) or 0.5% *R. solani* cell wall (VMR-N). Listed at the top are hours after transferring to the various media. 15µg of total RNA was loaded per lane. Top panel, autoradiograph of RNA gel blot hybridized with ^{32}P-dCTP-labeled *SM1* probe; bottom panel, autoradiograph of the same RNA gel blot hybridized with ^{32}P-dCTP-labeled *actin* probe (control) showing equal loading of RNA samples.
(B) RT-PCR analysis of *SM1* expression during different developmental stages of fungal growth. Developmental stages tested were: *T. virens* Gv29-8 spores germinating for 12h on solid VMS (lane 1) or PDA (lane 2) medium; 2-day-old non sporulating mycelia cultured on solid VMS (lane 3) or PDA (lane 4); 5-day-old sporulating mycelia cultured on solid VMS (lane 5) or PDA (lane 6); *T. virens* mycelia indirectly confronted with *R. solani* (lane 7); 5-day-old VMS liquid culture of Gv29-8 (lane 8) (For treatment details see Methods). *T. virens* genomic DNA (gDNA) was included as positive control (lane 9). Top panel, amplification of a 264 bp product using *SM1* specific primers; bottom panel, fungal actin was amplified as control for equal amounts of cDNA yielding a 500 bp fragment from cDNA, and for positive control for RT-PCR a 1kb fragment from gDNA. PCR products were run on 2 % agarose gels in TAE buffer 1x, and band intensities compared within each experiment after ethidium bromide staining.

The second experiment examined *SM1* expression during different developmental stages of fungal growth (Figure 2.4B). The gene was expressed at all stages and under all conditions tested. *Sm1* was expressed in germinating spores, with higher levels of transcription in spores germinating on a complex medium (PDA) than on a minimal medium (VMS). As described for other fungi, germination is earlier on complex than minimal media (data not shown), and that could account for the higher transcription levels on PDA at this stage. In both sporulating and non-sporulating (growing) mycelia, *SM1* was expressed to a higher level in the defined medium (VMS) than in a complex one (PDA), coherently with the catabolic repression observed in the previous experiment. *SM1* was also found to be expressed during indirect confrontation with *R. solani*. Fungus grown in VMS liquid medium as submerged mycelia was used as positive control, since Sm1 protein isolated from culture filtrates showed maximum levels of expression under this condition.

Finally, we examined whether *SM1* gene expression was regulated by the presence of the plant using a hydroponic growth system that allowed the co-culture of *T. virens* and cotton seedlings (Figure 2.5A). RNA samples from *T. virens* growing in the hydroponic system with or without the seedlings were compared. *SM1* transcript levels were higher when *T. virens* was growing in contact with the plant than when growing alone (Figure 2.5B). In agreement, the analysis of the secreted proteins in the medium showed that Sm1 was more abundant in the filtrate from the plant-fungal co-culture than in the filtrate from the fungus growing alone (Figure 2.5C).

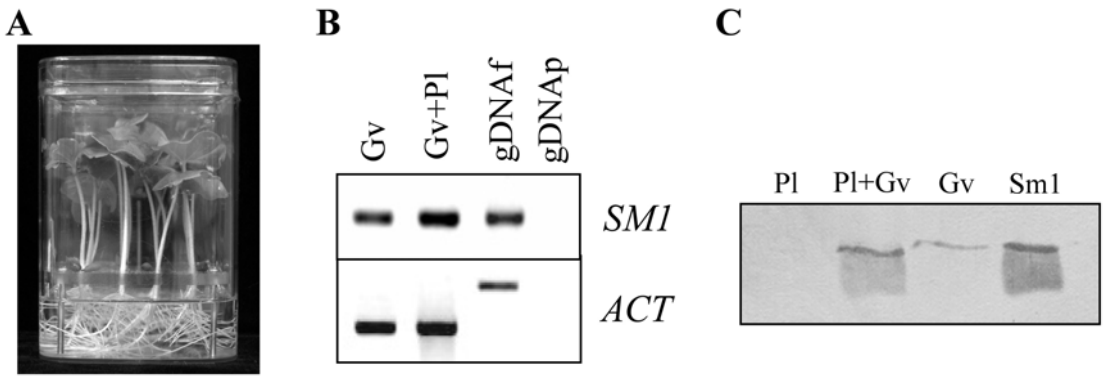

Figure 2.5. Expression Analysis of *T. virens SM1* in the Presence of a Host Plant.

(A) Experimental design for the hydroponic system for plant-fungal co-culture. Six-day-old cotton seedlings (*G. hirsutum* cv Paymaster 2326 BG/RR) were aseptically grown in MS medium (300 mL) in Lifeguard culture boxes. Then boxes were inoculated with a fungal preparation of *T. virens* Gv29.8 and incubated for 48 h (Gv+Pl) (See Methods). Two controls were included: hydroponic systems containing only the fungus, *T. virens* growing in MS in the absence of cotton seedlings (Gv); and control plants growing in MS without *T. virens* (Pl).
(B) *SM1* expression in fungus growing in presence (Gv+Pl) or absence (Gv) of the host. *T. virens* genomic DNA (gDNAf) was used as positive control for the RT-PCR and cotton genomic DNA was included to confirm the specificity of the primers to *T. virens* (gDNAp). Top panel, amplified *SM1* products after RT-PCR using *SM1* specific primers. Bottom panel, amplification of the constitutively expressed actin gene for control of equal cDNA amounts.
(C) Immunoblot analyses for detection of Sm1 secreted in the hydroponic growth medium. Equal volumes of concentrated samples equivalent to 300 mL medium from the hydroponic system (Pl, Gv+Pl, Gv) were loaded on a 15 % SDS-PAGE and electroblotted to a nitrocellulose membrane. Pure Sm1 was included as positive control (Sm1). The polyclonal antibody raised against Sm1 was used.

Sm1 Purification and MALDI/TOF Analysis

To characterize the function of Sm1, we proceeded to purify Sm1 in its native form from *T. virens* Gv29-8 culture filtrates. Several purification steps were required beginning with the protein extracts being subjected to anion exchange chromatography (AEX). The AEX elution profile is shown in Figure 2.6A. Sm1 was eluted at approximately 80 mM NaCl (fraction 5, under the major peak). The AEX fraction containing Sm1 was further submitted to gel filtration chromatography (GFC) and pure Sm1 was eluted in fractions 48-55 (the major peak) (Figure 2.6B). The successive purification step performed with GFC allowed isolation of a protein purified to homogeneity as demonstrated by SDS-PAGE (Figure 2.6C). Concentration of pure Sm1 protein was determined based on molar absorption coefficient (Pace et. al., 1995). The final yield of protein obtained from 1 liter of starting culture filtrate was ~ 1.6 mg.

The mass spectrum obtained by MALDI/TOF spectrometer of the pure native Sm1 protein revealed a single peak at 12,611.79 *m/z* (data not shown). This mass determination is in agreement with the predicted molecular weight of the mature protein according to the deduced amino acid sequence, suggesting that no post-translational modifications occur.

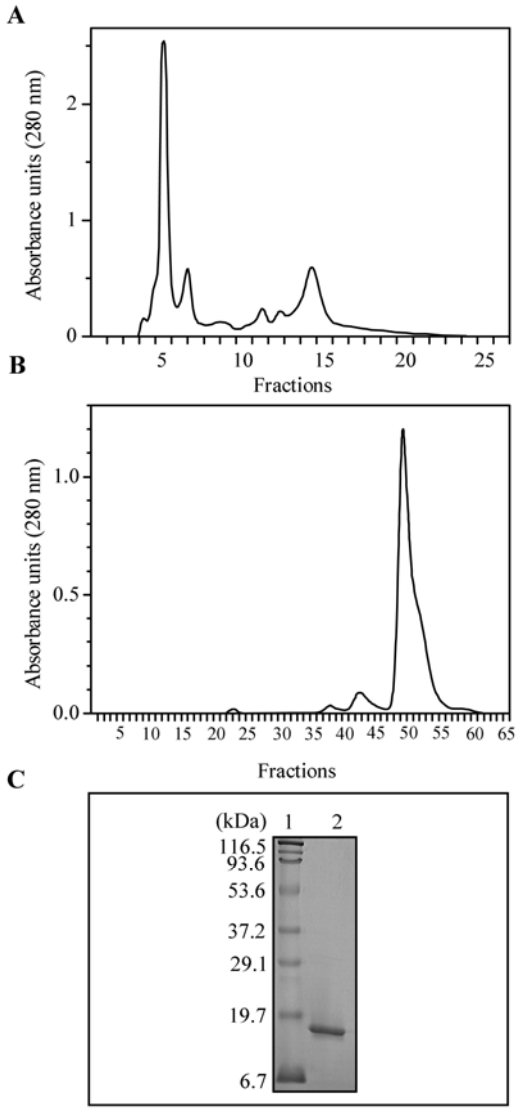

Figure 2.6. Purification of Native Sm1 from Culture Filtrates of *T. virens* Strain Gv29-8.

(A) Anion exchange chromatography. Concentrated culture filtrate, loaded on Sepharose Q column, was eluted by high-pressure liquid chromatography using sodium chloride gradient (0-1M). The divisions on *x axis* indicate 5 ml elution fractions. The fraction containing Sm1 (# 5) was pooled, dialyzed against 50 mM Tris, 100 mM NaCl, pH=7.5, concentrated and applied to the Superdex 200 column.
(B) Gel filtration chromatography. The column (Superdex 200) was eluted with 50 mM Tris, 100 mM NaCl at a flow rate of 0.5 ml/min. The divisions on *x axis* indicate 2 ml elution fractions. The 2 ml-fractions 48-54 (the major peak) were pooled and analyzed by SDS-PAGE and silver and Coomassie staining for protein purity.
(C) Electrophoretic profile of the purified Sm1. 2 µg of protein were analyzed by SDS-PAGE (13.5%) followed by Coomassie staining. A single band of Sm1 was detected, indicating a pure protein (lane 2). Lane 1, molecular mass marker: prestained SDS-PAGE broad range standards (Bio-Rad).

Enzymatic and Toxic Activity Tests

The properties of Sm1 were defined by conducting different activity tests with the purified protein. First, putative toxic activity against bacteria and fungi was tested. Sm1 was not found to inhibit the growth of any pathogenic bacteria, fungi or oomycete tested (*C. michiganensis*, *B. cereus*, *X. campestris* and *A. tumefaciens*; *M. hiemalis*, *S. rolfsii*, *B. cinerea*, *F. oxysporum*, *R. solani* and *C. heterostrophus*; *P. ultimum* and *P. infestans*, respectively) in either dual culture or antibiotic disk assays, even when the highest dose was applied. Additionally, the application of Sm1 did not result in any significant phytotoxicy against cotton, tobacco, peanut or rice leaves as evidence of necrosis was not observed even though the leaves were wounded by needle to provide access to the mesophyll cells. Putative enzymatic activity of the pure protein for protease, chitinase, β-1,3-glucanase and β-1,6-glucanase were not detected when Hide Azure powder, chymotrypsin substrate, 4-methylumbelliferyl-β-D-*N,N',N*-triacetylchitotriose or 4-methylumbelliferyl-β-D-glucosaminide, laminarin and pustulan were used as substrates, respectively (data not shown).

Sm1 Induces Early Plant Defense Reactions in Cotton and Rice

The lack of direct phytotoxic activity suggested a possible role of Sm1 as an elicitor of plant defense reactions. As the production of reactive oxygen species (e.g. hydrogen peroxide) and accumulation of phenolic compounds (usually associated with autofluorescence of these compounds) are the early responses in the plant-pathogen/elicitor recognition (Dixon et al., 1994; Hammond-Kosack and Jones, 1996; Hutcheson, 1998), we tested the ability of Sm1 to elicit these responses. The production of hydrogen peroxide in rice leaves (monocot) and cotton cotyledons (dicot) after exposure to Sm1 was examined. Application of a small amount of Sm1 (1nmol) resulted in the production of hydrogen peroxide in both plants. This reaction is illustrated by the appearance of a brownish-red precipitate in the treated tissues, generated by polymerization of the hydrogen peroxide with nitro 3,3'-diaminobenzidine (Figure 2.7). The induction of autofluorescence was tested in cotton cotyledons. A very slight response was found in water treated leaves, probably due to the wounding of the leaf during the treatment application. In contrast, high levels of autofluorescence are apparent after treatment with 1nm of Sm1 or 52nmol of INA, a SA analog used as positive control (Figure 2.8).

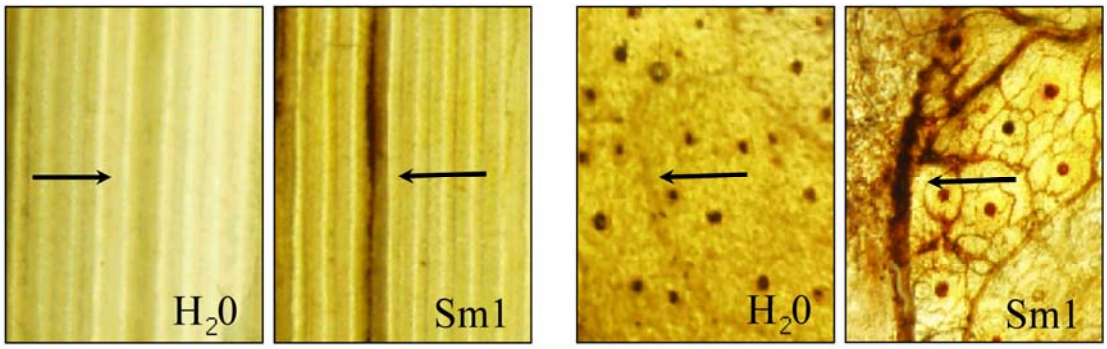

Figure 2.7. H_2O_2 Accumulation in Rice and Cotton.

Rice leaves (left) or cotton cotyledons (right) were treated with Sm1 (1 nmol) or H_2O (negative control). Treated tissues were harvested 24 h after treatment, and excised leaf/cotyledon sections were infiltrated with a solution of 3,3'-diaminobenzidine (DAB). In the presence of H_2O_2, DAB polymerizes, forming a dark red-brown precipitate staining the leaf vines (indicated by arrows). Microscopy was performed using Olympus Stereoscope SZX-9 at 40X magnification.

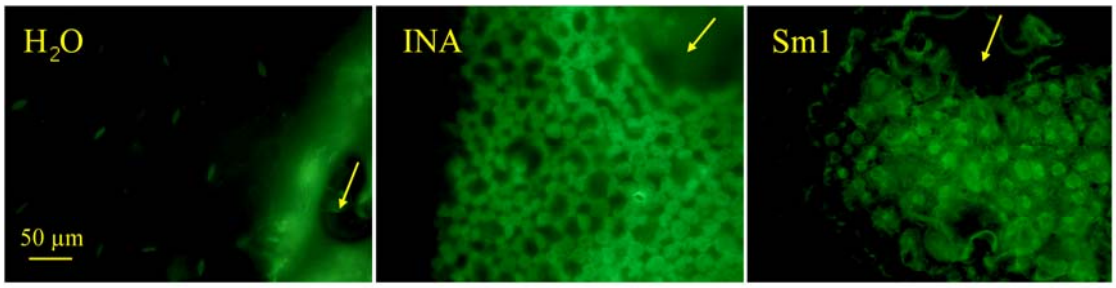

Figure 2.8. Induction of Autofluorescence in Cotton Cotyledons.

Cotton cotyledons treated with Sm1 or INA were assayed for induction of autofluorescence. Droplets of Sm1 (1nmol), INA (52 nmol, positive control) or water (negative control) were placed on the upper surface of slightly punctured cotyledons. Presence of autofluorescence in treated cotyledons was assessed after 24h incubation. Micrographs are centered on the treated region surrounding the application area (indicated by arrows). Microscopy was performed using Olympus BX-51 fluorescent microscope with excitation at (BP =360-379 nm), emission at (BA=420 nm) and 200X magnification.

Sm1 Induces the Expression of Plant Defense Genes Locally and Systemically

To gain further insight into the plant response to the Sm1 elicitor, and to determine if the induction is local (at the site of application) or systemic, several experiments were performed. The expression profile of several defense related genes in different cotton genotypes was determined by RT-PCR, using specific primers (Table 2.1). Six genes related to different plant defense pathways were selected: *GLU* and *CHT* (pathogenesis related proteins), *CAD1-C* and *HMG* (terpenoid phytoalexin pathway), and *POD6* and *GhLOX1* (related to oxidative burst and hypersensitive reactions) (Chen et al., 1995; Jalloul et al., 2002; Delannoy et al., 2003; Dowd et al., 2004). SA or INA was used as a positive control as both are known to resemble defense related signal compounds for systemic acquired resistance to pathogen infection (Ward et al., 1991).

Table 2.1. List of Primers of the Cotton Defense Genes Used in RT-PCR and Their Sources

Primer name and sequence (5' to 3')		Gene amplified	Product size (bp) cDNA/gDNA	Primers source[a]
CHTf	ACCAAGCTACTCGCAAGAGG	pathogen induced class I chitinase	158/278	CD485880
CHTr	CGGAAGCGCAGTAAGATGA			
GLUf	CATTGATATGACCTTGATCG	pathogen induced glucanase	171/171	CD486342
GLUr	GTGAGATATCCCTTGGATTG			
CAD1-Cf	ATAAGGATGAAATGCGTCC	elicitor induced δ-cadinene synthase	433/675	Chen et.al., 1995
CAD1-Cr	GAAGCTTGGTAAAGTTCCA			
HMGf	GATTTGAAGTTGTATTTGGAG	pathogen induced HMG-CoA reductase	216/316	CD486522
HMGr	GAAATCAGTTTGAAGGAAA			
POD6f	CGCTGCTCGTGATTCTGTAG	pathogen induced class III peroxidase	362/~600	Delannoy et.al., 2003
POD6r	CCTGTGTCCAATCCAATCCT			
LOX1f	GCATGGAGGACTGATGAAGAGTT	pathogen induced lipoxygenase	1060/~1500	Jalloul et.al., 2002
LOX1r	GCATGGAAGGCTGAAGCCACCCATAT			
ACTf	CCTCCGTCTAGACCTTGCTG	*G. hirsutum* actin *ACT9*	416/416	AY305737
ACTr	TCATTCGGTCAGCAATACCA			

[a] The primer pairs used for amplification of *CHT*, *GLU*, *HMG*, and *ACT* gene fragments were designed based on the sequences of pathogen- or elicitor-induced cotton or other plant genes available in the GeneBank database, and the corresponding accession number is indicated. The sequence of primer pairs used for amplification of *CAD1-C*, *POD6*, and *L0X1* gene fragments were obtained from the studies cited.

The expression of these defense-related genes was first examined locally at the site of application. Figure 2.9A shows gene expression 12 hours after application of Sm1, SA or H_20 to cotton roots. Based on RNA accumulation profiles, the expression of pathogenesis related genes (*GLU* and *CHT*) was induced by Sm1 application in all three genotypes, with the highest induction in Deltapine-50. *GLU* and *CHT* expression was also induced by SA in all genotypes. Interestingly, expression levels of both genes were higher after induction with Sm1 than with SA in Deltapine-50 and CA3274. Sm1 treatment also induced *POD6* and *LOX1* expression in all genotypes. Although *LOX1* was induced by SA treatment, *POD6* was not. *CAD1-C* and *HMG* were constitutively expressed in the roots and showed no response to the Sm1, SA or H_20 treatment. The effect on gene expression was similar when a much lower concentration of Sm1 (8 nmol /15 roots) was applied (Figure 2.10A, lane $Sm1^a$). For most genes, the basal levels of expression (in the water-treated controls) were higher in the breeding line CA3274 that shows a greater level of resistance to pathogens. When Sm1 was applied to cotton cotyledons, local induction of defense genes was similar to the response observed in roots (Figure 2.9B). *GLU* and *CHT* showed the highest induction in expression levels in response to the treatments. Interestingly, *CAD1-C*, which showed constitutively high expression in roots, showed induction by SA and Sm1 treatments in the cotyledon tissue. A similar pattern was observed for *HMG*, a gene involved in the same pathway, although constitutive expression was notably higher. Induction of *CAD1-C*, *HMG*, *LOX1* and *POD6* by Sm1 was much higher than by INA. In summary, the treatment of plant roots or cotyledons with pure native Sm1 protein resulted in local induction of defense related

genes, to a similar or even greater extent than treatment with SA, despite the low doses of Sm1 applied.

The possible induction of systemic defense reactions was analyzed in a system in which cotton roots (Deltapine-50) were exposed to Sm1 but contact of the protein with the leaves was avoided (Figure 2.10A). Treatment with SA was also included as positive control. Gene expression was analyzed in the cotyledons after application of the treatments to the roots, and a clear systemic response was observed 24h after application of Sm1 or SA (Figure 2.10B). Similar to the local response observed in the treated tissues (roots and cotyledons), PR genes *GLU* and *CHT* were the most highly induced. Induction of *LOX1* and *POD6* by both treatments was also observed. Both *CAD1-C* and *HMG* were induced upon Sm1 treatment but not by SA. Gene expression profile of directly inoculated roots (data not shown) was similar to the pattern corresponding to roots sprayed with Sm1 and SA (Figure 2.9A). Again, treatment with Sm1 efficiently induced expression of defense-related genes to similar or higher levels than SA.

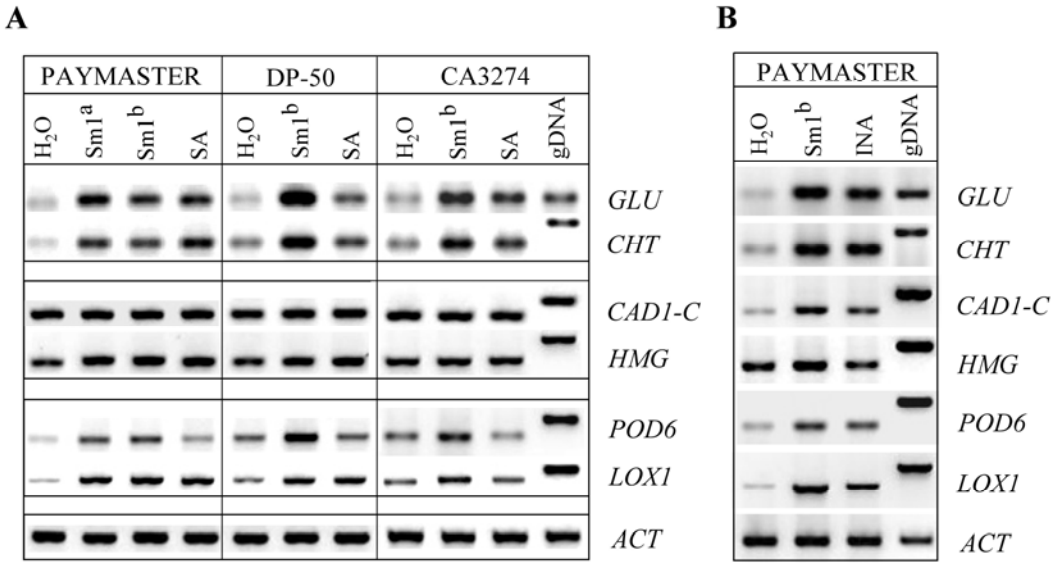

Figure 2.9. Local Induction of Defense-Related Gene Expression in Cotton.

(A) Defense gene expression in cotton roots 12h after treatment with SM1. Two different *G. hirsutum* varieties, Paymaster 2326 BG/RR and Deltapine-50, and a breeding line, CA3274, were used (listed on the top). Two-day-old cotton roots were sprayed with H_20 (negative control), salicylic acid (SA) (positive control), or pure Sm1. Three ml of 12 nmol Sm1 was applied per 10 roots ($Sm1^b$), or a lower dose (8 nmol/15 roots) was applied to Paymaster ($Sm1^a$). Application of 3 mL of 750 nmol/10 roots of SA was used for positive control (SA). Defense-related gene expression 12 hours after treatment was analyzed by RT-PCR. Gene specific primers for the following cotton defense genes were used: *GLU* (β-1,3-glucanase), *CHT* (chitinase), *CAD1-C* (+)-δ-cadinene synthase), *HMG* (3-hydroxy-3-methylglutaryl CoA reductase), *POD6* (peroxidase), *GhLOX1* (lipoxygenase). Cotton actin gene (*ACT*) was used as control for equal amounts of cDNA. For positive control, genomic DNA (gDNA) was included. See Table 1 for PCR product sizes. PCR was performed within the linear amplification range for each gene.
(B) Defense gene expression in cotton cotyledons. Cut petioles of 1-week-old cotton cv Paymaster cotyledons were treated with a 5 µL droplet of water, Sm1 (1 nmol) or INA (52 nmol), and the expression of defense genes was assessed after 24 hours. See (A) for description of the genes analyzed and RT-PCR conditions.

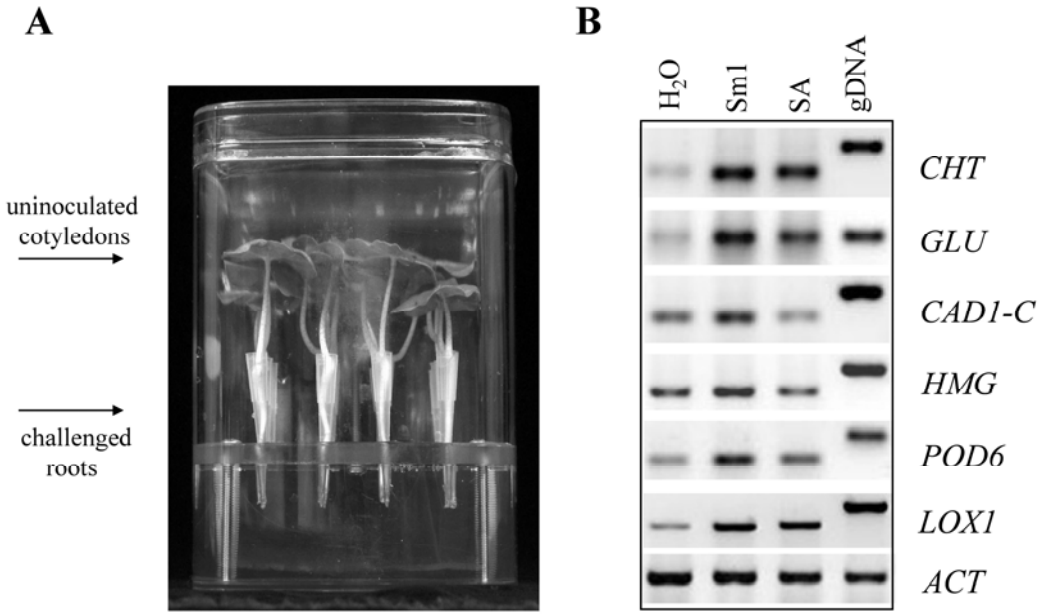

Figure 2.10. Induction of Cotton Defense Genes in Systemic Tissues.

(A) Experimental design for testing systemic induction of cotton defense genes. Four-day-old seedlings with the fully open cotyledons were placed into new vials containing 0.2 mL of sterile water, 3.2 nmol of Sm1 and of 600 nmol of SA. After 24h of elicitation, the root and the distal cotyledons (indicated by arrows) were harvested separately and RNA extracted. For growth conditions see Methods.
(B) Defense-related gene expression was analyzed in the non-treated cotyledons 24h after application of the treatments to the roots (see A). For RT-PCR conditions, refer to Figure 2-9A.

Finally, to assess the biological relevance of these results, we sought to determine if the defense response detected after treatment with Sm1 reflects the plant reaction to the living fungus. The ability of *T. virens* to induce the expression of plant defense genes, such as *GLU* and *CHT*, in hydroponicaly grown cotton was examined. As observed in Sm1-induced cotton (Figures 2.9 and 2.10) expression of both PR genes was higher in the plants growing in co-culture with *T. virens* than in plants growing alone (Figure 2.11). *T. virens* treatment also induced *POD6* expression, whereas *CAD1-C* and *HMG* were constitutively expressed.

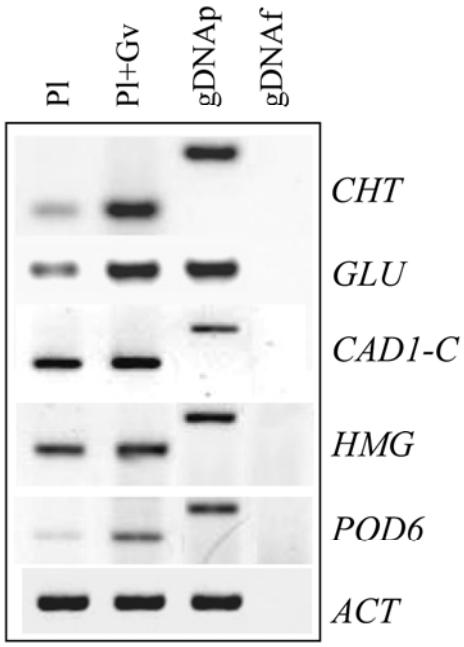

Figure 2.11. Induction of Cotton Defense Related Genes by Co-Culture with *T. virens.*

Expression analysis of pathogenesis related (PRs) genes, *CHT* (chitinase) and *GLU* (β-1,3-glucanase), *CAD1-C* (+)-δ-cadinene synthase), *HMG* (3-hydroxy-3-methylglutaryl CoA reductase), *POD6* (peroxidase), in roots of cotton seedlings grown without (pl) or with (Tv+pl) *T. virens* Gv29.8 in the hydroponic growth system (See Figure 5 for treatments). RT-PCR conditions as in Figure 9A.

DISCUSSION

Sm1 Is a Member of the Cerato-Platanin Family

Small, cysteine-rich proteins (including avirulence gene products, elicitins and hydrophobins) have been shown to play very important roles in the specificity, pathogenicity, recognition and adhesion of some symbiotic and/or pathogenic fungi (Templeton et al., 1994; Talbot, et al., 1993; Temple et al., 1997; Wosten, 2001; Tagu et al., 2002). Recently, a new family of hydrophobin-like proteins has been described based on the toxin cerato-platanin from the tree pathogen *Ceratocystis fimbriata* f. sp. *platani (*Pazzagli et al., 1999). The family consists of small (~150 amino acids) secreted proteins, mainly associated with toxicity and infection processes, produced by plant and human fungal pathogens (Hall et al., 1999; Hemmann et al., 1997; Wilson et at., 2002; Pan and Cole, 1995; Pazzagli et al., 1999). Despite the difference in total number of conserved cysteines (8 in hydrophobins, 4 in cerato-platanins), the cerato-platanin family shares several features in common with the hydrophobins, such as low molecular weight, moderate to high hydrophobicity and presence of an amino-terminal secretion signal (Marchler-Bauer et al., 2004; Wosten, 2001).

Here we report the purification and functional characterization of Sm1, a 12.6 kDa protein isolated from culture filtrates of *T. virens*. As deduced from the characteristics of the purified protein and its amino acid sequence, Sm1 has all the features described for the cerato-platanin family: size, hydrophobicity, signal peptide and conserved cysteine

pattern. Sequence alignments of conserved domains confirmed that Sm1 belongs to this family (E value = 2e-43).

Sm1 Is Not a Toxin but Displays Strong Elicitor Activity

Since members of this family are associated with toxicity, we first determined whether pure Sm1 in its native form displayed toxic activity towards other microbes or plants. Antibiotic or toxic effects were not observed against any of the diverse collection of bacteria or fungi tested. Additionally, no necrosis was found in any of the different plants treated with Sm1 (cotton, rice, tobacco, and peanut), even when high doses, if a protein was a toxin, were applied (2 nmol). This is in contrast to cerato-platanin, as an application of 0.8 nmol of the purified protein was sufficient to induce leaf cell necrosis (Pazzagli et al., 1999).

Similar to Sm1 produced by the avirulent *T. virens,* only a few members of the cerato-platanin family are produced by non pathogens. For example, Sm1 has high similarity (70%) to an immunomodulatory protein (Aca1) from the non-pathogen *Antrodia camphorate,* used in traditional Chinese medicine for treating cancer and inflammation (Hsu et al., 2005). In planta, fungal metabolites from non-pathogens have been shown to elicit plant defense responses and increase resistance against various pathogens without the induction of hypersensitive response or necrosis in the host (Madi and Katan 1998; Chang et al., 1997). As *T. virens* is shown to induce plant resistance (Howell et al., 2000), we tested the capacity of Sm1 to act as an elicitor of plant defense reactions.

We examined early cellular events in plant-elicitor interactions such as the generation of reactive oxygen species (ROS) during the oxidative burst (Mittler et al., 2004). ROS control multiple cellular functions in plants, including the oxidative cross-linking of cell-wall proteins, alteration of the redox status to regulate specific plant transcription factors, direct antimicrobial activity and initiation of the hypersensitive response (HR) (Bolwell and Wojtaszek, 1997; Bowler and Fluhr, 2000; Mittler et al., 2004). Sm1 induced significant production of H_2O_2 in both rice and cotton leaves (Figure 2.7), but did not cause HR-associated necrotic lesions. Previous studies have demonstrated that resistance and extracellular accumulation of ROS are not necessarily associated with HR (Baker and Orlandi, 1995; Glazener et al., 1996). Another early event associated with a plant response to pathogen attack is accumulation and oxidation of phenolic compounds such as phytoalexins and lignin, usually associated with autofluorescence of these compounds (Nicholson and Hammershmidt, 1992; Heath, 2000). Cerato-platanin was shown to induce autofluorescence 24 h after infiltration into tobacco leaves (Pazzagli et al., 1999). Similarly, Sm1 induced a significant autofluorescence response when applied to cotton cotyledons (Figure 2.8). The dose applied (1 nmol) was comparable to that reported for cerato-platanin, elicitins and other elicitors (Huet et al., 1995; Pazzagli et al., 1999; Scala et al., 2004).

We also investigated the transcriptional regulation of plant defense mechanisms in response to Sm1. Upland cotton (*G. hirsutum*) was chosen as the host plant since *T. virens* is rhizosphere competent on cotton, and the induction of phytoalexins and peroxidase activity in cotton roots treated with *T. virens* protein fractions have been

reported (Hanson and Howell, 2004). Pathogenesis-Related (PR) proteins are often used as markers of plant response to pathogens and their elicitor molecules (Bowles, 1990; Somssich and Hahlbrock, 1998). Recently, a large number of PR genes, including those encoding for glucanases and chitinases, have been identified in cotton upon infection with *F. oxysporum* (Dowd et al., 2004). In our study, both glucanase and chitinase genes were highly induced in all cotton genotypes, both locally and systemically, in response to Sm1 (Figure 2.9). Remarkably, glucanase and chitinase induction was much higher when treated with Sm1 than with SA in Deltapine-50.

Peroxidases are also related to resistance responses, including lignification and suberization, cross-linking of cell wall proteins, generation of reactive oxygen species, and phytoalexin synthesis, and they posses antifungal activity themselves (Quiroga, et al., 2000; Bradley et al., 1992; Bolwell and Wojtaszek, 1997; Caruso et al., 2001; Sasaki et al., 2004). Recently, several different peroxidases were cloned and studied in cotton plants during compatible and incompatible interactions with the bacterial pathogen *Xanthomonas campestris* pv *malvacearum (Xcm)* (Delannoy et al., 2003). Among them, *POD6* appeared to be related to resistance. In our study, *POD6* was specifically induced by Sm1, both locally and systemically, and only slightly induced by water or SA. Evidence for a SA-independent regulation of defense-related peroxidases has been previously reported (Martinez et al, 2000, Sasaki et al., 2004). Similarly, the involvement of lipoxygenases in conferring resistance against pathogens has been demonstrated for several plant-pathogen systems, including cotton-*Xcm* (Feussner and Wasternack, 2002; Rance et al., 1998; Jalloul et al., 2002; Wilson et al., 2001). Here we

show that *LOX1,* as with *POD6,* is upregulated after application of Sm1, both locally and systemically.

Several cotton sesquiterpenoids, with known fungistatic properties and insecticidal activities, can be induced by pathogen infection (Chen et al., 1995, 1996; Stipanovic et al., 1999; Abraham et al., 1999; Tan et al., 2000), but also by interaction with avirulent *Trichoderma* strains (Howell et al., 2000). We have investigated the transcriptional regulation of two genes encoding major enzymes of the mevalonate pathway leading to sesquiterpenoid biosynthesis in cotton, HMG-CoA reductase (*HMG*) and (+)-δ-cadinene synthase (*CAD1-C*), upon Sm1 elicitation. Expression of both genes was induced specifically by Sm1 in cotton cotyledon tissue both locally and systemically. Induction of these genes correlates well with the reported induction of phytoalexins (Hanson and Howell, 2004) and with the observed autofluorescence response. Recently, induction of phytoalexin synthesis by cerato-platanin in host and non-host plants has been also shown (Scala et al., 2004). In summary, all bioassays performed confirmed that Sm1 constitutes a very potent elicitor of plant defense responses, acting either locally at the site of application, or systemically in distant tissues.

Sm1 Is Common to Different *Trichoderma* Species and Exists in Different Conformational Forms

In an attempt to define the role of Sm1 in the biology of *T. virens*, we analyzed i) the distribution of the protein in different *Trichoderma* species, and ii) its transcriptional regulation. Screening of different *Trichoderma* species and strains for production of Sm1 homologues revealed that Sm1 is common to all of them, although the amount of the protein secreted appears to differ among the different species. The observation that under the same experimental growth conditions, some strains produced the protein in monomeric form, whereas the others produced both monomeric and dimeric forms, may be related to the presence of 4 conserved cysteines in Sm1 that may form intermolecular disulfide bridges. The existence of Sm1 monomer and dimer forms could be important in its biological activity. There are reports of key regulators of defense responses whose activity *in vivo* is regulated by redox changes, converting an inactive oligomer (formed through intermolecular disulfide bridges) to active monomers (Mou et al., 2003; Durrant and Dong, 2004). The monomeric form of Sm1 was the major form purified from *T. virens* culture filtrates and, as confirmed in the bioassays performed, it effectively elicits defense responses in cotton. In addition, the monomer is the major form detected when the fungus grew in the presence of the plant in the hydroponic system. These results strongly suggest that the activity of Sm1 in *Trichoderma*-plant interactions resides in the monomeric form.

_SM1_ Is Transcriptionally Regulated and Involved in _Trichoderma_-plant Interaction

SM1 expression seems to be subject to complex regulation, both during development and in response to external stimuli. We show here that *SM1* gene is under catabolic repression both in liquid and solid media, as indicated by the induction of *SM1* expression by carbon starvation. This repression is coherent with the presence in the promoter region of putative cis-acting elements involved in carbon and nitrogen regulation. Since the soil is generally considered as a nutritionally sparse environment, *SM1* expression is expected to be de-repressed in exploratory mycelia of *Trichoderma.* The presence of several other putative regulatory sequences in the promoter region, such as pH and stress response elements, is also consistent with a complex transcriptional regulation and deserves further experimental analysis. In contrast to most *Trichoderma* genes involved in mycoparasitism (Baek, et al., 1999; Cortés et al., 1998; Pozo et al., 2004), expression of *SM1* is not significantly induced by the presence of cell walls from a fungal host (*Rhizoctonia)* during simulated parasitism. Indeed, only two of the four putative mycoparasitism responsive elements (MYRE) described in *Trichoderma* cell-wall degrading enzymes (Cortes et al, 1998) were found in the *SM1* promoter. The low induction in gene expression in the presence of a fungal host, and the lack of enzymatic or fungitoxic activity do not support a direct role of Sm1 in mycoparasitism.

Analysis of s*m1* transcript levels during different fungal developmental stages *in vitro* revealed that the gene was expressed in all developmental stages tested, with higher expression during mycelial growth and conidiation than in germinating spores.

Developing or sporulating mycelia are actively growing structures, and in rhizosphere competent fungi, these are the structures that will establish contact with its host plant. Using a hydroponic system that allows co-culture of *Trichoderma* and cotton seedlings, we have shown the induction of *SM1* transcription in the presence of a host plant, coupled with higher Sm1 protein levels in the medium. Our results, namely the large amount of protein secreted, the ability of Sm1 to act as an elicitor of plant defense reactions, and the induction of *SM1* expression during growth in the presence of a host plant, indicates the role of Sm1 in the plant-fungal interaction. Recently, immunolocalization of cerato-platanin protein in *C. fimbriata* showed that, in addition to being released abundantly into media, the protein is located in the cell walls of ascospores, hyphae and conidia, as described for hydrophobins (Boddi et al., 2004). The localization at the fungal surface and the ability to induce defense reactions is consistent with a primary role involving direct contact with the plant recognition system. The role of hydrophobins in several mutualistic symbioses has been reported, including those established between fungi and plants, and algae and cyanobacteria (Tagu et al., 1996; Honegger, 1991; Scherrer et al., 2000). For example, a role for hydrophobins *hydPt-1* and *hydPt* has been proposed during early stages of *Pisolithus-Eucalyptus* ectomycorrhizae formation, related to the adhesion of the mycelium to the root surface (Tagu et al., 1996). Interestingly, transcripts coding for a homolog of the cerato-platanin SnodProt1 protein were among the most abundant in the free-living mycelium of this ectomycorrhizal fungus (*P. microcarpus)* (Peter et al., 2003). Recently, transcriptional regulation of this gene (*SnodProt1*) during the establishment and functioning of the

mycorrhizal association has been demonstrated (Duplessis et al., 2005). There is, therefore, strong evidence supporting the role of these types of molecules in fungal-plant interactions. The ability of Sm1 to act as an elicitor of plant defense reactions supports its involvement in the plant-fungus recognition and in the induction of systemic resistance against pathogens.

Conclusions and Further Work

The importance of induction of plant defense responses by *Trichoderma* spp. in biocontrol is now widely accepted; however, the process is largely undefined, as compared to the well documented responses by other beneficial organisms such as rhizobacteria (Pieterse et al., 2003). Our study provided direct evidence that rhizosphere competent *T. virens* produces and secretes a very potent elicitor which activates, both locally and systemically, most of the major cotton defense mechanisms. Sm1 does not possess toxic or enzymatic activity, and does not appear to be directly involved in mycoparasitism. On the contrary, our studies suggest a role in fungal morphogenesis and/or fungal-plant communication. Sm1 deletion- and over-expression strains are currently being characterized, and fusion of Sm1 with the green fluorescent protein is underway to determine cellular localization of the protein within the fungus and during interaction with the plant (Djonovic, Wiest, and Kenerley, unpublished). The spectrum of effectiveness of *Trichoderma*-induced systemic resistance and the defense reactions triggered by Sm1 combined with potential of increasing induced resistance to seedling

pathogens by *SM1* over-expressing strains may offer new directions for the biocontrol of plant pathogens.

METHODS

Fungal and Plant Materials

Three strains of *Trichoderma virens* [Gv29-8 (Baek and Kenerley, 1998), G-6 (Q strain) and G-9 (P strain) (Howell, 1999)], two strains of *T. harzianum* [T35, and YF] and one of *T. atroviride* [(IMI206040 (IMI)] (Gomez et al., 1997) were used in this study. The strains were routinely maintained on potato dextrose agar (PDA, Difco).

Plants used for elicitor activity tests were rice (*Oryza sativa* cv M-202), two cotton (*Gossypium hirsutum*) varieties, Paymaster 2326 BG/RR, and Deltapine-50, and the cotton breeding line CA3274. Paymaster 2326 BG/RR and Deltapine-50 are commercially available varieties, and susceptible to soil-borne pathogens such as *Pythium ultimum* and *Rhizoctonia solani* (Thaxton and El-Zik, 2004). CA3274 is a reselection that displays partial resistance to *P. ultimum* and *R. solani* (Dr. Wheeler, Texas Agricultural Experimental Station, Lubbock, TX, *personal communication*). For phytotoxicity tests, leaves of approximately 3-week-old cotton (*G. hirsutum* cv Paymaster 2326 BG/RR), rice (*O. sativa* cv M-202), tobacco (*Nicotiana tabacum* cv Glurk), and peanut (*Arachis hypogaea* cv Flowrunner) were used.

Fungal Culture Filtrates

Vogel's minimal medium (Vogel, 1956) supplemented with 1.5% sucrose (VMS) was inoculated with a conidial suspension of the appropriate fungal strain, and incubated on a rotary shaker at 160 rpm for 5 days at 23 ^{o}C. Culture filtrates were obtained by filtration through a 10 µm NITEX nylon cloth (TETKO Inc., Depew, NY).

Plant-Fungal Co-Culture Filtrates from a Hydroponic Growth System

We have developed a hydroponic system similar to the one described by Yedidia and colleagues (Yedidia et al., 1999; Viterbo et al., 2004) to evaluate the resistance response of cotton seedlings stimulated by *T. virens*. Polycarbonate culture boxes (Lifeguard (Sigma)), 10.9 x 10.9 x 15.7 cm) were each provided with a polycarbonate stand to support 16 seedlings, approximately 1 cm above the level of the growth medium (~ 300 mL). Plant nutrient solution was 1/2 strength Murashige and Skoog (MS) basal medium amended with Gamborg's vitamins (pH=5.6) (Sigma). Seeds were surface sterilized according to Dowd et al. (2004). Treated seeds were placed in sterile Petri dishes containing moistened filter paper and incubated in the dark for two days at 27 ^{o}C to allow germination. Germinated seeds with similar sized roots were placed on the polycarbonate stands in each culture box. The aseptic boxes were maintained in a controlled environment at 23 C ± 1 C and a 14 hour photoperiod with slight agitation on the rotary shaker (45 rpm).

The mycelial inoculum of *T. virens* Gv29-8 was prepared by inoculating 100 mL of GYEC media (Thomas and Kenerley, 1989) with $3x10^7$ spores /mL and incubating for 24 h on rotary shaker at 120 rpm and 23 C. The mycelia were harvested on two layers of sterile Miracloth (Calbiochem, San Diego, CA), washed with 200 mL of sterile water, and inoculated aseptically into 300 mL of MS medium containing 6-day-old cotton seedlings. For *Trichoderma* growing in the absence of cotton seedlings, MS, supplemented with 0.05% sucrose (300 ml medium) was inoculated with the same mycelial biomass. Control plants were grown in 300 mL MS medium without *Trichoderma*. In treatments of control plants and plants inoculated with the fungus, the growth medium was replaced with fresh MS to reduce the extracellular proteins accumulated during plant growth. Two days later, media from all treatments was collected, filtered through a 10 μm NITEX nylon cloth, and 10 mL of each treatment was concentrated by using 10 kDa cutoff Millipore microcon filter devices (Bedford, MA).

Protein Extracts and Polyacrylamide Gel Electrophoresis

Proteins in the culture filtrates were precipitated by 80% ammonium sulfate (Fisher Sci., Hampton, NH). The pellet was resuspended in 10 mM ammonium bicarbonate (NH_4HCO_3) (ICN Biomedicals), dialyzed against the same buffer (10-kDA mwco, Pierce, Rockford, IL), and successively filtered through a 0.45 μm filter (Fisher Sci., Hampton, NH). The dialyzed fraction was concentrated by lyophilization, and the

resulting dry protein pellet was stored at -20^{o}C. For protein analysis, pellets were resuspended in small amounts of 10 mM Tris, pH=7.8 and concentrations were determined by Bio-Rad Bradford microassay, using BSA as a standard. Protein extracts were subjected to SDS-PAGE. Silver (Blum et al., 1987) and Coomassie brilliant blue R-250 staining and prestained SDS-PAGE broad range molecular weight (MW) standards (Bio-Rad, CA) were used for protein visualization and molecular mass determination, respectively.

N-terminal Sequencing and Antibody Production

The identified protein band (Sm1) was excised and electro-eluted in the Electroeluter 422 (Bio-Rad, CA). This fraction was used to produce a polyclonal antibody in rabbits (Sigma, Woodlands, TX). N-terminal sequencing of the protein was performed by automated Edman chemistry on a Hewlett Packard G1005A Protein Sequencher (Protein Chemistry Laboratory, Texas A&M University).

Computational protein analyses were performed using the ExPASy proteome server at the Swiss Institute of Bioinformatics (http://us.expasy.org/), and EMBOSS at European Bioinformatics Institute (http://www.ebi.ac.uk/emboss). Multiple sequence alignments were performed using ClustalW at Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

Cloning of the *SM1* Gene

The sequence of the first 44 amino acid (aa) residues obtained by N-terminal sequencing was blasted against GeneBank and a *T. reseei* EST database (http://trichoderma.iq.usp.br). After similarity searches were performed, the homologous nucleotide sequence of a *T. reseei* EST was used to design specific primers for PCR amplification of *T. virens* genomic DNA. The nucleotide sequences of primers were: forward, 5'- GTCTCCTACGACACCGGCTA-3' (SmF), and reverse 5'-GTCGAGCGCAATGTTGAA-3' (SmR). *T. virens* genomic DNA, isolated as previously described (Xu et al., 1996), was PCR amplified using an Invitrogen *Taq*DNA Polymerase kit (Carlsbad, CA). The 264 bp SmF-SmR PCR product was subsequently purified (MinElute^{TM} PCR Purification Kit, Valencia, CA) and sequenced. The purified product was used to probe our *T. virens* bacterial artificial chromosomal (BAC) library (Grzegorski, 2001) and 33 positive BAC clones were identified. One of the positive clones was further digested with several restriction enzymes and the fragments subcloned into a pBluescript II SK (+/-) vector. Sequencing of the subclones was performed by a primer-walking strategy (Sambrook et al., 1989). All sequencing reactions were performed at the Gene Technologies Laboratory (Texas A&M University). DNA sequences were analyzed by DNA Strider 1.2 (Marck, 1988), and Sequencher 4.1 (GCC, Ann Arbor, MI).

SM1 Gene Expression Analysis

Gene expression analysis was performed with fungal tissue grown under different environmental and developmental conditions and in the presence of cotton seedlings.

To assess nutritional regulation of *SM1*, Vogel's minimal medium without a carbon source (VM) or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *R. solani* (VMR) as carbon sources was used (Pozo et al, 2004). Cell walls of *R. solani* as the sole carbon source were used to simulate mycoparasitic conditions and were prepared according to Ren and West (1992). Regulation of *SM1* expression by nitrogen was examined with the same carbon source conditions in Vogel´s minimal medium in the absence of nitrogen sources (VMG-N and VMR-N).

To study *SM1* expression during different developmental stages of fungal growth, samples included: germinating spores (GS); non-sporulating mycelia (nSM) and sporulating mycelia (SM) grown on solid VMS or PDA medium; mycelia grown in liquid VMS medium; and mycelia of *T. virens* indirectly confronting *R. solani* mycelia (Cortes et al., 1998; van West et al., 1998; McLeod et al., 2003). For GS stage, the conidia were obtained from 5-day-old cultures on PDA by gently rubbing with a sterile glass rod and collecting the conidia by centrifugation at 5000 rpm for 3 minutes. Collected spores were spread onto cellophane covering VMS or PDA, and incubated for 12h at 27 ^{o}C, in the dark, to allow germination. After the incubation period, the spores were scraped off the cellophane, frozen in liquid nitrogen, and stored at -80 ^{o}C until RNA extraction. For the nSM developmental stage, a mycelial plug from a 5-day-old

PDA culture was inoculated onto cellophane overlaying VMS or PDA, incubated at 27 ^{o}C for two days in the dark, and then harvested. For the SM stage, mycelia were collected after 5 days growth on VMS and PDA plates. To obtain mycelia grown in liquid VMS medium, 10^6 spores /ml (final concentration) of Gv29-8 were inoculated and then incubated for 5 days at room temperature with shaking at 160 rpm. The indirect confrontation assay was performed as described in Cortes et al. (1998). Briefly, a plug of *R. solani* was covered with two cellophane disks before placing a *T. virens* agar plug on top. After ~3 days of growth at 27 ^{o}C, in the dark, the ~ 1 cm overlapping mycelia of *T. virens* and *R. solani* were recovered and harvested separately. All the samples were immediately frozen in liquid nitrogen.

A hydroponic system (described in **Plant-Fungal Co-Culture Filtrates from a Hydroponic Growth System**) was used to compare the expression of *SM1* in the fungus growing in the presence or absence of cotton seedlings. Cotton seedlings were grown for 6 days, inoculated with a mycelial preparation of Gv29-8 and incubated for 48 hours. The mycelia from the culture filtrate and adhering to the roots in the hydroponic culture boxes were collected on 10 µm NITEX nylon cloth.

Total RNA was extracted following the protocol of Jones et al. (1985). *SM1* expression under different nutritional conditions was analyzed by Northern blot using the 264 bp (SmF-SmR) PCR product as described below. For analysis of expression during different developmental stages and from the hydroponic system, reverse transcriptase (RT)-PCR analysis was performed. Extracted RNA was DNase- treated and cleaned using a DNA-free^{TM} kit (Ambion, Austin, TX). Total RNA (2.5 µg) was reverse-

transcribed with First-Strand cDNA Synthesis Kit (G.E. Healthcare, Piscataway, NJ) using random hexa-mer pd$(N)_6$ as a primer. The *SM1* gene specific primers were SmF-SmR, and the actin specific primers, used as an internal standard, were, forward: 5'-AAGAAGTTGCTGCCCTCGT- 3' and reverse: 5'-GCTCAGCCAGGATCTTCATCATC- 3'. PCR amplification of *SM1* fragments comprised of 23 and 27 cycles for *SM1* (each cycle: 30sec at 94 ^{o}C, 20sec at 55C, and 20sec at 72 ^{o}C) for the hydroponic system and developmental expression analysis, respectively. Actin PCR amplification consisted of 25 cycles for both experiments (each cycle: 30sec at 94 ^{o}C, 30sec at 58C, and 40sec at 72 ^{o}C). For *SM1* expression analysis in the hydroponic system, the specificity of primers to *Trichoderma* was examined by using cotton and *Trichoderma* genomic DNA. PCR products were electrophoresed on 2 % agarose gels, and band intensities compared within each experiment after ethidium bromide staining.

DNA, RNA and Protein Gel Blot Analysis

The 264 bp (SmF-SmR) PCR product was used as probe for DNA and RNA gel blot analysis using standard procedures (Sambrook et al., 1989). Hybridizations were performed overnight at 42 ^{o}C using Ultrahyb as hybridization buffer (Ambion).

For Sm1 protein detection, 1 µg of total protein from culture filtrates corresponding to different *Trichoderma* strains (for comparison of Sm1 production among the different strains), or proteins from concentrated samples corresponding to equal volume

equivalent to 300 mL medium from the hydroponic system (for comparison of Sm1 production by *T. virens* growing in the presence or absence of cotton seedlings) were electrophoresed on SDS-PAGE gels and electroblotted to a nitrocellulose membrane (Osmonics Inc., Gloucester, MA). Sm1 protein was detected using Sm1 polyclonal antibodies (dilution 1: 1000) in a standard Western blot procedure (Sambrook et al., 1989).

Sm1 Protein Purification and Mass Spectrometry

Sm1 was purified from protein extracts of Gv29-8 culture filtrates. The purification procedure consisted of two steps: 1) Anion exchange chromatography (AEX) performed using a Sepharose Q column (10 x 20 cm, G. E. Healthcare, Piscataway, NJ), and 2) Gel filtration chromatography (GFC) using Superdex200 column (16 x 60 cm, G. E. Healthcare, Piscataway, NJ). For AEX the protein pellet was resuspended in a 2-ml aliquot of 10 mM Tris, pH=8.2, and loaded on a column previously equilibrated with the same buffer. Material bound to the AEX column was eluted using a linear gradient of solvent A (10 mM Tris, pH=8.2) and solvent B (1M NaCl, 10 mM Tris, pH=8.2), at a flow rate of 5ml/min with an Acta Explorer 10 (G. E. Healthcare, Piscataway, NJ). Sm1 was eluted at approximately 80 mM NaCl. This fraction was pooled, concentrated to 2 ml, dialyzed against 50 mM Tris, 100 mM NaCl, pH=7.5, and chromatographed on a Superdex 200 column, previously equilibrated with the same buffer. The Superdex 200 column was calibrated using the following molecular weight markers: blue dextran (MW

2,000,000), bovine serum albumin (MW 67,000), myoglobine (MW 16,900), aprotinin (MW 6,500) and potassium ferricyanide (MW 329.25). The column was eluted with 50 mM Tris, 100 mM NaCl at a flow rate of 0.5 ml/min. The Superdex 200 fraction which showed maximum A_{280} was isolated, and 2 ml-fractions dialyzed against 10 mM NH_4HCO_3. After removal of NH_4HCO_3 under vacuum, the purity of protein was assessed by gel electrophoresis (SDS-PAGE) followed by silver- and Coomassie brilliant blue R-250 staining.

The pure protein was then subjected to a matrix-assisted laser desorption ionization-time of flight (MALDI/TOF) mass spectrometry (Laboratory for Biological Mass Spectrometry, Texas A&M University) for the determination of molecular weight.

Enzyme and Toxin Activity Tests

One μg of pure Sm1 resuspended in water was tested for putative protease, glucanase and chitinase activity. General and basic protease activities were measured using Hide Azure powder (Calbiochem, San Diego, CA) and chymotrypsin substrate (Sigma) according to Flores et al. (1997) and Geremia et al. (1993), respectively. β-1,3-glucanase and β-1,6-glucanase activity were determined by liberation of reducing sugars from laminarin and pustulan, respectively (Nelson, 1957). For chitinase activity the procedure of Ren et al. (1992) was followed, using the substrates 4-methylumbelliferyl-β-D-*N,N',N*-triacetylchitotriose (Sigma) for endochitinase and 4-methylumbelliferyl-β-D-glucosaminide (Sigma) for n-acetyl glucosaminidase activity.

Pure Sm1 resuspended in water was bioassayed for toxicity against several plant pathogenic bacteria and fungi as described by Park et al. (1992) and Howell et al. (1993), respectively. Bacteria tested included the gram positive *Clavibacter michiganensis* subsp. *michiganensis*, and *Bacillus cereus*, and the gram negative *Xanthomonas campestris* pv. *vesicatoria* and *Agrobacterium tumefaciens*. Fungi tested were: *Mucor hiemalis*, *Sclerotium rolfsii*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Cochliobolus heterostrophus*, and the Oomycetes *Pythium ultimum*, and *Phytopthora infestans*. Ten µL droplets containing 0.05, 0.1, 0.5, 1, 2 nmol of Sm1 were applied to each antibiotic disk for inhibition assays.

To assess phytotoxicity of Sm1, leaves of approximately 3-week-old cotton, tobacco, peanut and rice were treated with 0.1, 1, or 2 nmol of purified Sm1 protein resuspended in water. The protein was applied either by infiltration (20 µL) using a Hagborg (1970) device, or as a 3 µL droplet after slightly puncturing the leaves with a needle. After 24, 48, and 96 h of incubation, the leaves were monitored for appearance of necrotic lesions.

Elicitor Activity Tests: Production of H_2O_2 and Autofluorescence

The production of H_2O_2 in three-week old rice leaves and one-week old cotton cotyledons was examined 24 h after application of Sm1 (1 nmol) or H_2O (negative control) following the procedure of Fitzgerald et al. (2004). Briefly, drops (2 µl) of Sm1 or water were placed on the upper surface of the leaves which were previously slightly punctured. Leaves were then vacuum infiltrated with nitro 3,3'-diaminobenzidine (DAB)

(Sigma), incubated over night, fixed and cleared in alcoholic lacto-phenol solution, and examined for the formation a red-brown precipitate. Microscopy was performed using an Olympus Stereoscope SZX-9 at 200X magnification.

To test for autofluorescence, 1 nmol of Sm1, 52 nmol of INA (2,6-dichloroisonicotinic acid) (Sigma) or sterile H_2O was applied to *G. hirsutum* leaves, as described above. After 24h of incubation, the induction of autofluorescence in leaves was assessed. Treated leaves were cleared in alcoholic lacto-phenol solution, incubated in 70% glycerol, and mounted on slides (Fitzgerald et al., 2004). Microscopy was performed using Olympus BX-51 fluorescent microscope at 20X magnification (excitation at BP =360-379 nm, and emission at BA=420 nm).

Elicitation of Plant Defense Genes by Sm1

Local induction of plant defense genes by Sm1 was examined in the roots of two different cotton varieties: *G. hirsutum* cv Paymaster 2326 BG/RR and Deltapine-50, and the cotton breeding line CA3274. Surface sterilized and pre-germinated seeds (as described previously) were collected, placed into sterile Petri dishes with filter paper, and treated by spraying with a Preval sprayer (Yonkers, NY). The treatments were: sterile H_20 (negative control), salicylic acid (SA) (positive control), or pure Sm1. Two different concentrations of Sm1 were tested: i) 3 mL containing 12 nmol of Sm1 was applied to 10 roots placed on a regular plate (100 x 15 mm) to all genotypes and ii) an additional lower doses consisting of 5 mL of 8 nmol Sm1 applied to 15 roots in a large

plate (150 x 15 mm) to cv Paymaster. For all genotypes, 3 mL containing 750 nmol of SA was applied. Three replications of each treatment were performed. Cotton roots were harvested at 3, 6, 9, 12, 24 and 48 h after spraying.

Local induction of plant defense genes was determined in cotyledons of one week old Paymaster seedlings. Cotyledons were detached from the seedling and placed into sterile Petri dishes containing moistened filter paper. A 5 μL droplet of water, Sm1 (1 nmol) or INA (52 nmol) was applied as a hanging droplet to the cut petiole. The droplets were absorbed within 5 min of application. Additional water droplets were added to prevent the petiole from drying. After 24h of incubation the cotyledon tissue was harvested.

Systemic induction of plant defense genes was assessed using Deltapine-50. Surface sterilized and pre-germinated cotton seeds were placed into small sterile vials (containing MS medium) held by a polycarbonate stand. The polycarbonate stand, with the cotton roots, was then placed into a sterile polycarbonate culture box. Seedlings were provided with additional growth medium, if needed, on a daily bases. The seedlings were grown at 25 ^{o}C in a 14 h photoperiod until the cotyledons were fully open (two-three days). The seedlings were then transferred to fresh vials containing 0.2 mL of sterile water, 0.2 mL of water containing 3.2 nmol of Sm1 or of 600 nmol of SA per plant. After 24h the root and the cotyledons were harvested separately and RNA extracted.

Expression of defense related genes was analyzed in *G. hirsutum* cv Paymaster seedlings hydroponicaly grown with or without *T. virens* (see **Plant-Fungal Co-Culture**

Filtrates from a Hydroponic Growth System). Plant root tissue was harvested from the hydroponic system previously described, after 48h of incubation.

All harvested samples were immediately frozen in liquid nitrogen. Total RNA was extracted by the method of Wang et al. (2000).

Expression Analysis of Cotton Defense-Related Genes

Expression of genes corresponding to different plant defense pathways were examined: *GLU* (β-1,3-glucanase), *CHT* (chitinase), *CAD1-C* ((+)-δ-cadinene synthase), *HMG* (3-hydroxy-3-methylglutaryl CoA reductase), *POD6* (peroxidase), and *GhLOX1* (lipoxygenase). *G. hirsutum* actin gene (*ACT*) was used as comparison for quantitative gene expression. All primers used in this study are presented in Table 1. The primers to specifically amplify *HMG*, *GLU*, *and ACT* were designed based on available pathogen- or elicitor-induced cotton and/or other plant gene sequences available in the GeneBank database. Primer sequences for *CAD1-C*, *POD6* and *GhLOX1* were obtained from reports of Chen et al. (1995), Delannoy et al. (2003) and Jalloul et al. (2002), respectively. Following amplification of *G. hirsutum* DNA, PCR products were sequenced to confirm their gene specific identities. Extracted RNA was DNase- treated and cleaned using DNA-free™ Ambion kit. RNA (2.5 µg) was then reverse-transcribed with a First-Strand cDNA Synthesis Kit using random hexa-mers $pd(N)_6$ as primers. For each gene, a range of PCR amplifications were performed, and subsequently PCR products were electrophoresed, stained with ethidium bromide and the band signals

quantified by phosphorimaging (Fujifilm BAS 1800 II PhosphorImager, Japan) to determine the number of cycles at which linear amplification occurred. PCR amplification of *GLU*, *CHT*, *CAD1-C*, *HMG*, and *ACT* fragments comprised of 23 and 25 cycles (each cycle: 30sec at 94 ^{o}C, 20sec at 52 ^{o}C, and 30sec at 72 ^{o}C); *POD6*: 25 and 27 cycles (each cycle: 30sec at 94 ^{o}C, 20sec at 58 ^{o}C, and 15sec at 72 ^{o}C); and *GhLOX1* 25 cycles and 27 (each cycle: 30sec at 94 ^{o}C, 20sec at 58 ^{o}C, and 40sec at 72 ^{o}C) for root and leaf RNA, respectively. PCR products were electrophoresed on agarose gels and band intensities compared within each experiment after ethidium bromide staining.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number DQ121133 (*SM1*). Accession numbers for the sequences shown in Figure 2.2 are as follows: AY826795 (*Gp*SNODPROT-FS), AF074941 (*Pn*SNODPROT1), AY099225 (*Lm*SP1), AY569691 (*Ac*ACA1), Q00398 (*Ci*CS-AG), AJ002026 (*Af*rAspf13), AJ311644 (*Cf*Cerato-platanin), AACM01000460 (FG11205.1), AACU01000497 (MG05344.4) and AL513410 (NCsnodprot1).

CHAPTER III

MOLECULAR CHARACTERIZATION OF COTTON/MAIZE-*TRICHODERMA VIRENS* INTERACTION

INTRODUCTION

Plants have evolved complex and active defense mechanisms to protect themselves against numerous pathogens. Induced defense responses are not just initiated by pathogens, but may result from interactions with avirulent microbes. The colonization of the rhizosphere by certain strains of plant growth-promoting rhizobacteria (PGPR) results in induced systemic resistance (ISR) (Pieterse et al., 2003; Van Loon et al., 1999). In addition to PGPR, there is another group of organisms that have been found to induce plant resistance to pathogens. They represent the anamorphic stages of several fungi including *Trichoderma* spp., *Fusarium* spp., binucleate *Rhizoctonia* and *Pythium oligandrum*, and are commonly found in most soils throughout the world (Fravel et al., 2005; Harman et al., 2004b; Hwang et al., 2003; Le Floch et al., 2005).

In recent years, the induction of plant resistance by *Trichoderma* spp. has been demonstrated to be a very important mechanism involved in biological control (De Meyer, 1998; Howell et al., 2000; Yedidia et al., 2000, 2003; Harman et al., 2004a; Khan et al., 2004; Shoresh, et al., 2005). The induction of host defense responses complete the mechanisms of mycoparasitism, antibiosis and competition afforded by *Trichoderma spp.* in preventing pathogen attacks (Park et al., 1992; Baek et al., 1999;

Howell et al., 1993; Pozo et al., 2004). Defining the elements of this communication and the signals that determine the final outcome of the plant-fungal interaction (parasitic to mutualistic) still need to be resolved. *Trichoderma* hyphae are able to penetrate the root tissue, colonizing several epidermal layers, but are further stopped from spreading by formation of plant cell wall appositions enriched with callose. This phenomenon results in both local and systemic induced resistance to pathogen attack (Yedidia et al., 1999, 2000, 2003). Similarly, it has been demonstrated that the biocontrol agent *P. oligandrum* is able to penetrate the tomato root system without significant cell damage (Rey et al., 1998) and to trigger structural defense-related responses upon pathogen attack. The formation of plant structural and biochemical barriers was found to be a major component of the observed induced resistance (Benhamou et al., 1997).

Once the fungus ceases from invading the plant and a mutualistic relationship is established, extensive molecular communication occurs, usually through highly coordinated cellular processes (Nimchuk et al., 2003; Bais et al., 2004; Pozo et al., 2005). While signaling during plant-pathogen associations has been studied for many years, more recent efforts are being made to understand the communication processes involved in plant interactions with non-pathogenic microorganisms, especially those improving plant fitness or inducing systemic resistance (Pieterse et al., 1998; Geurts and Bisseling, 2002; Limpens and Bisseling, 2003; Parniske, 2004, Levy et al., 2004; Harrison, 2005). The resistance responses mediated by *Trichoderma* species are commonly found to be associated with increased levels of expression of defense related genes or their products, or the accumulation of plant antimicrobial compounds (Howell

et al., 2000; Elad, 2000; Yedidia et al., 1999, 2000, 2003; Harman et al., 2004a; Shoresh, et al., 2005). Yedidia et al. (2000) demonstrated that cucumber roots grown in aseptic conditions and inoculated with *T. harzianum* T-203 (*T. asperellum*) exhibited higher levels of chitinase, β-1,3-glucanase, cellulase and peroxidase than control plants. In the same biological system following interaction with *T. asperellum*, subsequent challenge with the leaf bacterial pathogen, *Pseudomonas syringae* pv. *lachrymans*, resulted in reduction of disease symptoms by as much as 80% compared to the control (Yedidia et al., 1999). An accumulation of the mRNA of two defense genes, phenylalanine ammonia lyase (PAL) and hydroxyperoxide lyase (HPL), was found to be associated with disease protection. There was also an increase in the production of phenolic secondary metabolites (phytoalexins) that were found to have *in vitro* antibacterial activity. In addition to *T. asperellum*, there is evidence that *T. harzianum, T. atroviride* and *T. virens* are capable of inducing defense responses in plants (Howell et al., 2000; Harman et al., 2004a; Harman et al., 2004b). Cotton roots grown from seeds treated with *T. virens* showed elevated levels of terpenoid synthesis and peroxidase activity compared to untreated control plants. Disease control was highly correlated with the ability of strains of *T. virens* to induce the production of these terpenoids (phytoalexins) in cotton roots (Howell et al., 2000).

In an attempt to bring new insights into mechanisms underlying the processes of plant-*Trichoderma* recognition and elicitation, we have previously identified, purified and characterized a proteinaceous elicitor, Sm1, secreted by *T. virens* (Chapter II). Sm1 induced expression of pathogenesis related (*PR*) genes, glucanase (*PR2*) and chitinase

(*PR3*), class III peroxidase, 9S-lipoxygenase and genes encoding major enzymes of sesquiterpenoid phytoalexins pathway, HMG-CoA reductase and (+)-δ-cadinene synthase both locally and systemically in cotton plants. To correlate these findings in plant-fungal system, we examined the expression of plant defense genes when *T. virens* was grown with cotton seedlings in an aseptic hydroponic system. The induction of *PR* genes, glucanase and chitinase was detected in roots of hydroponicaly grown cotton seedlings with *T. virens*.

In this study (Chapter III) the expression of cotton defense related genes in systemic tissue (cotyledons) as a response to inoculation of roots with *Trichoderma* is presented. To characterize the role Sm1 may have in *T. virens* physiology and further elucidate its importance during plant-fungal interactions, *SM1* deletion- and over-expression strains were generated. The deletion or over-expression of *SM1* did not affect fungal growth or phenotype. To investigate if plant defense responses are unique to the cotton–*Trichoderma* interaction or appear in other plant species, maize (*Zea mays* inbred line B73) was selected as another host for hydroponic experiments. By choosing maize, we also sought to gain further insights into plant defense responses in monocotyledonous plants triggered by beneficial organisms, as there is very little information available (Harman et al., 2004a). *T. virens* was shown to induce expression of defense genes locally and systemically in both hosts. Using *SM1* transformants of *T. virens*, the impact of Sm1 in *T. virens*/cotton or *T. virens*/maize interaction is complex, affecting both plant transcriptome and proteome.

RESULTS

Identification of *SM1* Deletion- and Over-expression Transformants

The *SM1* disruption vector pSZD25 was constructed for replacement of 0.483 kb of the *SM1* ORF with a selectable marker (Baek and Kenerley, 1998). The construct comprised the selectable *ARG2* gene flanked by 2.1 and 1.7 kb segments of the *SM1* sequence (Figure 3.1). The linearized plasmid was used for transformation of an arginine auxotrophic Tv10.4 strain (Baek and Kenerley, 1998), and total of 147 stable transformants were tested for a gene disruption by PCR (data not shown). Nine PCR-selected candidates were further analyzed by Southern hybridization to verify the gene disruption. After digestion of the genomic DNA with *Hind*III, and hybridization with the 264 bp *SM1* PCR product, wild-type DNA yields a band of 1 kb corresponding to the native *SM1* gene (Figure 3.1A). Transformants that have undergone a homologous recombination event should yield a band of 2.8 kb which replaces the 1 kb wild-type band. Isolates that have gone heterologous integration reveal both bands, as the disruption vector did not target the *SM1* gene. Three strains, SKO25A, SKO46, SKO70A, were clearly disrupted in the gene, and strain 147 contained both 1 and 2.8 kb bands, indicating ectopic integration of pSZD25 (Figure 3.1B). After probing with a 710 bp *Xho*I fragment from the *ARG2* gene, a single integration event was detected in disruption transformants (data not shown).

The over-expression vector pSZD26 was constructed for constitutive over-production of the *SM1* gene. A 1 kb *Hind*III insert containing *SM1* ORF was placed between the promoter and the terminator regions of the *T. virens GPD* (glyceraldehydes-3-phosphate dehydrogenase) gene (Figure 3.2A, top panel). To obtain over-expression strains, Tv10.4

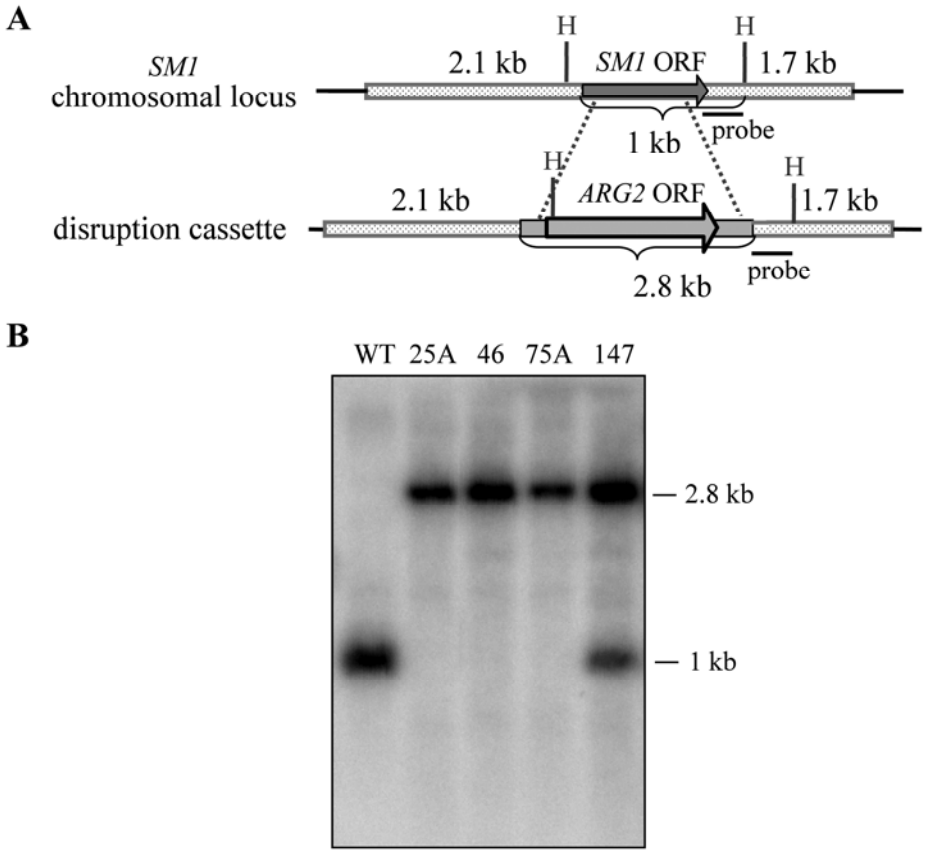

Figure 3.1. Southern Analysis and Confirmation of *SM1* Disruptants.

(A) Scheme of the gene deletion strategy. A homologous integration event where the native *SM1* is replaced with the 3.0 kb fragment of the *T. virens ARG2* gene of the targeting vector yielding a 2.8 kb band versus a 1 kb band in the wild-type genomic DNA digested with *Hind*III in Southern analysis.
(B) Southern analysis of *T. virens* wild-type (WT) strain and *sm1* deletion transformants (SKO25A, SKO46, SKO70A). Autoradiograph of DNA gel blot hybridized with ^{32}P-dCTP-labeled *sm1* probe indicated in the figure. 15µg of genomic DNA was digested with *Hind*III and loaded per lane. Numbers on the left indicate expected size in native and deletion events. Strain 147 contains the native and over-expression bands indicating ectopic integration.

was co-transformed with pSZD26 and a plasmid containing *ARG2* gene as a selectable marker (Baek and Kenerley, 1998). From fourteen randomly selected putative over-expression transformants (SOEs), ten demonstrated the presence of the over-expression cassette (Figure 3.2A). The expected 1 kb PCR product was observed in ten isolates and the positive control suggesting that they were transformed with the over-expression construct (pSZD26) (Figure 3.2A). These ten candidates were further verified by Southern blotting analysis. After double-digestion of genomic DNA with *Mlu*I/*Eco*RV, the *SM1* probe (the 264 bp PCR product) was expected to hybridize to the native gene yielding a 4 kb band. In over-expression strains the hybridization with the same probe would yield a 1.5 kb band (Figure 3.2B, top panel). Additionally, in wild-type strain two fragments of higher molecular weight (over 12 kb) are expected to hybridize to the probe due to double digestion with *Mlu*I/*Eco*RV (Figure 3.2B, bottom panel). The intensity of hybridization of 1.5 kb band in some of the transformants, such as SOE38 and SOE39, may suggest integration of the multiple copies of the construct.

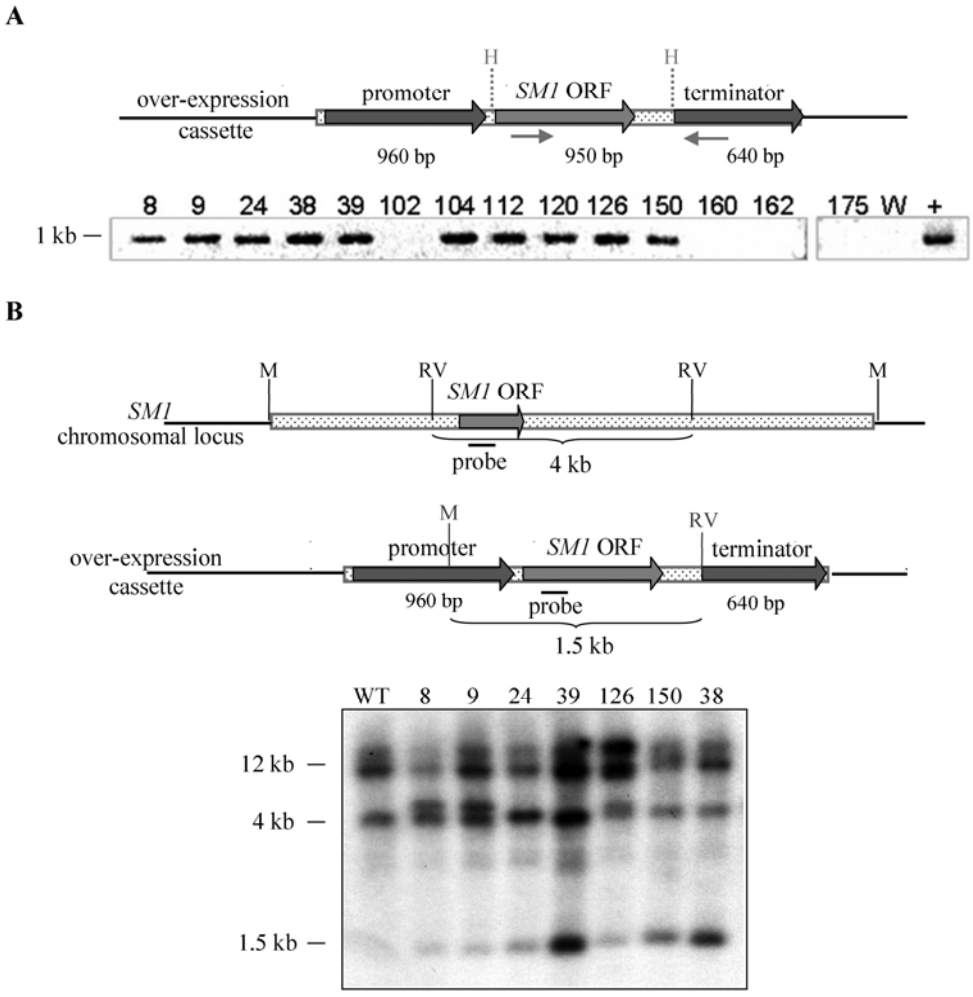

Figure 3.2. Confirmation of *SM1* Over-expression Transformants.

(A) Gene over-expression strategy and selection of transformants by PCR. Top panel, an over-expression construct: a 1 kb *Hind*III fragment (indicated by dashed lines) containing *SM1* ORF was placed between promoter and terminator regions of the *T. virens gpd* gene. Arrows indicate primers used for screening of putative transformants. Bottom panel, gel electrophoresis of 1 kb PCR products amplified by indicated primers with genomic DNA from fourteen putative *T. virens* transformants. Lanes 1-5 and 7-11 putative transformants (SOE 8, 9, 24, 38, 39, and 104, 112, 120, 126, 150); Lane 15, *T. virens* WT genomic DNA (negative control); Lane 16, pSZD26 (positive control).
(B) Gene over-expression strategy and Southern analysis of *T. virens* WT strain and *SM1* over-expression transformants (SOE8, SOE9, SOE24, SOE39, SOE126, SOE150, SOE38). Top panel, the diagram of the over-expression strategy; indicated in letters enzyme restriction sites, *Mlu*I (M) and *Eco*RV (RV); Numbers over brackets indicate expected sizes in wild-type and over-expression genomic DNA double-digested with *Mlu*I/*Eco*RV and probed with indicated probe in Southern analysis. The dimensions are not drawn to scale. Bottom panel, 15µg of genomic DNA was double digested with *Mlu*I/*Eco*RV, blotted on a membrane and probed with ^{32}P-dCTP-labeled 264 bp PCR product amplified from *SM1* ORF. Numbers on the left indicate expected size in over-expression and wild-type sequences.

SM1 Expression and Protein Production in Transformants

Northern analysis of selected transformants grown in VM liquid medium supplemented with 1.5 % sucrose (VMS) for five days (deletion strains, SKOs) or in GYEC liquid medium for three days (over-expression strains, SOEs) is presented in Figure 3.3. No transcripts of *SM1* were detected in any of the SKO transformants (SKO25A, SKO46, SKO70A), confirming the replacement of the gene (Figure 3.3A). Examination of the seven over-expression strains (SOE8, SOE9, SOE24, SOE38, SOE39, SOE126, SOE150) revealed higher levels of expression than for the wild-type, confirming the constitutive expression of the constructs (Figure 3.3B). However, at the protein level, no apparent increase of SM1 production was detected in any of the SOEs compared to the wild-type. This was apparent whether the equal volumes of concentrated samples equivalent to the starting volume were loaded per lane (Figure 3.4, left panel) or when equal amounts of total protein (quantified by Bradford) were loaded per well (Figure 3.4, right panel). Coomassie- or silver stain profile of secreted proteins of *T. virens* WT and deletion transformants demonstrated the absence of the protein encoded by *SM1* gene (Figure 3.5A, B-top panel), as confirmed by Western analysis (Figure 3.5B-bottom panel). Additionally, there was no difference in the extracellular protein profile of wild-type and the SKOs except for the 12.6 kDa Sm1 band (Figure 3.5A).

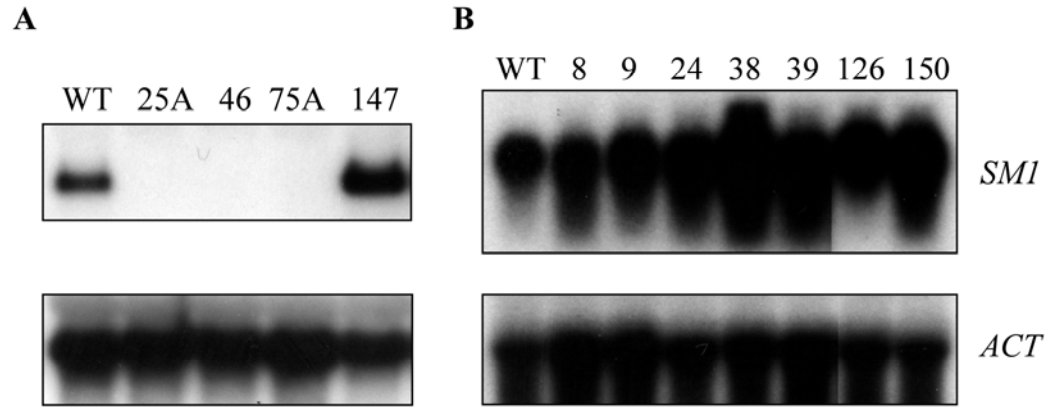

Figure 3.3. Northern Analysis of *SM1* Expression in Transformants.

(A) Northern blot of *SM1* disruptants strains. Total RNA was extracted from wild-type (WT), deletion strains (SKO25A, SKO46, SKO70A) and stain with ectopic integration of the construct (147) cultured in VMS media for 5 days. 15µg of total RNA was separated on formaldehyde-agarose gel, transferred to a Hydrobond-N^+ nylon membrane, and hybridized with the 264 bp PCR product of *SM1* gene (Top panel). Bottom panel, the same membrane was hybridized with the 600 bp actin fragment PCR amplified from *T. virens* wt cDNA as a control for even loading.
(B) Northern blot of *SM1* over-expression strains. Total RNA was extracted from wild-type (WT), over-expression strains (SOE8, SOE9, SOE24, SOE38, SOE39, SOE126, SOE150) cultured in GYEC media for 3 days. Total RNA samples (10 µg) were prepared, separated on formaldehyde-agarose gel, and transferred to a nylon membrane. The *SM1* specific probe (Top panel) and actin probe (Bottom panel) were the same as in A.

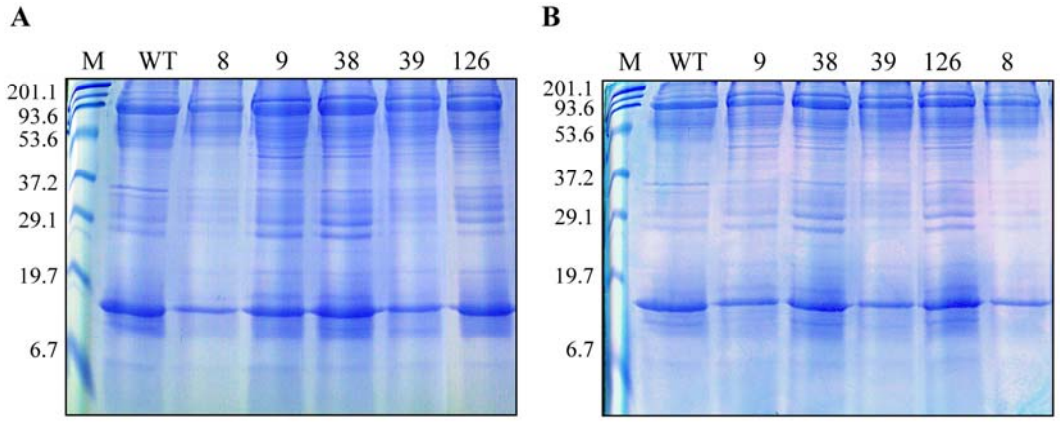

Figure 3.4. Sm1 Production in Over-expression Strains.

Protein profile of culture filtrates from *T. virens* WT and over-expression strains. Coomassie stained pattern of the SDS-PAGE analysis. Equal volumes of concentrated samples equivalent to the starting volume (left panel) or 6 µg of total protein were loaded per lane (right panel). Lanes, from left to right: Prestained broad range, molecular weight standards in kiloDaltons (M); *T. virens* WT, over-expression strains (SOE8, SOE9, SOE38, SOE39, SOE126).

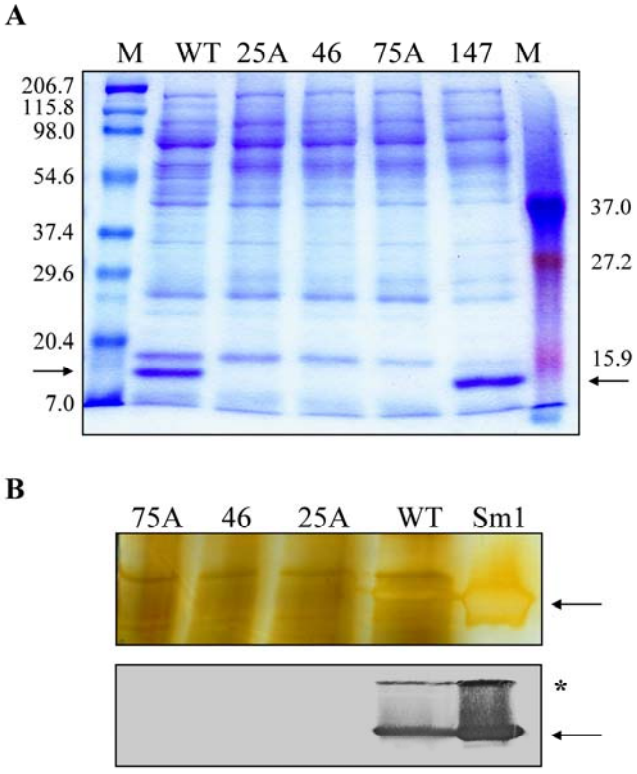

Figure 3.5. Sm1 Production in Disruptant Strains.

(A) Protein profile of culture filtrates from *T. virens* WT and deletion strains. Coomassie stained pattern of the SDS-PAGE analysis. 6 µg of total protein were loaded per lane. Lanes, from left to right: Prestained broad range, molecular weight standards (Bio-Rad, CA)(M); *T. virens* WT, deletion strains (SKO25A, SKO46, SKO75A), and stain with ectopic integration (147); Kaleidoscope polypeptide molecular weight standards (Bio-Rad, CA) (M). Molecular weights, in kiloDaltons, are indicated on the left and right. The 12.6 kDa-Sm1 is indicated by arrows.
(B) SDS-PAGE and Immunoblot analysis of protein extracts. Lanes, from left to right (SDS-PAGE and Western blot): deletion strains (SKO25A, SKO46, SKO75A); *T. virens*WT and a pure Sm1 as a positive control. 6 µg of total protein (were loaded per lane of concentrated culture filtrates and 2 µg of pure SM1. A polyclonal antibody raised against Sm1 was used. Indicated by *arrows*, the 12.6 kDa monomeric form of the protein, and indicated by an asterisk, a SDS-resistant 25.2 kDa dimeric form.

Growth Assays of Transformants

There were no phenotypic changes in the appearance of transformants compared to the wild-type with respect to the production of aerial hyphae and pigmentation during sporulation. *T. virens* WT, SKOs (SKO25A, SKO46, SKO70A) and SOEs (SOE38, SOE39), were selected for growth analysis. Growth area was compared after one and two days of growth on VMS, PDA, or WA plates. Based on analysis of variance test (ANOVA; $P<0.05$) there was no significant differences in growth among WT, GKOs, and GOEs strains in any of the media tested. (Figure 3.6).

Hydroponic System and Expression Analysis of Cotton Defense-Related Genes

It was previously shown (Chapter II) that Sm1 induces expression of cotton defense genes locally and systemically. Additionally, it was demonstrated that *T. virens* Gv29-8 wild-type strain induces the expression of plant defense genes pathogenesis related (*PR*) genes, glucanase (*PR2*) and chitinase (*PR3*), in roots of hydroponicaly grown cotton seedlings. Further experiments were designed to examine if these responses are induced systemically in cotyledon tissue when roots are treated with *T. virens* WT. Also, the effect on the induction of plant defense responses by over-expressing or deleting *SM1* was assessed.

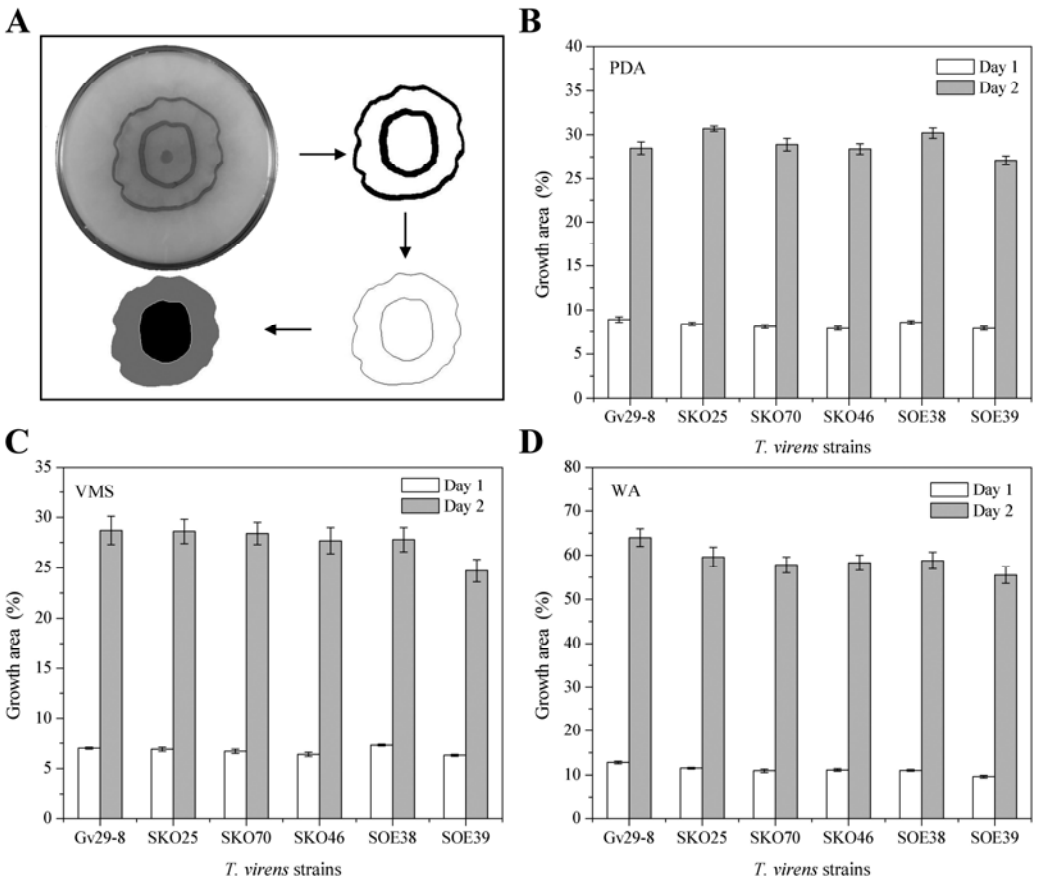

Figure 3.6. Growth Analysis of Transformants.

(A) The image of fungal radial growth on PDA plate after two days of incubation, as an example for processing of data. After incubation period, the surface area of growth for each day was marked and the area of growth calculated using a Matlab code capable of independently processing large number of consecutively numbered images.
(B) to **(D)** The surface area of growth of transformants and WT in PDA, VMS and WA plates. The data are represented as the ratio of fungal growth area to the total area of plate. Each bar represents mean growth area of four replicates from two independent experiments with the standard error.

The aseptic hydroponic growth system was used (described previously in Chapter II) to spatially separate the root-inducing agent from the distal cotyledon tissue. To verify that the strains were not present on the cotyledons, cotyledon homogenates were plated onto a *Trichoderma* selective medium (GVSM) (Park et al., 1992). *Trichoderma* was not detected from any cotyledon tissue samples assayed (data not shown).

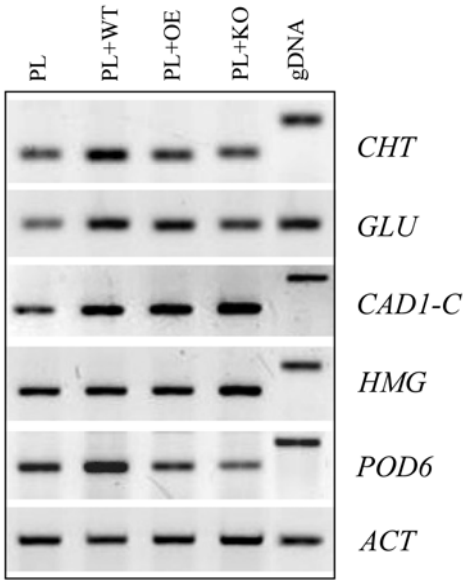

Figure 3.7. Systemic Induction of Cotton Defense Related Gene Expression by Co-Culture with *T. virens* Strains.

Six-day-old cotton seedlings (*G. hirsutum* cv Paymaster 2326 BG/RR) were aseptically grown in MS medium (300 mL) in Lifeguard culture boxes. Then boxes were inoculated with a fungal preparation of either *T. virens* Gv29-8 (PL+WT), the over-expression strain SOE38 (PL+OE) or the disruptant strain SKO25A (PL+KO) and incubated for 48 h. Positive control was cotton seedlings growing in MS without *T. virens* (PL).
Defense-related gene expression in cotyledon tissue 48 hours after treatment was analyzed by RT-PCR. Gene specific primers for the following cotton defense genes were used: *GLU* (β-1,3-glucanase), *CHT* (chitinase), *CAD1-C* ((+)-δ-cadinene synthase), *HMG* (3-hydroxy-3-methylglutaryl CoA reductase), and *POD6* (peroxidase). Cotton actin gene (*ACT*) was used as control for equal amounts of cDNA. For positive control, genomic DNA (gDNA) was included. See Table 2-1 for PCR product sizes. PCR was performed within the linear amplification range for each gene. PCR products were run on 2 % agarose gels in TAE buffer 1x, and band intensities compared within each experiment after ethidium bromide staining.

The expression profile of several defense related genes (Table 2.1, Chapter II) in hydroponicaly grown cotton (*G. hirsutum* Paymaster 2326 BG/RR) with *T. virens* wild-type or transformed strains was determined by RT-PCR. Five genes related to different plant defense pathways were selected: *GLU* and *CHT* (pathogenesis related proteins), *CAD1-C* and *HMG* (terpenoid phytoalexin pathway), and *POD6* (related to oxidative burst and hypersensitive reactions) (Chen et al., 1995; Jalloul et al., 2002; Delannoy et al., 2003).

Figure 3.7 shows gene expression in cotyledon tissue of 6-day-old cotton seedlings at 48h post-application of *T. virens* WT or transformants to the root system. The expression of pathogenesis related genes, *GLU* and *CHT*, was clearly higher in the plants growing in co-culture with *T. virens* WT than in plants growing alone. When the plants were growing with over-expression (SOE38) strain, the level of expression of *GLU* was slightly lower than with the wild-type, but clearly higher than plant growing without *Trichoderma*. The level of expression of *GLU* in response to *SM1* deletion strain (SKO25A) was lower then to the WT or SOE strain, and just slightly higher than basal expression observed in plants grown alone. There was no clear difference in levels of expression of *CHT* in response to transformed strains (SOE and SKO) compared to the basal expression. *CAD1-C* showed induction following inoculation with WT or transformants compared to the untreated plants, but no apparent difference in gene expression was detected among the strains of *Trichoderma*. *HMG* was constitutively expressed in all treatments. Interestingly, expression *POD6* was induced by treatment with the WT, but repressed by treatments with SOE and SKO.

Hydroponic System and Expression Analysis of Maize Defense-Related Genes

To investigate if the defense responses described above were unique to the cotton–*Trichoderma* interaction or could be observed in other plant species, maize (*Zea mays* inbred line B73) was selected for examination.

The expression of the following genes corresponding to different plant defense pathways in maize was examined: *PR1* and *PR5* (pathogenesis-related genes), *PAL* (phenylpropanoid pathway), *LOX10* (a closest maize homolog of a bean *LOX* gene associated with systemic resistance by a nonpathogenic *Pseudomonas* strain), and *OPR7* (a gene most likely involved in JA biosynthesis) (Farag et al., 2005; Morris et al., 1998; Ongena et al., 2004; Zhang et al. 2005). The maize housekeeping gene, glycerol phosphate dehydrogenase cytosolic form (*GAP C*) (Farag et al., 2005), was used to ensure equal loading. The sequences of primers used in this study are presented in Table 3.1.

Table 3.1. List of Primers of the Maize Defense Genes Used in RT-PCR and Their Sources

Primer name and sequence (5' to 3')		Product size cDNA (bp)	Primers source
ZmPAL-F	CGAGGTCAACTCCGTGAACG	320	Farag et al., (2005)
ZmPAL-R	GCTCTGCACGTGGTTGGTGA		
GAPc-F	GCTAGCTGCACCACAAACTGC	520	Farag et al., (2005)
GAPc-R	TAGCCCCACTCGTTGTCGTAC		
ZmPR-1 F	AACAATGGCACCGAGGCTAGCGT	510	U82200 Kolomiets et al., unpublished
ZmPR-1 R	GTATGCATGACAGTCTAGTAGGG		
ZmPR-5 F	TAGCTCTATAGCTCGAGTATTGCT	625	U82201 Kolomiets et al., unpublished
ZmPR-5 R	TCACTAGCCCATGCATGCAGAGC		
OPR7-F	CGGCTGTTCATCGCTAATCCCGA	240	Zhang et al., (2005)
OPR7-R	CAATCGCGGCATTACCCAGATGT		
ZmLOX10-F	CGCCATCGACGACCTCTTCA	150	Kolomiets et al., unpublished
ZmLOX10-R	TCCGCACCTTGTGCACGTA		

Figure 3.8 illustrates gene expression in root and leaf tissue of 4-day-old maize seedlings at 48h post-application of *T. virens* WT or transformants to the root system. Expression of *PAL* was found to be induced by WT and SOE strains both locally (roots) and systemically (leaves) and down-regulated by the treatment with the deletion strain. The expression of pathogenesis related genes, *PR1* and *PR5*, was not detected in any of the treatments, neither in the plant growing with the *Trichoderma* nor in plant growing alone. Thus, the effect of transformants compared to the wild-type on expression of these two genes in maize could not be evaluated. *OPR7* and *LOX10* showed similar pattern of expression in both roots and leaves. At the time points tested, *OPR7* or *LOX10* transcripts were not detected in roots of plants growing with or without *Trichoderma*. Low expression of both genes was observed in leaves in all treatments. However, *OPR7* appears to be slightly induced by the treatment with SOE. Also, *LOX10* transcripts accumulate to slightly higher levels in leaves of plants treated with *Trichoderma* WT.

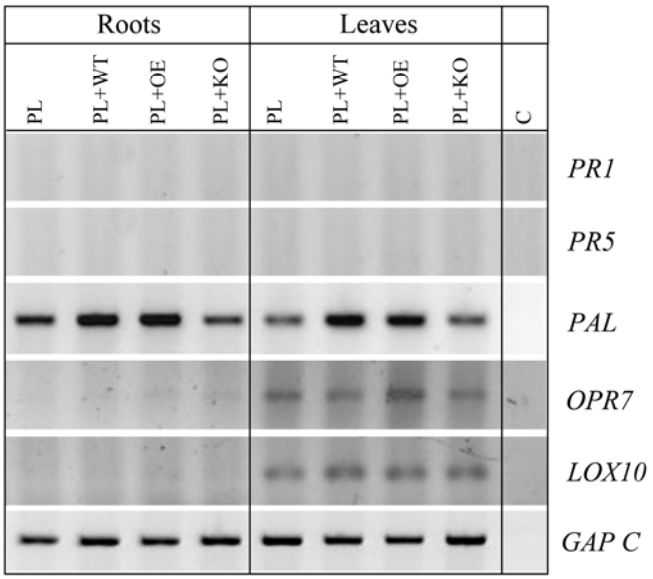

Figure 3.8. Local and Systemic Induction of Maize Defense Related Genes Expression by Co-Culture with *T. virens* strains.

Defense-related gene expression in maize root and leaf tissue 48 hours after inoculation with *T. virens* strains (see Figure 3.7) was analyzed by RT-PCR. Gene specific primers for the following maize defense genes were used: *PR1* and *PR5* (pathogenesis-related genes), *PAL* (phenylalanine ammonia-lyase), *LOX10* (lipoxygenase 10) and *OPR7* (12-oxo-phytodienoic acid reductase 7). The maize glycerol phosphate dehydrogenase, cytosolic form (*GAP C*) was used to as control for equal amounts of cDNA. See Table 3-1 for PCR product sizes. The positive control for RT-PCR (C) was DNase- treated and cleaned RNA reaction without reverse transcription. PCR products were run on 2 % agarose gels in TAE buffer 1x, and band intensities compared within each experiment after ethidium bromide staining.

Extracellular Proteins from Plant-Fungal Co-Culture Filtrates from the Hydroponic Growth System

At least ten specific extracellular proteins were detected in hydroponic growth medium of cotton or maize seedlings grown in the presence of *T. virens* WT for 48h as compared to the fungus growing alone in a hydroponics medium supplemented with 0.05 % sucrose (indicated by numbers, Figure 3.9). When the cotton or maize seedlings were grown without the fungus and the medium replaced 48h prior to harvest, very few proteins were detected. This protein pattern was reproducible in several independent experiments.

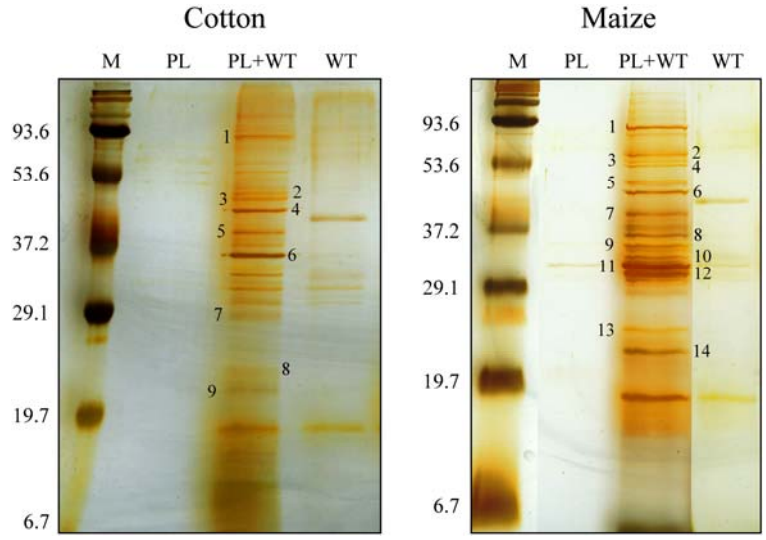

Figure 3.9. Silver Stained Pattern of SDS-PAGE of Proteins Specific for Cotton/Maize-*T.virens* WT Interaction.

Protein extracts were obtained by ammonium sulfate precipitation from hydroponics medium of *T. virens* WT grown with cotton (left panel; lane PL+WT) or *T. virens* WT grown with maize (right panel; lane PL+WT). Two controls were included: *T. virens* WT grown in MS (supplemented with 0.05% sucrose) in the absence of seedlings (lane WT, both panels); and control plants grown in MS without the fungus (lane PL, both panels). Equal volumes of concentrated samples equivalent to 300 mL medium from the hydroponic system were loaded per lane. Proteins specific for fungus-cotton/maize interaction are indicated by numbers. Molecular weights (M), in kiloDaltons, are indicated on the left of each panel.

To compare extracellular proteins specific to the cotton- or maize – *T. virens* WT interaction, proteins from hydroponic growth medium of cotton or maize seedlings grown in the presence of *T. virens* WT for 48h were precipitated or concentrated. At least one highly produced protein was found to be specific to the cotton- or maize-*T. virens* WT interaction (Figure 3.10, indicated by asterisks), regardless of the method for obtaining protein extracts.

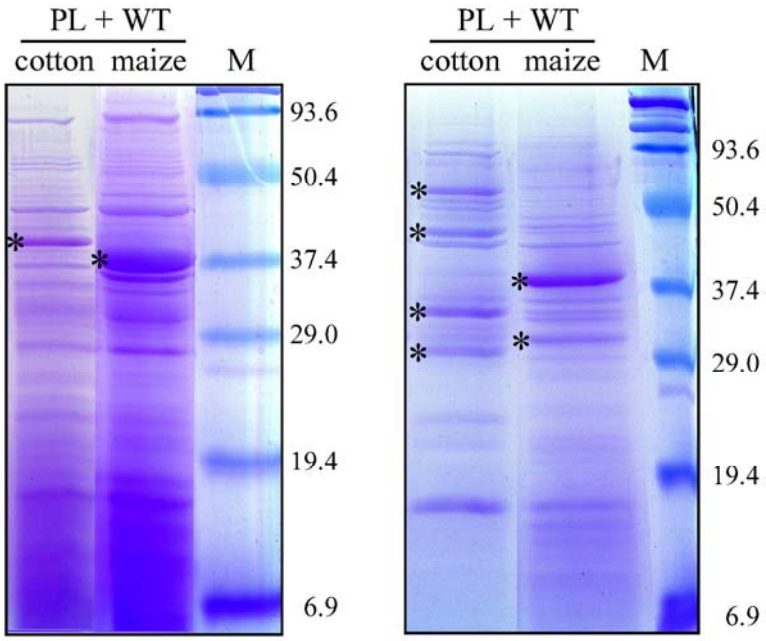

Figure 3.10. Comparison of Proteins Specific for Cotton-*T. virens* WT vs. Maize-*T.virens* WT Interaction.

Coomassie stained profile of the SDS-PAGE analysis of protein extracts obtained from growth media from cotton/mazie-*T.virens* WT treatments (see Figure 3.9). Protein extracts were either concentrated using 10 kDa Millipore Amicon Ultra centrifugal filter devices (Bedford, MA) (left panel) or precipitated by ammonium sulfate (right panel). Proteins specific for *T. virens* WT-cotton/maize interaction are indicated by *asterisks*. Molecular weights (M), in kiloDaltons, are indicated on the right of each panel.

Proteins secreted into the hydroponic growth medium when *T. virens* WT, *SM1* deletion (SKO25A) or over-expression (SOE38) strains were grown with the cotton or maize seedlings for 48 h were obtained by either precipitation or concentration. When proteins of cotton-*Trichoderma* interaction were either precipitated (Figure 3.11) or concentrated (Figure 3.12), there was no significant difference in the protein profile between cotton grown with WT or with SKO (the 12.6 kDa Sm1 band was absent in the cotton-SKO treatment). This was additionally confirmed by Western analysis (Figure 3.12, bottom panel). The protein pattern of cotton-SOE differed from either cotton-WT or cotton-SKO by having highly pronounced protein of approximately 32 kDa (Figures 3.11, 3.12). When proteins from the hydroponics medium of maize-*Trichoderma* interaction were concentrated, no significant difference was observed among the fungal isolates (WT, SKO, and SOE) (Figure 3.12). However, when proteins were precipitated, several bands were more pronounced in plant-WT interaction, than in SOE- or SKO-plant interaction (Figure 3.11). As expected, SM1 was absent in maize-SKO interaction.

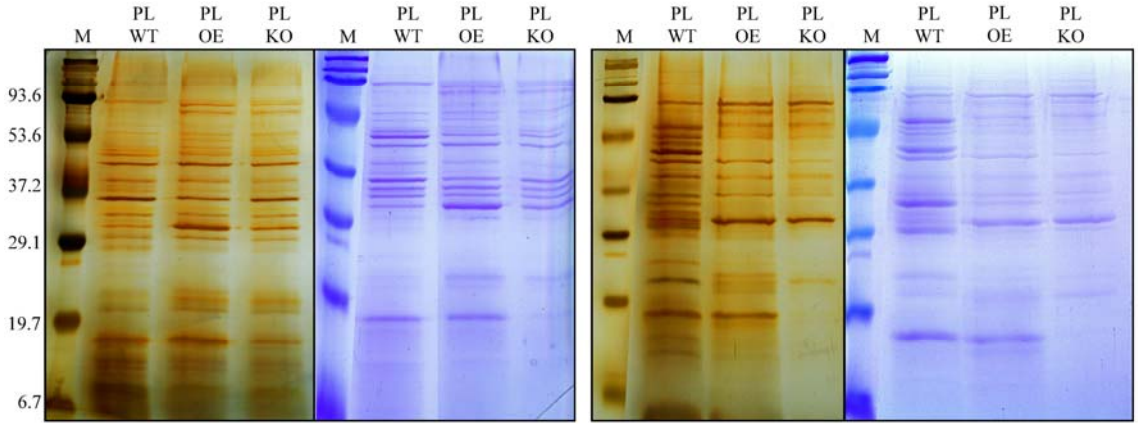

Figure 3.11. Comparison of Proteins Obtained by Precipitation from Hydroponic Medium When Cotton or Maize Was Grown with *T. virens* WT and Transformants.

Coomassie and silver stained profile of the SDS-PAGE analysis of protein extracts obtained by precipitation from cotton/*Trichoderma* (left panel) or maize/*Trichoderma* (right lanel) hydroponics growth medium. Molecular weights (M), in kiloDaltons, indicated on the right correspond to markers of other panels.

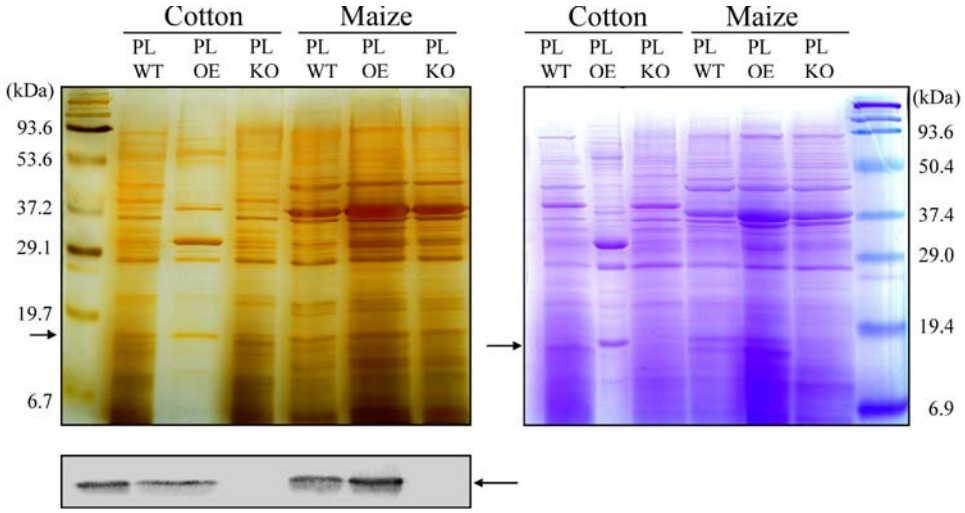

Figure 3.12. Comparison of Proteins Obtained by Concentration from Hydroponic Medium When Cotton or Maize Was Grown with *T. virens* WT and Transformants.

Silver and Coomassie stained profile of the SDS-PAGE analysis of protein extracts obtained by concentration using 10 kDa Millipore Amicon Ultra centrifugal filter devices from cotton/*Trichoderma* or maize/*Trichoderma* hydroponics growth medium. Molecular weights (M), in kiloDaltons, are indicated on the left and right of each panel. Bottom panel, Immunoblot analysis of proteins extracts corresponding to SDS-PAGE on left and right using Sm1 antibody. Indicated by arrows, the 12.6 kDa monomeric form of Sm1. The protein band that corresponds to the Sm1 dimeric form (25.2 kDa) was not detected.

Western analysis were performed with proteins extracts from cotton/ maize – *T. virens* WT or from the fungus grown in absence of the seedlings, with the antibodies for T. *virens* serine protease (Pozo et al., 2004). The protein blot analysis revealed the presence of serine protease in plant-fungal interaction at much higher levels than in fungus growing without the plants (Figure 3.13). Two bands of protease were observed, a 29 kDa mature protein and a slightly higher molecular weight band which may correspond to not fully processed mature protein as two KR sites are detected in the protein sequence (Pozo et al., 2004). KR sequence is a recognition site for a subtilisin-like endoproteinase (Kex2) known to processes secreted proteins in different organisms (Thomas et al., 1991; Fuller et al., 1989; Tao et al., 1990; Goller et al., 1998).

Five proteins unique for cotton-*T.virens* WT interaction were selected for further identification by N-terminal sequencing and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The sequences identified by N-terminal sequencing were (Figure 3.9): protein # 3 [(T)-A-Q-P-G-S-L-P-I-(Q/V)-(G/F)-V-N-N-(Q)], protein # 4 [(T)-A-Q-P-G-H-L-P-I-Q-(I)], protein # 6 [X-L/X-Q/L-D/P-G/V-H/L-I/L-P/I-G/F-Q/G-I/X-P/V-N/X-N/X-F/L]. The sequences identified by MALDI-TOF MS/MS for corresponding proteins were (Figure 3.9, left panel): protein # 3 [NDVI/LQ/K], and protein # 6 [PGSVVDI/LF/MA; K/QDI/LI/LSTW; TGVDATHPE]. Letters without parentheses are high confidence sequence. Letters with parentheses are less confident and may be one or the other (if more than one amino acid is designated). Letter X designed not known. Similarity searches of generated sequence using MPsrch at the European Bioinformatics Institute (http://www.ebi.ac.uk/MPsrch/)

revealed homology of the most pronounced, ~37 kDa protein to different classes of proteases. The highest sequence similarity matches for this protein were: a 39.1 kDa cuticle-degrading protease (Q6QZW9_9HYPO) (E=1.51e-03); a 40 kDa subtilisin-like protease PR1B (Q9P3Y1_METAN) (E=1.60e-02); and a 38.3 kDa serine proteinase precursor (P87203_AGABI) (E=3.68e-01). Protein #4 was identified as a 46.3 kDa glycosyltransferase ID (Q8UA24_AGRT5) (E=2.26e-03). Sequencing of other proteins did not yield sequence sufficient for identification.

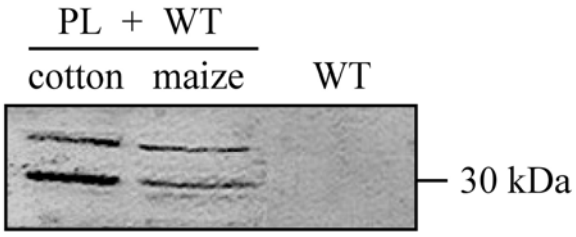

Figure 3.13. Detection of *T. virens* Serine Protease in Hydroponic Growth Medium.

Immunoblot analysis of proteins extracts corresponding to SDS-PAGE in Figure 3.12 using polyclonal antibody raised against *T. virens* serine protease (Tvsp1). Molecular marker is indicated on the right.

DISCUSSION

Growth/Survival of Genetically Modified Strains

Sm1 is a member of a new family of hydrophobin-like proteins described based on the toxin cerato-platanin secreted by the pathogen *Ceratocystis fimbriata* f. sp. *platani* (Pazzagli et al., 1999). The family consists of small (~150 amino acids) secreted proteins, mainly associated with toxicity and infection processes, produced by plant and human fungal pathogens (Hall et al., 1999; Hemmann et al., 1997; Wilson et at., 2002; Pan and Cole, 1995; Pazzagli et al., 1999). In spite of those similarities, relatively little is known about their regulation, or their role in fungal development and physiology. The cerato-platanin protein from *C. fimbriata*, in addition to being released abundantly into media, was shown to be located in the cell walls of ascospores, hyphae and conidia (Boddi et al., 2004). The authors proposed that this protein may have some new structural functions for *C. fimbriata* (Boddi et al., 2004). However, neither deletion nor over-expression of *SM1* affected cell functions such as viability, growth, or conditiation. Recently, the application of *T. virens* Sm1: GFP (green fluorescent protein) fusion strains demonstrated that Sm1 is located in the fungal cell wall (Djonovic, Wiest, and Kenerley, unpublished). Using Sm1: GFP transformants the localization of this fungal elicitor can be studied during all stages of the life cycle, i.e., during saprophytic growth as well as during colonization of a host plant. This will be of great interest as Sm1 has some characteristics of hydrophobins which are known to be located on the surface of

the conidial or hyphal walls and implicated in many aspects of fungal development (Martin et al., 1995; Temple et al., 1997; Wosten, 2001; Tagu et al., 2002). One very interesting function of hydrophobins is that some may be responsible for a parasitic fitness factor; the ability of a given pathogen genotype to survive and reproduce within the pathogen population (Shaner et al., 1992; Andrivon et al., 1993). The disruptant of cerato-ulmin, a phytotoxic hydrophobin protein produced by *Ceratocystis ulmi*, revealed a very different function than as a wilt toxin (Temple et al., 1997). Its function was shown to be a parasitic fitness factor that increased the hydrophobic and adherent qualities of yeast-like cells which protected infectious propagules from desiccation providing better transmission (Temple, 1997). In nature isolates of *C. ulmi* producing cerato-ulmin appear to be replacing the non-producers (Brasier, 1991; Tegli et al., 1994). The construction of strains disrupted in Sm1 can be used to address whether Sm1 is also involved in fitness. Their ability to colonize roots and survive in soil compared to indigenous strains can be compared. Further experiments can be performed to determine whether other strains of *Trichoderma* can replace the disrupted strains in the soil environment.

Another aspect of the localization of hydrophobin proteins at the fungal surface is that they mediate adhesion of the conidia or hyphae to the hydrophobic surface of the host (Doss et al., 1993; St Leger et al., 1992; Martin et al., 1995; Wosten, 2001; Tagu et al., 2002). The ability of an elicitor to induce defense reactions is consistent with a primary role involving direct contact with the plant recognition system. Therefore, it is hypothesized that the deletion of *SM1* from *T. virens* will affect the communication

between the plant and the fungus. Reduced induction signals lead to the accumulation of fewer transcripts of plant defense genes and consequently lower levels of protection against pathogen attack.

T. virens Induces Expression of Defense Related Genes in Cotton Locally and Systemically

Considerable progress has been made in elucidating the pathways of plant resistance triggered by *Trichoderma* species (Yedidia et al., 2000, 2003; Martinez et al., 2001; Harman et. al., 2004b; Shoresh et al., 2005). An interesting concept has been proposed recently by Shoresh et al. (2005) for the mode of action of systemic resistance induced by *T. asperellum* T203 in cucumber plants. It was shown that the main signal transduction pathway responsible for *T. asperellum* induced systemic responses in cucumber is mediated though jasmonic acid/ethylene pathway that resembles rhizobacteria-induced ISR. However, elevated levels of SAR-related genes, such as chitinase, β-1,3-glucanase, and peroxidase were detected after subsequent challenge of *Trichoderma*-preinoculated plants with a bacterial leaf pathogen. The high expression of SAR-genes induced by *Trichoderma* prior to pathogen challenge was related to the recently described mechanism for rhizobacteria-induced ISR in *Arabidopsis* plants termed ‘sensitization’ or ‘priming’ (van Wees et al., 1999). They proposed that induction of these defense related genes by *Trichoderma* ‘sensitizes’ the plant to respond more efficiently to the pathogen attack. Since the plant ‘sensitization’ resulted in reduced

disease symptoms, it was concluded that it has a crucial role in ISR mediated by *T. asperellum* (Shoresh et al., 2005).

Previously (Chapter II) *T. virens* Gv29-8 was shown to induce localized defense responses in hydroponicaly grown cotton seedlings). The expression of cotton defense genes was induced in distant cotyledon tissue 48 h after inoculation of *T. virens* Gv29-8 to the root system. *PR* genes, glucanase (*GLU*) and chitinase (*CHT*) and peroxidase (*POD6*) were found to be induced at the highest levels by Gv29-8. The high basal levels of the expression of (+)-δ-cadinene synthase (*CAD1-C*) and HMG-CoA reductase (*HMG*) masked the ability to evaluate the levels of induction by *T. virens*. Since *Trichoderma* was not detected on the cotton cotyledons of inoculated plants, this response appeared to be systemic. This is the first report of systemic induced responses in cotton mediated by *T. virens*. Clearly, the next step is to determine if this induction is sufficient to provide protection against foliar pathogens as reported in other studies (De Meyer et al., 1998; Koike et al., 2001; Harman et al., 2004b). *SM1* targeted disruption had an effect on expression of *GLU*, *CHT* and *POD6*. These genes seem to be less expressed in the interaction of cotton with the *T. virens* disrupted strain than with the wild-type. However, since the differences are not prominent, a more sensitive method is required to better quantify these responses. The observation that over-expression of *SM1* did not affect transcription level of defense genes, may be explained simply by the over-expression event did not lead to the increased levels of protein in any of the transformants (Figure 3.6). Interestingly, transcription of *POD6* was negatively affected by either *SM1* over-expression or deletion strains. Future experiments will be performed

where the *Trichoderma* induced plants will be challenged with a foliar pathogen. If the *PR* genes are induced to levels that 'prime' the plant (Conrath et al., 2002), the plant will react more efficiently to the pathogen challenge in *Trichoderma*-induced than non-induced plants.

T. virens Induces Expression of Defense Related Genes in Maize Locally and Systemically

Resistance responses triggered by defense-related signal compounds such as SA, JA or wounding has been described in maize (Morris et al., 1998; Bravo et al., 2003; Zhang et al., 2005). However, very little is known about induction of resistance in maize by beneficial root colonizing microbes (Harman et al., 2004b; Ongena et al., 2004). Moreover, there is only one report describing systemic resistance induced in maize by *Trichoderma* (Harman et al. 2004a). A reduction in disease symptoms was observed on maize plants inoculated with *Colletotrichum graminicola* grown from seeds treated with *T. harzianum* T22. The resistance responses were correlated with increased protein levels and activities of β-1,3-glucanase, exo- and endochitinase in both roots and shoots.

An important if not required prerequisite for the initiation of this interaction is demonstration of rhizosphere competence. *T. virens* was found to colonize maize root system very well as inspected visually since the plants were grown in transparent culture boxes (data not shown). To characterize the *T. virens*-maize interaction at the molecular

level, expression of several defense related genes that were reported to be regulated by major defense signals including SA and JA were examined.

As with tobacco and *Arabidopsis*, maize *PR1* and *PR5* genes can be induced locally by pathogen infection and functional mimics of SA (Morris et al., 1998). However, it is not known whether the maize *PR1* and *PR5* genes can be induced systemically following pathogen infection (Morris et al., 1998). Accumulating evidence suggest that the involvement of PR1 and PR5 proteins in plant defense may be related to their antifungal properties. The PR5 proteins, also referred as thaumatin-like proteins, were found to cause leakage of fungal cytoplasmic material and hyphal rupture, whereas PR1 protein from tobacco and tomato inhibited growth of oomycetes fungi (reviewed in Muthukrishnan et al., 2001). In this study, expression of pathogenesis related genes, *PR1* and *PR5*, was not detected in either *Trichoderma*-treated or non-treated plants. These two genes have not been reported to be induced by beneficial organism suggesting that they are not responsible for *Trichoderma* mediated defense responses in maize. Expression of *PR1* and *PR5* is usually regulated by SA-mediated signal transduction pathways (Morris et al., 1998; Van Loon and Van Strien, 1999). SA is a major signal required for systemic acquired resistance induced by necrotizing pathogens. However, this molecule is proposed to have little or no signaling role during induced systemic resistance (ISR) associated with beneficial organisms. Therefore, the finding that these two proteins are not induced either locally or systemically by *T. virens* supports the hypothesis that SA is not a major player in ISR.

The involvement of JA-mediated pathway in the maize interaction with *Trichoderma* was examined by determining the expression of genes implicated in JA biosynthesis, a 13-lipoxygenase gene *LOX10* (Kolomiets et al., unpublished) and *OPR7* (Zhang et al., 2005). Moreover, these two genes have been shown to be JA- inducible. In agreement with findings of Zhang et al. (2005) and Kolomiets et al. (unpublished), these two genes were expressed in leaves but not roots of seedlings inoculated with *T. virens*. However, expression of *OPR7* and *LOX10* was only slightly induced systemically by the treatments with *Trichoderma* strain over-expressing *SM1* gene or by WT strain, respectively. These data suggest that JA-mediated pathways may be be activated during the interaction of maize with *Trichoderma*. However, given the low level of expression, other putative JA-biosynthetic genes such as allene oxide synthase and allene oxide cyclase need to be tested to provide further evidence for this hypothesis.

PAL is the first enzyme in the phenylpropanoid biosynthesis pathway, which provides precursors for the formation of monolignols/lignin, coumarins, benzoic acids, stilbenes, and flavonoids/isoflavonoids. These phenylpropanoids exhibit broad-spectrum antimicrobial activity and are believed to assist the plant defend against infection (Dixon et al., 2002). PAL has been considered a major enzyme in SA synthesis (Pallas, 1996), although growing evidence indicate that the phenylpropanoid pathway is not the only, or even the most important, route to the biosynthesis of SA (Dixon et al., 2002). Conversely, some studies have reported that synthesis of PAL is activated by the JA/ethylene signaling pathway (Diallinas and Kanellis, 1994; Kato et al., 2000; Shoresh et al., 2005). Expression of *PAL* in maize was found to be upregulated by *T. virens* WT

and OE strain and downregulated by the disrupted strain. The clear pattern of expression in both roots and leaves may indicate that *T. virens* mediates defense responses in maize through this pathway. In addition, this finding supports the hypothesis that ability of *Trichoderma* to communicate with the plant is affected by Sm1 disruption.

Cotton/Maize-*T. virens* Proteomics

Several findings were obtained from profiling extracellular proteins from cotton/maize-*Trichoderma* interaction. A higher number of protein bands were detected in plant-fungal treatments for both hosts than in fungus alone treatment. This is probably the result of a mutualistic effect. These proteins may be signaling molecules involved in early stages of *Trichoderma* plant recognition, as demonstrated by increased levels of Sm1 in cotton/maize-*Trichoderma* interaction. By identifying these proteins and their mode of action, a larger picture of the complex processes of signaling and recognition among plants and microbes will be obtained. Many proteins known as virulence factors are secreted. By a comparison of protein profile between symbiotic plant-microbe interactions to the pathogenic ones, unknown virulence factors may be identified. By the same approach, clues may be found that will help to explain why *Trichoderma* develops symbiotic and not parasitic relation with the plant.

The presence of a serine protease was identified in cotton/mazie-*Trichoderma* interaction. The identification of serine protease corroborate previous report by Viterbo et al. (2004) whom identified two aspartyl proteases in culture filtrates of *T. asperellum*

grown aseptically with cucumber plants. This is consistent with the numerous roles of serine proteases in biological processes, including fungal development (Reichard et al., 2000) pathogenic processes (Kolattukudy et al., 1993; Sreedhar et al., 1999), and biocontrol activity (Flores et al., 1997; Pozo et al., 2004). When proteins obtained from growth medium of the cotton/*T.virens* WT or mazie/*T. virens* WT were compared, at least one highly produced protein was found to be specific for each plant-fungal system. These proteins may be host determinants that are important in plant-microbe recognition.

In both biological systems, using two different techniques for obtaining protein extracts, most proteins were found to be present in all treatments but in different amounts. For example in the cotton-*Trichoderma* system the most prominent band was ~ 37 kDa in WT treatment, and the ~30 kDa band in OE treatment. In maize, a similar patter of proteins was obtained when the proteins where concentrated, but precipitated proteins yielded a slightly different pattern between transformants and the wild-type. This discrepancy is most likely the result of the difference in obtaining protein extracts by two techniques. The general observation that there was no drastic difference in protein profile among treatments may be due to the low resolution of 1-D gel. Protein synthesis is a complex, dynamic process of constant production and turnover. Sampling earlier in the interaction and over more frequent intervals would result in a larger, more complete picture.

In summary, *T. virens* mediated responses in two hosts, monocot (maize) and dicot (cotton), were characterized for the first time at the molecular level. The evidence was provided that the expression of defense related genes was induced in foliar tissue by

application of *Trichoderma* to the roots. These data strongly suggest that rhizosphere competent *Trichoderma* spp. play a significant role in protection against a broad range of pathogens through the induction of non-specific resistance. Additionally we identified some gene-markers of resistance that may be crucial for the each plant-fungal system. Gene expression and proteomics studies with strains disrupted in *SM1* generally confirmed the importance of Sm1 as an elicitor in defence related responses. However further analysis by challenging 'primed' plants with a foliar pathogen are necessary to demonstrate the biological relevance. Targeted gene disruption will provide opportunity to determine the role Sm1 has in fungal metabolism and ecology. Evidence that Sm1 is an effective elicitor secreted by *T. virens* (Chapter II and III) affords new opportunities to investigate alternative approaches for biocontrol. The generation of transgenic plants expressing *SM1* or combining two different mechanisms of protection by constructing *Trichoderma* strains that constitutively over-express *SM1* and cell wall degrading enzymes presents new applications in biological control.

METHODS

Fungal and Plant Materials

Two strains of *Trichoderma virens* were used in this study, wild-type strain, Gv29-8, and an arginine auxotrophic strain, Tv10.4, as the recipient for fungal transformation (Baek and Kenerley, 1998). The strains were routinely maintained on potato dextrose agar (PDA, Difco). For screening of transformants, Vogel's minimal medium (Vogel, 1956) supplemented with 1.5 % sucrose (VMS) and PDA were used.

Cotton (*G. hirsutum* Paymaster 2326 BG/RR) and maize (*Zea mays* inbred line B73) seedlings used in this study were grown in a hydroponics system (see Chapter II). Cotton and maize were kindly provided by Dr. Terry Wheeler (Texas Agricultural Experimental Station, Lubbock, TX), and Dr. Mike Kolomiets (Dept. of Plant Pathology and Microbiology, Texas A&M University), respectively.

DNA Manipulation

T. virens genomic DNA was isolated as previously described (Xu et al., 1996). Southern hybridization analyses were carried out according to Sambrook et al. (1989). Probes were random labeled using Random Primer DNA Labeling Kit (Takara, Madison, WI) and hybridizations were performed overnight at 42 ^{o}C with Ultrahyb as hybridization buffer (Ambion, Austin, TX). QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was

used for plasmid DNA purification. Nucleotide sequencing was performed by a primer-walking strategy (Sambrook et al., 1989). All sequencing reactions were performed at the Gene Technologies Laboratory (Texas A&M University). DNA sequences were analyzed by DNA Strider 1.2 (Marck, 1988), and Sequencher 4.1 (GCC, Ann Arbor, MI).

Construction of *SM1* Disruption- and Over-expression Vectors

Probing a *T. virens* bacterial artificial chromosomal (BAC) library (Grzegorski, 2001) with a purified 264 bp PCR product of the *SM1* gene (as described in chapter II), yielded 33 positive BAC clones. One of the positive clones (E4) was further digested with several restriction enzymes, and the fragments subcloned into pBluescript II SK (+/-) vector to obtain the following vectors: a *Sac*I subclone (pSZD14), a *Hind*III subclone (pSZD15), and a *Cla*I subclone (pSZD21).

The *SM1* disruption vector (pSZD25) was constructed by replacing the 483 bp ORF (open reading frame) with a 3.0 kb *SmaI/EcoRV* fragment of the *T. virens ARG2* gene (Baek and Kenerley, 1988), which served as a selectable marker. The resulting vector contained 2.1 kb and 1.7 kb fragments from pSZD21 flanking the selectable *ARG2* gene. The 5.6 kb *Pst*I/*Xba*I deletion cassette was isolated from pSZD25 and used for fungal transformation. An over-expression vector (pSZD26) was constructed for constitutive over-production of the *SM1* gene by placing a 1 kb *Hind*III insert from pSDZ15 subclone containing *SM1* ORF between the promoter and the terminator regions of the *T.*

virens GPD (glyceraldehydes-3-phosphate dehydrogenase) gene (Xu et al., 1996). To obtain over-expression strains, Tv10.4 was co-transformed with pSZD26 and pJMB4 which contains *ARG2* gene as a selectable marker.

Transformation and Screening of Transformants

Stable prototrophic transformants were selected by consecutive transfer of single colonies to VMS, PDA, and VMS. Screening of potential deletion and over-expression transformants was first performed by PCR. For deletion transformants, *SM1* specific primers, forward, 5'- GTCTCCTACGACACCGGCTA-3' (SmF), and reverse 5'-GTCGAGCGCAATGTTGAA-3' (SmR), were used to amplify the 264 bp PCR product from the wild-type genomic DNA. PCR amplification of *SM1* fragment comprised of 28 cycles (each cycle: 30sec at 94 ^{o}C, 20sec at 55 ^{o}C, and 20sec at 72 ^{o}C). Strains that yielded no PCR product were further screened by Southern analysis. For over-expression strains, forward (SmF) and reverse (R50) 5'- TACAGACAATGATTCATG-3' primers were designed to amplify a 1 kb fragment from the SM1 ORF and a *GPD* terminator region from the over-expression cassette. Strains that yielded the 1 kb PCR product were selected for further analysis by Southern blotting. PCR amplification comprised of 28 cycles (each cycle: 30sec at 94 ^{o}C, 20sec at 40 ^{o}C, and 30sec at 72 ^{o}C). PCR was performed using an Invitrogen *Taq*DNA Polymerase kit (Carlsbad, CA).

Northern Analysis of Transformants

To confirm that the over-expression transformants were functional, i.e. showing elevated levels of transcription compared to the wild-type, and that the deletion transformants were missing the *SM1* transcripts, Northern analyses were performed.

Conidia of 7-day-old *T. virens* wt and transformants cultured on PDA was used to inoculate VMS (for deletion transformants) or GYEC liquid media (Thomas and Kenerley, 1989) to a final concentration of 10^6 spores /ml. All cultures were incubated on an orbital shaker (130 rpm) at room temperature. After five and three days of growth for deletion and over-expression strains, respectively, the mycelia were harvested and rinsed thoroughly with sterile water. Total RNA was extracted from the harvested mycelia following the protocol of Jones et al, 1985. 15µg for deletion stains and 10 µg for over-expression strains of total RNA per sample was denaturated, resolved in 1.5 % agarose formaldehyde gel, transferred to a Hydrobond-N^+ nylon membrane (Amersham Biosciences, UK), and hybridized overnight at 42 ^oC using Ultrahyb (Ambion). The 264 bp (SmF-SmR) PCR product was used as *SM1* probe. A 600 bp actin fragment amplified from Gv29-8 cDNA [primers, forward: 5'- AAGAAGTTGCTGCCCTCGT- 3' (ActF) and reverse: 5'- GCTCAGCCAGGATCTTCATCATC- 3' (ActR)] was used as control for even loading.

SDS-PAGE and Western Analysis of Transformants

Fungal culture filtrates were obtained by inoculating 200 ml of VMS with a conidial suspension of the appropriate fungal strain to a final concentration of 10^6/mL conidia. Following incubation on a rotary shaker at 130 rpm for 5 days at 23 ^oC, culture filtrates by filtration through a 10 μm NITEX nylon cloth (TETKO Inc., Depew, NY).

Proteins in the culture filtrates were precipitated by 80% ammonium sulfate (Fisher Sci., Hampton, NH). The pellet was resuspended in 10 mM ammonium bicarbonate (NH_4HCO_3) (ICN Biomedicals), dialyzed against the same buffer (10-kDA mwco, Pierce, Rockford, IL), and successively filtered through a 0.45 μm filter (Fisher Sci., Hampton, NH). The dialyzed fraction was concentrated by lyophilization, and the resulting dry protein pellet was stored at -20^oC. For protein analysis, pellets were resuspended in small amounts of 10 mM Tris, pH=7.8 and concentrations were determined by Bio-Rad Bradford microassay using BSA as a standard. When purified SM1 was used as a positive control, the cconcentration of the protein was determined based on molar absorption coefficient (Pace et. al., 1995). Protein extracts were subjected to SDS-PAGE following silver (Blum et al., 1987) or Coomassie brilliant blue R-250 staining for protein visualization. Prestained SDS-PAGE broad range molecular weight (MW) standards (Bio-Rad, CA) or Kaleidoscope polypeptide molecular weight standards (Bio-Rad, CA) were used for molecular mass determination.

Protein extracts obtained as described above were compared among the wild-type, disruption and over-expression strains. Proteins produced by deletion strains were

quantified by Bradford microassay, electrophoresed on SDS-PAGE gel following Coomassie or silver staining or electroblotted to a nitrocellulose membrane (Osmonics Inc., Gloucester, MA) in a standard Western blot procedure (as described in Chapter II). To compare the production of SM1 in wild-type and over-expression strains, equal volumes of concentrated samples equivalent to the starting volume (200 mL) were loaded per lane or the samples were quantified by Bradford and equal amounts loaded per well.

Growth Assays of Transformants

Cultures of selected transformants were compared with the wild-type strain for radial growth. Agar plugs from actively growing colonies were inoculated in the center of VMS, PDA, or WA (water agar) plates, and the hyphal extension was recorded for three days of growth at 27C. After each day, growth area was marked with the red permanent marker. The photograph of the plate with the dark background was taken from the top from the constant distance between the plate and the camera, and pictures were saved in JPEG format. For extraction of surface growth areas for each day from the images, the Matlab code capable of independently processing large number of consecutively numbered images was developed. Each treatment contained four repetitions and each experiment was repeated at least twice. Data were analyzed by analysis of variance (ANOVA) and Fisher's PLSD test ($P<0.05$) ((Statview v 5.0.1, SAS Institute, Cary, NC).

Plant-Fungal Co-Culture Filtrates from a Hydroponic Growth System

A hydroponic system (previously described in Chapter II) was used to evaluate the resistance response of dicot-cotton and monocot-maize seedlings when inoculated with *T. virens* wt, *sm1* deletion (SKO25) or over-expression (SOE38) strains. Cotton and maize seeds were surface sterilized according to Dowd et al. (2004) with the percentage of hydrogen peroxide was increased to 10% for maize seeds.

The mycelial inoculum of *T. virens* Gv29-8 and transformants, prepared as previously described (Chapter II), was aseptically added into 300 mL of MS medium containing either 6-day-old cotton seedlings or 4-day-old maize seedlings. For *Trichoderma* growing in the absence of cotton/maize seedlings, MS, supplemented with 0.05% sucrose (300 ml medium) was inoculated with the same mycelial biomass. Control plants were grown in 300 mL MS medium without *Trichoderma*. In treatments of control plants and plants inoculated with the fungus, on a day of inoculation, the growth medium was replaced with fresh MS to reduce the extracellular proteins accumulated during plant growth. Two days later, media from all treatments were collected, filtered through a 10 μm NITEX nylon cloth, and successively filtered through a 0.45 μm filter (Fisher Sci., Hampton, NH). Then, the culture filtrates were treated with a protease inhibitor cocktail (0.05 % v/v, Sigma, St. Louis, MO) and allowed to incubate at room temperature for 20 minutes (Grinyer et al., 2005).

Expression Analysis of Cotton and Maize Defense-Related Genes

Expression of defense related genes was analyzed in cotton (*G. hirsutum* cv Paymaster) and maize (*Zea mays* inbred line B73) seedlings grown hydroponicaly with or without *T. virens* wt, over-expression or deletion strains. (see **Plant-Fungal Co-Culture Filtrates from a Hydroponic Growth System**). Cotton cotyledon and maize root and leaf tissue were harvested from the hydroponic system after 48h of incubation. All harvested samples were immediately frozen in liquid nitrogen. Total RNA from cotton was extracted by the method of Wang et al. (2000), and from maize using TRI reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocols.

Expression of the following defense related genes of maize was examined: *PR1* and *PR5* (pathogenesis-related genes), *PAL* (phenylalanine ammonia-lyase), *LOX10* (lipoxygenase 10) and *OPR7* (12-oxo-phytodienoic acid reductase 7). The maize glycerol phosphate dehydrogenase, cytosolic form (*GAP C*) (Farag et al., 2005), was used to insure equal loading of the lanes. The primers for amplification of *PR1* and *PR5* were designed (Kolomiets and Gao, unpublished) based on available elicitor-induced maize gene sequences from the GeneBank database (Morris et al., 1998). The gene-specific primers for amplification of *LOX10* were designed (Nemchenko and Kolomiets, unpublished). This gene was selected for our analysis because it is the closest maize homologue of a bean lipoxygenase gene that was reported to be associated with systemic resistance induced in by a nonpathogenic *Pseudomonas* strain (Ongena et al., 2004).

Primer sequences for *PAL* and *OPR7* were obtained from reports of Farag et al. (2005) and Zhang et al. (2005), respectively. The sequences of primer pairs described above are presented in Table 2.1.

RT-PCR analyses were performed as previously described (Chapter II). PCR amplification of *PR1*, *PR5*, *PAL*, *LOX10*, *OPR7*, and *GAP C* fragments comprised of 25 cycles (each cycle: 30sec at 94 ^{o}C, 30sec at 58 ^{o}C, and 40sec at 72 ^{o}C) for both root and leaf RNA and 23 cycles for *PAL* root RNA. The positive control for RT-PCR was DNase- treated and cleaned RNA reaction without reverse transcription. PCR products were electrophoresed on agarose gels and band intensities compared within each experiment after ethidium bromide staining.

Extracellular Proteins from Plant-Fungal Co-Culture Filtrates from the Hydroponic Growth System

To determine proteins unique for cotton/maize-*T.virens* wt interaction, two controls were included: *T. virens* wt growing in hydroponic growth medium (supplemented with 0.05% sucrose) in the absence of seedlings; and control plants growing in growth medium without *T. virens* wt. Proteins extracts from these treatments were obtained by 95 % ammonium sulfate precipitation as described above.

Proteins secreted into the hydroponic growth medium when *T. virens* wt, *sm1* deletion (SKO25) or over-expression (SOE38) strains were growing with the cotton or maize seedlings were obtained by either 95 % ammonium sulfate precipitation or concentration

using 10 kDa cutoff Millipore Amicon Ultra centrifugal filter devices (Bedford, MA). Protein extracts were analyzed by SDS-PAGE followed by silver staining, for sensitivity, and Coomassie staining, for representation of relative amounts of proteins. Western analyses (as described in Chapter II) were conducted for detection of Sm1 and a *T. virens* serine protease (Pozo et al., 2004) from the culture filtrates mentioned above when the fungus was growing with or without cotton or maize seedlings.

Isolation and Identification of Secreted Proteins from the Hydroponic System

The proteins were electrophoretically separated on 10% or 15% SDS-PAGE gels, electroblotted to a PVDF (polyvinylidene difluoride) membrane, and Coomassie stained. Selected bands were excised from membranes or gels and prepared for N-terminal sequencing and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF). N-terminal sequencing of the selected proteins was performed by automated Edman chemistry on a Hewlett Packard G1005A Protein Sequencher (Protein Chemistry Laboratory, Texas A&M University). MALDI-TOF MS and MS/MS was performed on an Applied Biosystem 4700 mass spectrometer at Laboratory for Biological Mass Spectrometry, Texas A&M University. The samples for MALDI-TOF MS/MS were prepared by in gel digestion following the procedure of Stone and Williams, 1993 (Protein Chemistry Laboratory, Texas A&M University). The peptide mass fingerprints were searched against proteins from all fungal species using the Mascot peptide mass fingerprint and also MS/MS data was analyzed manually

(http://www.matrixscience.com/search_form_select.html). Data search was carried out with the BLAST program (http://www.ncbi.nim.nih.gov/BLAST/) against nonredundant databases and with MPsrch at the European Bioinformatics Institute (http://www.ebi.ac.uk/MPsrch/).

CHAPTER IV

FUNCTIONAL CHARACTERIZATION OF A BETA-1,6-GLUCANASE FROM *TRICHODERMA VIRENS*

INTRODUCTION

Filamentous fungi of the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. *Trichoderma* spp. can directly impact other fungi in the following maner: after sensing a suitable fungal host, *Trichoderma* responds with the production of antibiotic compounds, formation of specialized structures, and secretion of cell wall degrading enzymes followed by the assimilation of its host cellular content, a process known as mycoparasitism (Chet and Chernin, 2002; Steyaert et al., 2003; Benitez et al., 2004). The mycoparasitic activity of *Trichoderma* spp. against phytopathogenic fungi and oomycetes due to lytic activity of cell wall degrading enzymes (CWDE) has been widely studied (Baek et al., 1998; Pozo et al., 2004; Sanz et al., 2005; Fravel, 2005).

Chitinases and β-1,3-glucanase are considered the major enzymes responsible for the degradation of cell walls of filamentous fungi, as chitin and β-1,3-glucan are main fungal structural components (Bartnicki-Garcia, 1968; Papavizas, 1985). However, the cell wall of plant pathogenic oomycetes, such as *Pythium ultimum*, is composed of mainly β-1,3- and β-1,6-glucans and cellulose, instead of chitin (Bartnicki-Garcia, 1968). Furthermore, less abundant structural components, such as β-1,6-glucan, have been described in

budding yeast as the link between cell wall proteins and the main β-1,3-glucan/chitin polysaccharide (Kapteyn et al., 1996, 1997; Magnelli et al., 2002). Thus, hydrolytic enzymes, such as β-1,6-glucanase, may contribute to the efficient disorganization and further degradation of the host cell walls by mycoparasitism. In fact, β-1,6-glucanase has been described as lytic against yeast and fungal cell walls in filamentous fungi (Shibata and Fukimbar, 1973; Yamamoto et al., 1974; Lora et al., 1995; de la Cruz et al., 1995).

Even though β-1,6-glucanases have been purified from several filamentous fungi, including *Penicillium brefeldianum* (Schep at al., 1984), *Acermonium* sp. (Pitson et al., 1996; Jayus et al., 2001), *Neurospora crassa* (Oyama et al., 2002) and *Trichoderma harzianum* (Lora et al., 1995; de la Cruz et al., 1995; Soler et al., 1999; de la Cruz and Llobell, 1999; Montero et al., 2005) and their biochemical and lytic properties studied, the physiological function has not been conclusively established in any species. Only recently evidence has been provided indicating role for a β-1,6-glucanase in the mycoparasitic interaction between the parasite, *Verticillium fungicola*, and its host, *Agaricus bisporus* (Amey et al., 2003). In this process the parasite penetrates cell walls of its host by combined effect of hydrolytic enzymes and mechanical pressure, allowing infection to develop (Calonje et al., 1997, 2000; Dragt et al., 1996). The importance of β-1,6-glucanase in this process was demonstrated as the strain *V. fungicola* disrupted in the corresponding gene, *Glu1*, produced significantly reduced lesion sizes on its host and the mutant had reduced capacity to utilize chitin compared to the other isolates (Amey et al., 2003). However, the observation that complete reduction in virulence did not occur

suggests that successful cell wall degradation is likely to be achieved by the activity of multiple enzymes (Calonje et al, 1997; Amey et al., 2003).

Due to the complexity of fungal cell walls, multiple hydrolytic enzymes may be required for successful wall degradation. Synergistic effect of a β-1,6-glucanase with β-1,3-glucanase or chitinase from *Trichoderma* in the attack and effective digestion of the pathogen cell wall has been demonstrated (de la Cruz et al., 1995). A mutant of *T. harzianum* CECT 2413 with increased activity for chitinase, β-1,3- and β-1,6-glucanases overgrew, sporulated on and parasitized colonies of *Rhizoctonia solani* faster and exerted better protection on grapes against *Botrytis cinerea* than the wild type (Rey et al., 2001). Furthermore, a strategy to improve biological agents by constitutive over-expression of chitinase, glucanase, or protease has proven to be very effective (Flores et al., 1997; Limon et al., 1999; Baek et al., 1999; Pozo et al., 2004; Ahman et al., 2002). A similar strategy has been used successfully to improve plant resistance to pathogens by engineering a range of plant species to over-express cell wall degrading enzymes from *Trichoderma* spp. (Bolar et al., 2000, 2001; Mora et al., 2001; Lorito et al., 2001; Emani et al., 2003).

Several mechanisms have been described to explain the capability of the biocontrol agent *T. virens* to protect plants from pathogen proliferation and infection (Howell, 2003; Harman et al., 2004b). However, the success of a biocontrol agent is most likely the result of a combination of these mechanisms. *T. virens* is unique among mycoparasitic fungi as it produces metabolites with known antimicrobial activities including peptaibols, gliovirin and gliotoxin that have been shown to act synergistically

with lytic enzymes (Howell et al., 1993; Wiest et al., 2002). Additionally, the increased levels of bioprotection were demonstrated by genetically modified strains of *T. virens* strains constitutively over-expressing genes encoding for lytic enzymes, such as chitinase or protease (Baek et al., 1999; Pozo et al., 2004).

Previously in our lab, *TV-BGN3*, a gene encoding an β-1,6-glucanase from *T. virens* was cloned and its homology to glycosyl hydrolase family 5 reported (Kim et al., 2002). To elucidate the role this enzyme may have in biocontrol activity of *T. virens*, gene expression analysis were conducted, and a series of transformants in which the *TV-BGN3* gene was disrupted or constitutively over-expressed were constructed. In addition, double over-expression transformants were generated, constitutively over-expressing *TV-BGN3* and *TV-BGN2*, a gene encoding *T. virens* β-1,3-glucanase 2 demonstrated to be involved in mycoparasitism (Pozo, Djonovic and Kenerely, to be published elsewhere). Our results reveal induction of *TV-BGN3* in the presence of fungal cell walls and demonstrate enhanced ability of single or double over-expression transformants to inhibit fungal growth.

RESULTS

Isolation of *TV-BGN3* from a BAC Library and Nucleotide Sequence Analysis

A clone with high molecular weight insert containing the entire *TV-BGN3* open reading frame (ORF) was obtained from a *T. virens* bacterial artificial chromosomal (BAC) library (Grzegorski, 2001). Fourteen clones displayed strong hybridization signals after probing with 1.35 kb ORF of the β-1,6-glucanase cDNA. Sequencing the clone, 4L19, revealed the presence of entire *TV-BGN3* ORF. The 3.9 kb region was obtained including the 1.35 kb of coding region, 1.91 kb of 5' flanking sequence and 0.64 kb of 3' flanking sequence.

The DNA sequence, shown in Figure 4.1, includes 740 bp of 5'flanking region, an open reading frame of 1348 bp, and 636 bp of 3'flanking region. The *TV-BGN3* ORF, interrupted by a single intron, encodes a 429 amino acid polypeptide with a predicted molecular mass of 48.1 kDa, and a pI of 5.56 (Kim et al., 2002). Analysis of the 5' region of *TV-BGN3* revealed putative TATA boxes at positions -16, -91, and -540, and putative CAAT boxes at -23, -104,-114 and -445 upstream of the ATG codon. Additionally, several putative regulatory motifs in the promoter region of *TV-BGN3*, previously reported in other fungal genes, including *Trichoderma*, were identified (Figure 4.2). An *AceI* (5' AGGCA 3') motif, involved in the induction of cellobiohydrolase genes in response to cellulose (Saloheimo et al., 2000; Aro et al., 2001) was found at position -681.

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-740  GTTCGGAGAACAAATTCAGTCGCGGCAACATTCCTGGCAGGGGTAAAATAGCGGTAGGTAGGCAGTGCCA
-670  CAACCCCTTTTTAGATTAGTAGTGCAGAATACTGCAAGCTTTAAAGACGCCAGATGTGATGGCTGTACGG
-600  AGTACTCCACAAACCTCCGACAGCCCTCGATATTCCCGATCCCGGGCCTTATTAAGTTAATATAGTACGG
-530  AGTATGCCTTGTCTATTTATTGACAGCTTGTTGATTACCATGCGTCAGCGAGTGCTAAGATTGGCATCCC
-460  CCCTTGGGTGGAAGCTAGCACCTCTAGCGGGCACATAACGCATGCGTGTGGGCAATGGTGCCGAATTGAA
-390  GGACAGAAATCCACTCCAATTCCAAGCTATTTGTTACAGTAAGCTGAATCAGATTCTTATTCCGGCTAGC
-320  ACGGTTCTAACAAGTTGCGAATTAATAGGGATGCGACACTAATCGGGCCGGCGTCTTGTTTTTGTACAGG
-250  TGCGAGAGCCAGCCGGGCTTAATGAAATACCACATCATACCAAGCAAACAACATACGTAAACCAGGTACT
-180  AGGCTATCATTAATTCTCCTCGTAGCGCGTACTCCAAACGTTTACCAGGAAACAGCTGAAGCTTGTCAAT
-110  AAGGCTCAATTTAAGTCTGTATATAAGTCTGTATGGTTCCTGACAACTGAATTGAAGTTTTCCTTATCAT
 -40  CACCGACAACTCTTGGACAATTTCTATAGCTACCATCAAGATGCGATACTCCATCGTTGCCCCGGCTATT
                                          M  R  Y  S  I  V  A  P  A  I

  31  CTCGCCGGCACCGCCTTTGCGTGGCTTCCTCAAGACCGCGACCTGGCAGCCTTCAACCAGACCGCTCGT
  11  L  A  G  T  A  F  A  W  L  P  Q  D  R  D  L  A  A  F  N  Q  T  A  R

 100  TTTGAGCAGCTTGGCAAGCGTTTTGAGCCTTCACTTGCGTCTGGTATCACCAAGATCCGTGGTGTCAAC
  34  F  E  Q  L  G  K  R  F  E  P  S  L  A  S  G  I  T  K  I  R  G  V  N
                           ----------------------

 169  TTCGGTGgtaagtgaatatcctattacttcctgaagacgaagaaagccagcctcacacattgacatagG
  57  F  G  G

 238  ATGGCTTATTTGCGAACCATGGATGATGTCTAATGAGTGGAACAATGTTATGGGTTGCAATGGTGCTGC
  60   W  L  I  C  E  P  W  M  M  S  N  E  W  N  N  V  M  G  C  N  G  A  A

 308  ATCTGAGTTCGACTGCATGCTTCAACATTATATGGGCAACAATCGTGCGCTCGGAAACCAGAAGTTCCA
  83   S  E  F  D  C  M  L  Q  H  Y  M  G  N  N  R  A  L  G  N  Q  K  F  Q

 376  GAACCACTGGAGAGACTGGATCAACCCCGCCACCGTTCAGTCCGTTCATGATGTCGGCTTGAACACCAT
 106   N  H  W  R  D  W  I  N  P  A  T  V  Q  S  V  H  D  V  G  L  N  T  I

 445  CCGTATTCCCATCGGCTACTGGTCCTACACAGCCATTGTCGACACAGCGAGCGAGCCATTTGCCGATGG
 129   R  I  P  I  G  Y  W  S  Y  T  A  I  V  D  T  A  S  E  P  F  A  D  G

 514  CAACCTCATGCTCCCGTACCTTGACGCGGTTGTCCAGAAGGCTGCTGATCTCGGGATCTACGTCATCAT
 152   N  L  M  L  P  Y  L  D  A  V  V  Q  K  A  A  D  L  G  I  Y  V  I  I

 583  TGATCTCCATGGCGCTCCTGGTGGTCAGCAACAGGATGTCTTCACTGGCCAGAACCCCAAGCCCGCGGG
 175   D  L  H  G  A  P  G  G  Q  Q  Q  D  V  F  T  G  Q  N  P  K  P  A  G
```

Figure 4.1. Nucleotide and Deduced Amino Acid Sequences of Tv-bgn3.

The predicted amino acid sequence of Tv-bgn3 is shown below the nucleotide sequence. The dashed underlined peptide sequence corresponds to that obtained by N-terminal sequencing of the protein. The *TV-BGN3* open reading frame starts at position 1 and ends at 1348. The intron sequence (at positions 176-236) is marked in lower case. The consensus sequences for 5' and 3' intron splicing sites are marked in bold. The signal sequence cleavage site and the proenzyme cleavage site are indicated by dashed and solid arrows, respectively. Sites of putative regulatory motifs correspond to the numbers indicated in Results.

```
 652   TTTCTATAACTCATACGACTATGGCCGTGCCGAGAAGTGGTTGTCTTGGATGACAAACCGTATCCATAC
 198    F  Y  N  S  Y  D  Y  G  R  A  E  K  W  L  S  W  M  T  N  R  I  H  T

 721   TAACCCTGCATACAAAACTGTTGGTATGATTGAGGTTCTCAACGAGCCTGTCTCTAGACACGACGGGGG
 222    N  P  A  Y  K  T  V  G  M  I  E  V  L  N  E  P  V  S  R  H  D  G  G

 790   TGGTCGCTACCCTGCTCCTGGCCAAGACCCAAGTCTAGTGCAAACCTACTACCCAGGCGCTCTCAAGGC
 244    G  R  Y  P  A  P  G  Q  D  P  S  L  V  Q  T  Y  Y  P  G  A  L  K  A

 859   TGTCCGTGATGCTGAGGCTGCGCTGAATGTTCCCAGCAACAAGAAGCTGCACGTACAATTCATGTCCAG
 267    V  R  D  A  E  A  A  L  N  V  P  S  N  K  K  L  H  V  Q  F  M  S  S

 928   CAAGTGGGATTCTGGTGATGCTCGCACCAACGCAGCAGTCGCAAACGACCCCATGACTGGATTCGATGA
 290    K  W  D  S  G  D  A  R  T  N  A  A  V  A  N  D  P  M  T  G  F  D  D

 997   TCACAACTATATTGGTTTTGCTCTCATTAACACCGGTGATCAATACTCGGTGATGCACAGTGCATGCAC
 313    H  N  Y  I  G  F  A  L  I  N  T  G  D  Q  Y  S  V  M  H  S  A  C  T

1066   TGACTCAAGAGTTGTGAACGGCCAGAACTTCGCCATTACAGGCGAGTGGAGCATGACTTCTGGTGCTGA
 336    D  S  R  V  V  N  G  Q  N  F  A  I  T  G  E  W  S  M  T  S  G  A  D

1135   TTGGCACGACGCAAACTTCTTCAAGAAATTCTTTACAGCTCAGCAGCAGTTGTATGAGTCTCCTGGAAT
 359    W  H  D  A  N  F  F  K  K  F  F  T  A  Q  Q  Q  L  Y  E  S  P  G  M

1204   GGACGGATGGATCTACTGGACCTGGAAGACCGAGTTGAATGACCCTCGATGGACCTACTCCTATGCTAC
 382    D  G  W  I  Y  W  T  W  K  T  E  L  N  D  P  R  W  T  Y  S  Y  A  T

1273   CTACCTCAACTACATCCCAACGAACGCCGCTGCCCTGCAGCAGAACGTTTACCAGGATGTTTGCTCCGG
 405    Y  L  N  Y  I  P  T  N  A  A  A  L  Q  Q  N  V  Y  Q  D  V  C  S  G

1342   ATATAGGTAAAGCGTGAGCCATTGCTCACAAGGCGTTACCACTCAATTATCACTTCAAACTTGTATATT
 428    Y  R  *
1411   TTTTCATAGGACATATGTTTTTTTTTTTTTTTTTTGGCCTTGACACTCCCTGGTCAACTTAGTTTACAA
1480   ACTATGTGTCAATATCTCGCATTTTGGCGTCATACCACAAACGAGTGTTCAAGCTTACAAATTAAATGA
1549   TTAAGGTAAGGTTGACTCTAGGAGCTTCAAAGTAGTGCACTGAATGATACGCAACGGCCATGAATTCCA
1618   TATATTTGCTGAACACAATCAATTTCCCGGGGGGGGGGGGGGGTGGCGCCAGTGAGGTGGTGTCAAATA
1687   TTGCAATCTTGCTAACCTTTGCGCCTATGTATTGCACGGCATCTTAAGGCGAGTGCCATGTTTGAGCAA
1756   GCCCAAAACGAGCATTCTCAATATCCCAAACTGTATAATAATACTTCAGTAAGCTGCCACCAACTACCC
1825   ACAAGCTTTTCTCCTGATCGCCACCGCTACTGTTGATAAGCATCTGGCAGATCCCCGGCTGGCCTGAAA
1894   TTGTCCCAACATTGAGCTCTTGGGGAGGAATTATATACTTGTGGCCTCCAATCATGAAAGTAATTGATG
1963   ATTTGATATGTTCTAGTTTCGAACA
```

Figure 4.1. Continued.

Two stress-response sites, STRE (CCCCT) (Marchler et al., 1993; Cortes et al., 1998) were identified at -461 and -667. Some of the mycoparasitism response elements (MYRE4-MYRE1) described for *ech42* and *prb1* of *T. atroviride* (Cortes et al., 1998), and newly identified MYRE5 motif (CP4-CP1, CX3-CX1, or PX2-PX1) in the promoter region of *T. hamatum chit42*, *prb1*, and *xbg1.3-110* genes (Steyaert at al., 2004) were also found in *TV-BGN3* promoter. These include: MYRE3 (CGGCAC) at -502, PX2 (ATTTAAG) at -102 and CP2 (AACGTT) at -144. Interestingly, no CreA-binding motifs (5' SYGGRG 3') involved in catabolite repression in *A. nidulans* (Sophianopoulou et al., 1993; Kulmburg et al., 1993) or a HGATAR site, a putative binding consensus for nitrogen regulator such as AreA in *A. nidulans* (Ravagnani et al., 1997) were found in the *TV-BGN3* promoter region.

TV-BGN3 copy number was determined by Southern analysis of *T. virens* 29-8 genomic DNA digested with the restriction enzymes: *Hin*dIII, *Eco*RI, *Eco*RV, *Xba*I, *Bam*HI, *Apa*I, *Xho*I, *Sal*I, *Cla*I, *Spe*I, *Sac*I, *Kpn*I, *Bsph*I, *Pst*I, *Sma*I, *Not*I, and *Mlu*I. When the DNA was digested with restriction enzymes for which there are no sites in the glucanase genomic DNA (*Hin*dIII, *Eco*RI, *Eco*RV, *Bam*HI, *Apa*I, *Xho*I, *Cla*I, *Spe*I, *Sac*I, *Kpn*I, *Bsph*I, , *Sma*I, *Not*I, and *Mlu*I), only single hybridization bands were detected (Figure 4.2). When the DNA was digested with enzymes that have single restriction sites within the glucanase genomic DNA (*Xba*I, *Sal*I, and *Pst*I), two hybridization bands were seen as the entire *TV-BGN3* ORF was used as probe. These data indicate that *TV-BGN3* is a single copy gene in *T. virens* genome.

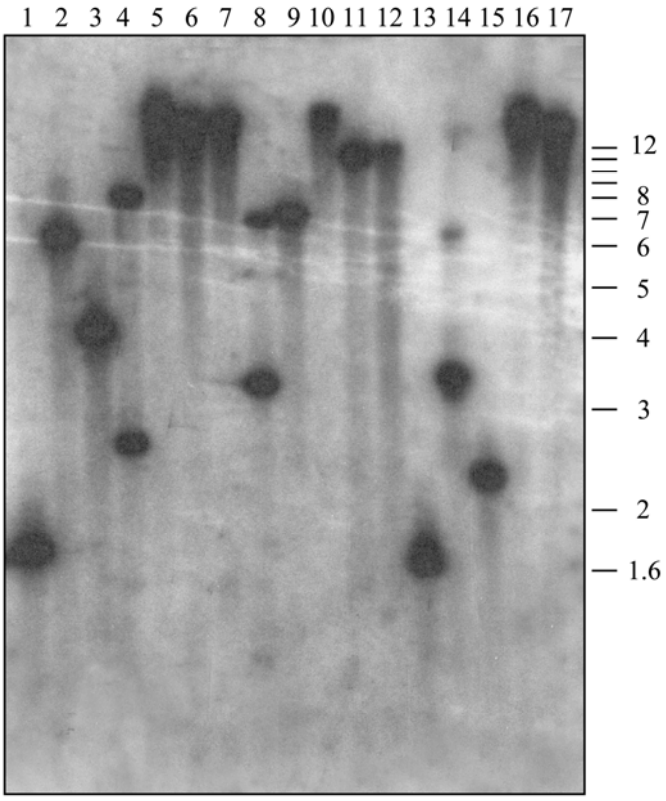

Figure 4.2. Southern Analysis of *TV-BGN3* in the *T. virens* Gv29-8 Genome.

Lanes 1-17, *T. virens* Gv29-8 genomic DNA (20 µg) digested with restriction enzymes *Hin*dIII, *Eco*RI, *Eco*RV, *Xba*I, *Bam*HI, *Apa*I, *Xho*I, *Sal*I, *Cla*I, *Spe*I, *Sac*I, *Kpn*I, *Bsph*I, *Pst*I, *Sma*I, *Not*I, *Mlu*I, respectively, and fractionated on a 1% agarose gel. The 1.35 kb PCR product of the β-1,6-glucanase cDNA clone was used as a probe. Size markers in kilobase pairs (kb) are indicated on the right.

Similarity of the *T. virens* β-1,6-Glucanase to Other Sequences

Tv-bgn3 shares high similarity to β-1,6-glucanases of different fungi including the biocontrol agent *T. harzianum* (92% identity)(Lora et al., 1995), grass endophytes *Neotyphodium* sp. (75% identity) (Moy et al., 2002) and *Acremonium sp*. (74% identity), and the fungal pathogen *Verticillium fungicola* (67% identity)(Amey at al., 2003)(Figure 4.3). Additionally, there is a high similarity of Tv-bgn3 to a hypothetical protein (XM_388441) from the cereal pathogen *Gibberella zeae* (identical 75%) (Figure 4.3). Even though *T. virens* Tv-bgn3 is homologous to β-1,6-glucanases from other fungi, its relationship to glycosyl hydrolase family 5, including the predicted catalytic residues (Mackenzie et al., 1997; Cutfield et al., 1999), was recognized previously (Lora et al., 1995; Kim et al., 2002) (indicated by asterisk, Figure 4.3). N-terminal amino acid sequence analysis using SignalP and the hydropathy plot calculated by the method of Kyte and Doolittle (1982) indicate that a putative cleavage site between the signal peptide and the mature, secreted protein is located between residues 17 and 18. The mature protein however starts at amino acid 41 as determined by N-terminal sequencing, indicating additional possessing of the preprotein (Figure 4.1). This is in agreement with the presence of the KR sequence at positions 39-40, which has been described in other proteins as a target sequence for a specific protease, Kex2 (Geremia et al., 1993; Lora et al., 1995). Thus, the mature protein contains 389 amino acids, a predicted molecular mass of 43.6 kDa and a pI of 5.31. This correlates with the size of the pure Tv-bgn3 observed in SDS-PAGE (Figure 4.4) which was used for N-terminal sequencing and

antibody production. The pure Tv-bgn3 was obtained by electro-elution from SDS-PAGE containing protein extracts of 5-days-old culture filtrates of *T. virens*. Several potential post-translational modifications are predicted from the sequence (ScanProsite) including N-glycosylation site (residue 29), amidation site (residue 37) and multiple phosphorylation and N-myristoylation sites.

Figure 4.3. Multiple Sequence Alignments of Tv-bgn3 and Its Homologues.

Comparison of the deduced amino acid sequence of the *T. virens* β-1,6-glucanase (*Tv*) and its homologues from *T. harzianum* (*Tz*), *G. zeae* (*Gz*), *Acremonium sp*. (*Asp*), *Neotyphodium* sp. (*Nsp*) and *V. fungicola* (*Vf*). The identical amino acid residues are shaded in black and similar residues are shaded in gray. The *asterisks* indicate the experimentally determined active site residues in the exo-β-1,3-glucanase. Alignments were obtained by using ClustalW at the Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

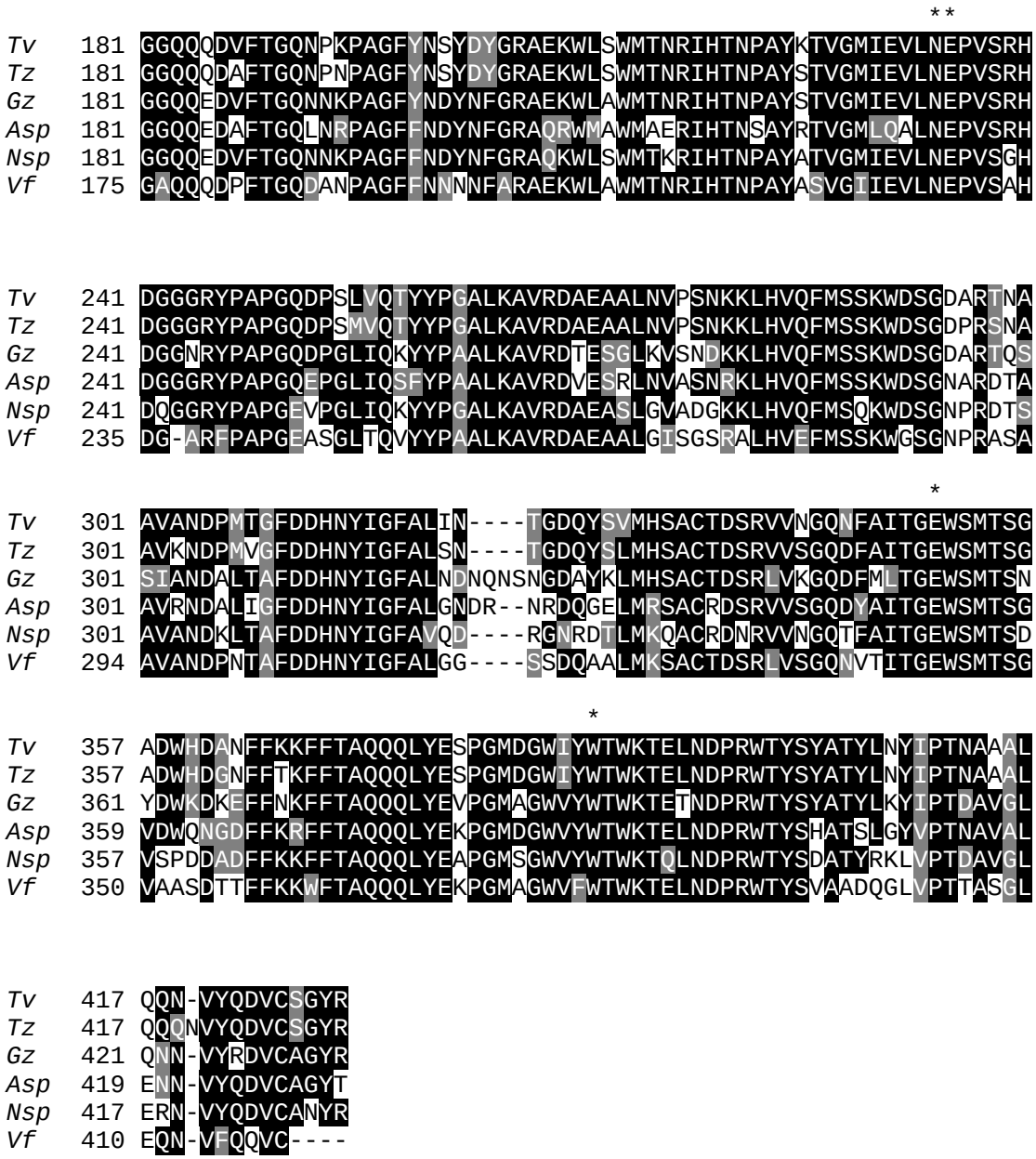

Figure 4.3. Continued.

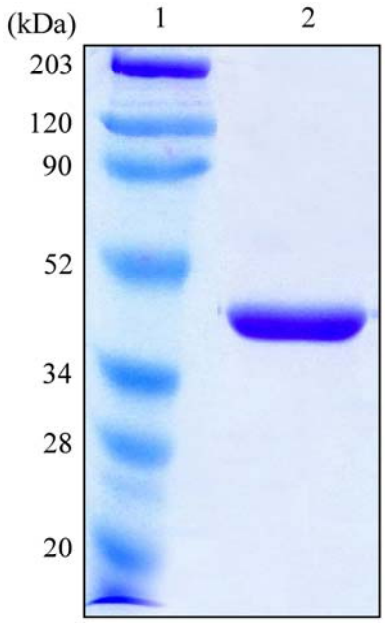

Figure 4.4. Electrophoretic Profile of the Tv-bgn3 Purified by Electro-Elution from SDS-PAGE.

Three micrograms of protein purified by electro-elution (see Methods) were analyzed by SDS-PAGE (12%) followed by Coomassie staining. Protein extracts were obtained by precipitation of 5-days-old culture filtrates of *T. virens.* A single band of Tv-bgn3 was detected, indicating a pure protein (lane 2). This protein fraction was used for production of polyclonal antibodies. Lane 1, prestained SDS-PAGE broad range standards (Bio-Rad); molecular weights in kiloDaltons are indicated on the left.

TV-BGN3 Gene Expression Analysis

To assess the nutritional regulation of *TV-BGN3*, Vogel's minimal medium without a carbon source (VM) or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *R. solani* (VMR) was used (Pozo et al, 2004). Cell walls of *R. solani* as the sole carbon source were used to simulate mycoparasitic conditions. RT-PCR analysis revealed very low basal levels of expression of *TV-BGN3* in minimal media, VM supplemented with glucose and nitrogen (VMG), or VM supplemented with glucose but depleted for nitrogen (VMG-N). When *R. solani* cell walls were used as a carbon source, expression of *TV-BGN3* was found to be induced, regardless the presence or absence of a nitrogen source (VMR, VMR-N). The induction was first observed at 6 h time point and the expression level remained similar after 24h incubation (Figure 4.5).

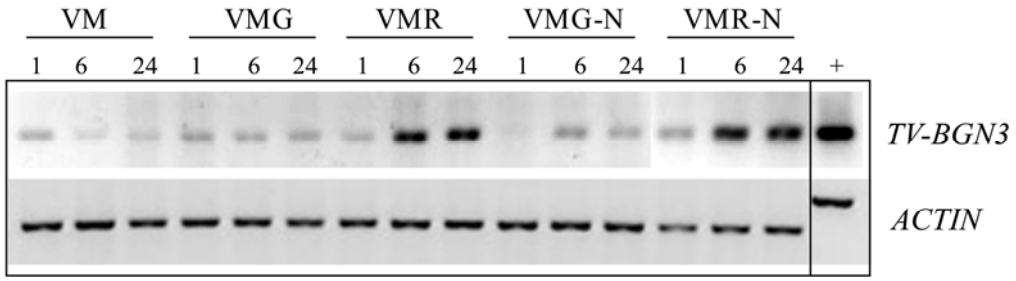

Figure 4.5. Gene Expression Analysis of *T. virens TV-BGN3.*

RT-PCR analysis of *TV-BGN3* expression during growth of *T. virens* in submerged culture under different nutritional conditions. Media used were: Vogel's minimal medium without carbon source (VM), or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *R. solani* (VMR); and VM lacking nitrogen but supplemented with either 1.5% glucose (VMG-N) or 0.5% *R. solani* cell wall (VMR-N). Listed at the top are hours after transferring to the various media. *T. virens* genomic DNA (+) was included as positive control for RT-PCR. Top panel, amplification of a 200 bp product using *TV-BGN3* specific primers; bottom panel, fungal actin was amplified as control for equal amounts of cDNA, yielding a 500 bp fragment from cDNA and 1kb fragment from gDNA. PCR products were run on 2 % agarose gels in TAE buffer 1x, and band intensities compared within each experiment after ethidium bromide staining.

Identification of β-1,6-Glucanase Deletion-, Over-expression-, and Double-over-expression (β-1,6-Glucanase and β-1,3-Glucanase 2) Transformants

The β-1,6-glucanase (*TV-BGN3*) disruption vector pSZD12 was constructed to replace the 1.35 kb *TV-BGN3* ORF with a selectable marker. The construct comprised the selectable *ARG2* gene (Baek and Kenerley, 1998) flanked by 1.2 kb and 3.8 kb segments of the *TV-BGN3* sequence (Figure 4.6A). The linearized plasmid was used for transformation of an arginine auxotrophic strain Tv10.4 (Baek and Kenerley, 1998). A total of 90 stable transformants were tested for a gene disruption by PCR. Six PCR-selected candidates were further analyzed by Southern hybridization to verify the gene disruption. As seen in Figure 4.6A, after double-digestion of the genomic DNA with *Bam*HI/*Xba*I and hybridization with the *TV-BGN3* probe (a 829 bp *Sal*I/*Pst*I fragment from pJCC6), a positive disruption event was expected to yield a 1.8 kb band versus a 2.5 kb band in the wild-type. Five strains, GKO100, GKO111, GKO112, GKO168, and GKO212 were clearly disrupted for the gene, while strain 25 contained both 1.8 kb and 2.5 kb bands, indicating ectopic integration of pSZD12 (Figure 4.6B).

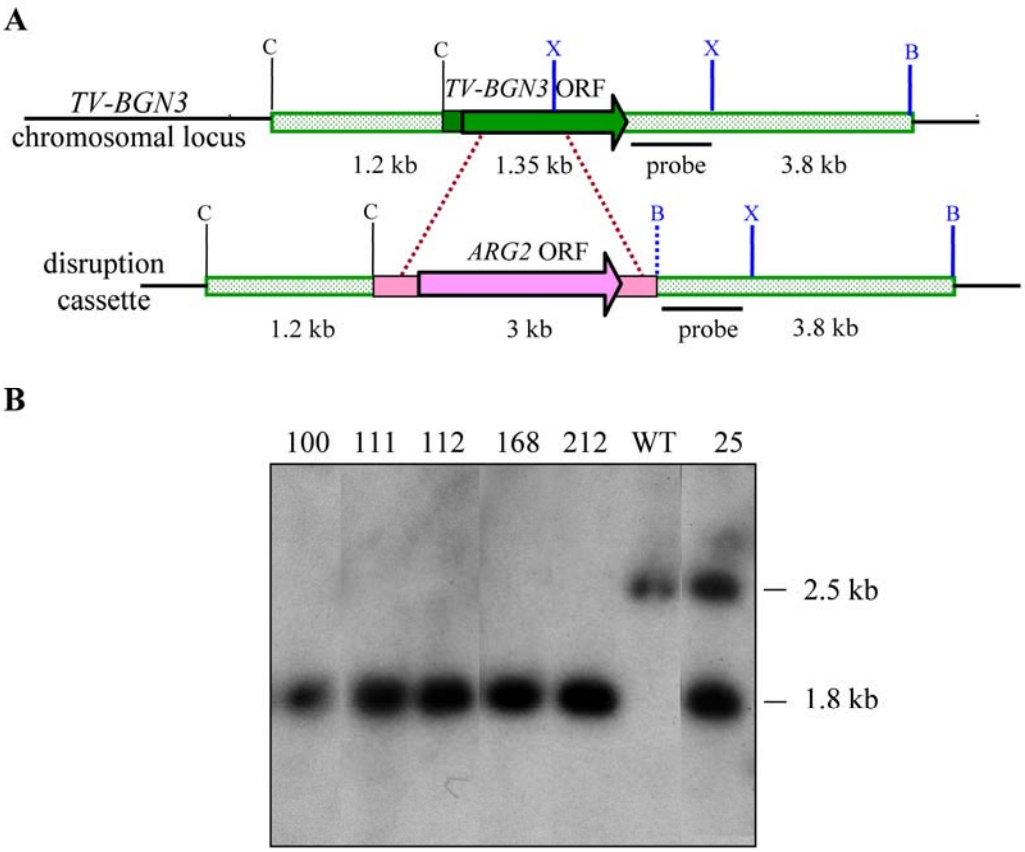

Figure 4.6. Southern Analysis and Confirmation of *TV-BGN3* Disruptants.

(A) Scheme of the gene deletion strategy. The *TV-BGN3* disruption vector comprised the selectable *ARG2* gene flanked by 1.2 kb and 3.8 kb segments of the *TV-BGN3* sequence. For PCR amplification of the 3.8 kb flanking region, *Bam*HI restriction site was introduced to the forward primer (indicated by dashed line, B). In a homologous integration event the native *TV-BGN3* will be replaced with the 3.0 kb fragment of the *T. virens ARG2* gene of the targeting vector yielding a 2.5 kb band versus a 1.8 kb band in the wild-type genomic DNA digested with *Bam*HI (B)/*Xba*I (X). The dimensions are not drawn to scale.
(B) Southern analysis of *T. virens* wild-type (wt) strain and *TV-BGN3* deletion transformants (GKO100, GKO111, GKO112, GKO168, GKO212). Autoradiograph of DNA gel blot hybridized with ^{32}P-dCTP-labeled *TV-BGN3* probe indicated in the figure. 15µg of genomic DNA was digested with *Bam*HI/*Xba*I and loaded per lane. Numbers on the left indicate expected size in native and deletion events. Strain 25 contains the native and over-expression bands indicating ectopic integration.

The over-expression vector pJCC6 was constructed for constitutive over-production of *TV-BGN3* gene. In the vector containing *ARG2* gene as a selectable marker (Baek and Kenerley, 1998), a 1.35 kb ORF of the *TV-BGN3* cDNA was placed between the promoter and the terminator regions of the *T. virens GPD* (glyceraldehydes-3-phosphate dehydrogenase) gene (Figure 4.7A). For constitutive over-production of β-1,3-glucanase 2 (*TV-BGN2*), pDXG13 plasmid was constructed. A 2.4 kb ORF of the *TV-BGN2* genomic clone was placed between the promoter and the terminator regions of the *T. virens GPD* gene in the vector containing *ARG2* gene as a selectable marker (Figure 4.8A). For double-over-expression of two glucanase genes, *TV-BGN3* and *TV-BGN2*, Tv10.4 strain was co-transformed with circular forms of pJCC6 and pDXG13.

Screening of stable putative *TV-BGN3* over-expression (GOE) and *TV-BGN3*, *TV-BGN2* double-over-expression transformants (dGOE) was initially performed by measuring enzymatic activity in the culture filtrates after two days of fungal growth in VMM media. For screening purposes, total enzymatic activity of culture filtrates from putative transformants was not measured. The transformants with the highest enzymatic activity were selected based on comparison of amounts of reducing sugars they released with the glucose standards (Somogyi, 1952; Nelson, 1957) (for example see Figure 4.7B).

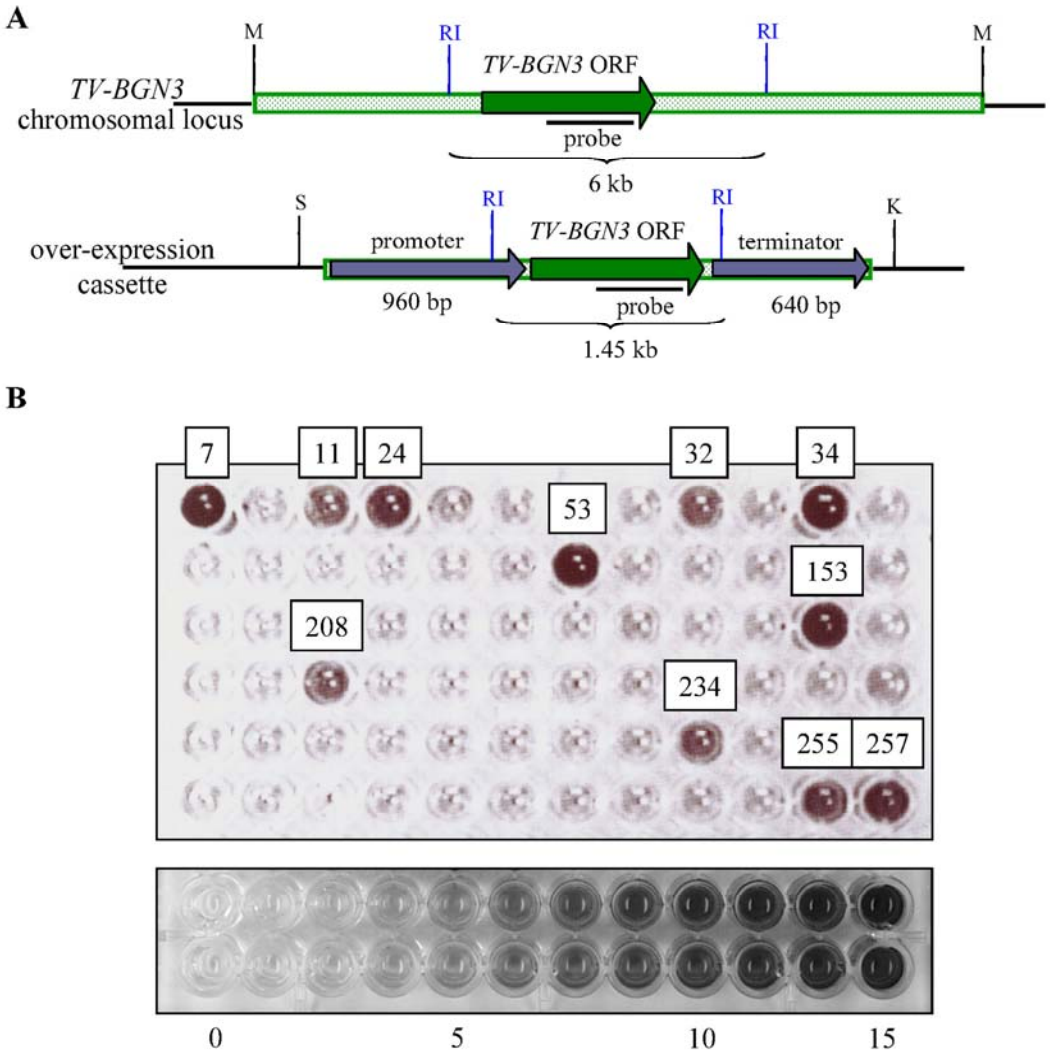

Figure 4.7. Confirmation of *TV-BGN3* Over-Expression Transformants.

(A) Gene over-expression strategy. The over-expression vector, pJCC6, was constructed by placing the 1.35 kb ORF of the *TV-BGN3* cDNA clone between the promoter and the terminator regions of the *T. virens gpd* (glyceraldehydes-3-phosphate dehydrogenase) gene. Indicated in letters are the enzyme restriction sites, *Mlu*I (M) and *Eco*RI (RI); numbers in brackets indicate expected sizes in wild-type and over-expression genomic DNA double-digested with *Mlu*I/*Eco*RI. The dimensions are not drawn to scale.
(B) Activity of β-1,6-glucanase in the culture filtrates of putative over-expression transformants. β-1,6-glucanase activity was determined by measuring the amount of reducing sugars released from pustulan, following the colorimetric procedure of Somogyi and Nelson (see Methods). Top panel, putative over-expression transformants; bottom panel, standards of glucose in µg.
(C) Southern analysis of *T. virens* WT strain and *TV-BGN3* over-expression transformants (GOE24, GOE234, GOE257, GOE53, GOE255, GOE7, GOE34, GOE153). 15µg of genomic DNA was double digested with *Mlu*I/*Eco*RI, and probed with ^{32}P-dCTP-labeled 829 bp *Sal*I/*Pst*I fragment of *TV-BGN3*. Numbers on the right indicate expected size in over-expression and wild-type sequences.

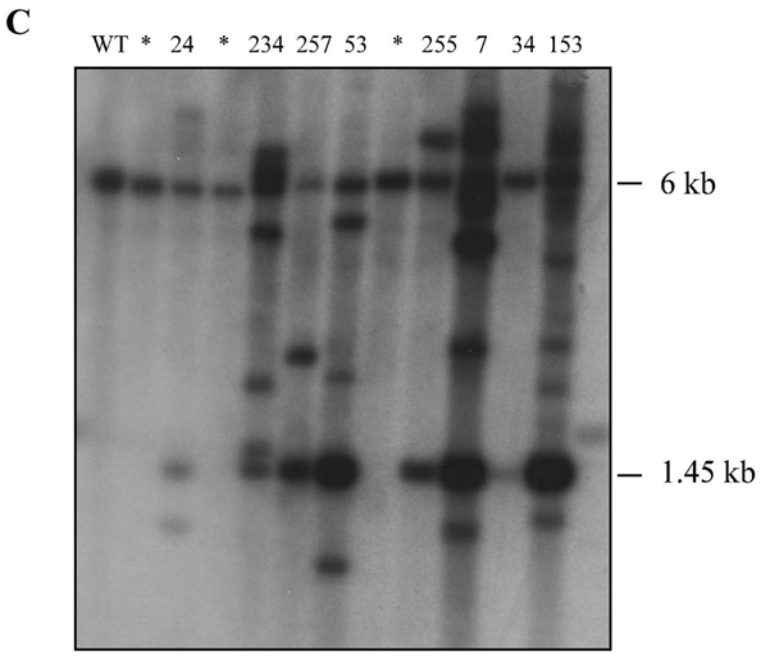

Figure 4.7. Continued.

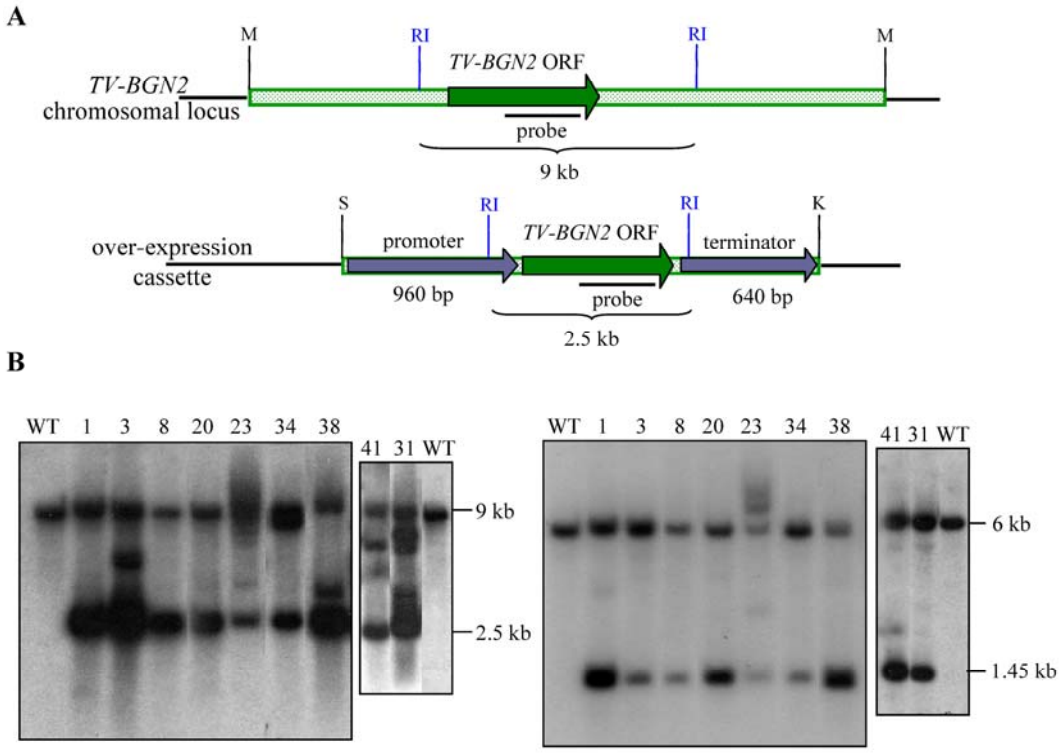

Figure 4.8. Confirmation of *TV-BGN3* and *TV-BGN2* Co-Expression Transformants.

(A) *TV-BGN2* over-expression strategy. An over-expression vector (pDXG13) was constructed by placing the 2.4 kb ORF of the *TV-BGN2* genomic clone between the promoter and the terminator regions of the *T. virens gpd* gene. Numbers over brackets indicate expected sizes in wild-type and over-expression genomic DNA digested with *Eco*RI (RI) and probed with indicated probe in Southern analysis. The dimensions are not drawn to scale. For over-expression strategy for *TV-BGN3*, please see Figure 4-8A.
(B) Southern analysis of *T. virens* WT strain and co-expression transformants. 15µg of genomic DNA was digested with *Eco*RI, and probed with ^{32}P-dCTP-labeled 572 bp *PstI*/*Xho*I fragment of *TV-BGN2* genomic clone (left panel). The same blot was used for probing with *TV-BGN3* probe (right panel). Nine positive transformants were identified (dGOE1, dGOE3, dGOE8, dGOE20, dGOE23, dGOE34, dGOE38, dGOE31, dGOE41) as constitutively over-expressing both genes.

Out of 72 putative GOEs and 46 dGOEs, 11 and 20 candidates, respectively were selected for Southern blotting analysis. Genomic DNA of putative β-1,6-glucanase over-expression and double-over-expression strains was digested with *Eco*RI. A positive *TV-BGN3* over-expression event was expected to yield a 1.45 band versus 6 kb in wild-type (Figure 4.7A), and a positive *TV-BGN2* over-expression event a 2.5 band versus 9 kb band in wild-type strain (Figure 4.8A) after hybridization with the gene specific probes. Those probes included a 829 bp *Sal*I/*Pst*I fragment from pJCC6 for *TV-BGN3* and a 572 bp *PstI*/*Xho*I fragment of *TV-BGN2* genomic clone. Eight β-1,6-glucanase over-expression transformants were identified (GOE24, GOE234, GOE257, GOE53, GOE255, GOE7, GOE34, GOE153), as demonstrated by Southern blotting analysis (Figure 4.7C). Most of the GOE transformants had additional bands indicating various integration invents with genomic rearrangements. The intensity of hybridization of the 1.45 kb band in some of the transformants, such as GOE7, GOE53 and GOE153, suggests integration of multiple copies of the construct (Figure 4.7C). Southern analysis revealed nine positive transformants for double-over-expression of *TV-BGN3* and *TV-BGN2* (dGOE1, dGOE3, dGOE8, dGOE20, dGOE23, dGOE34, dGOE38, dGOE31, dGOE41) (Figure 4.8B). Most of the dGOE transformants showed just one additional copy of *TV-BGN3* or *TV-BGN2*. Increased intensity of hybridization for 1.45 or 2.5 kb bands was observed as well (e.g. dGOE1, dGOE38, dGOE41) (Figure 4.8B).

β-1,6-Glucanase Expression and Protein Production in Transformants

To confirm that the *TV-BGN3* over-expression transformants were functional, i.e. showing elevated levels of transcription compared to the wild-type, and that the deletion transformants were missing the *TV-BGN3* transcripts, Northern analyses were performed. Figure 4.9 shows Northern analysis of selected transformants grown in VMS for two days, and then transferred to VM supplemented with *R. solani* cell wall for 18 h. No transcripts of *TV-BGN3* were detected in any of the GKO transformants (GKO100, GKO111, GKO112, GKO168, and GKO212), confirming the disruption of the gene by the selectable marker (Figure 4.9). In three selected over-expression strains (GOE7, GOE53, GOE153), the levels of expression were much higher than in the wild-type, confirming the constitutive expression of the constructs (Figure 4.9). Transcript profiling of these same GOEs correlated with protein production as these transformants produced the highest amounts of the *TV-BGN3* after 5 days of growth in VMS medium (Figure 4.10). The other four transformants (GOE24, GOE234, GOE255, GOE257) produced less Tv-bgn3 than GOE7, GOE53, GOE153, but more than wild-type (Figure 4.10).

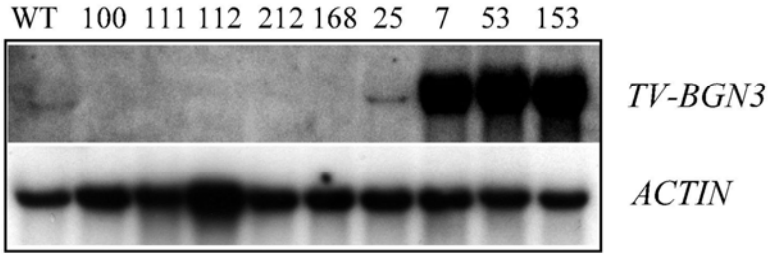

Figure 4.9. Northern Analysis of *TV-BGN3* Expression in Deletion and Over-Expression Strains.

Total RNA was extracted from wild-type (wt), deletion strains (GKO100, GKO111, GKO112, GKO168, and GKO212), stain with ectopic integration of the construct (25), and selected over-expression strains (GOE7, GOE53, GOE153) cultured in VM media supplemented with 0.3 % *R. solani* cell walls for 18 h. 5 µg of total RNA was separated on formaldehyde-agarose gel, transferred to a Hydrobond-N^+ nylon membrane, and hybridized with a 538 bp *Sal*I/*Pst*I *TV-BGN3* fragment (Top panel). Bottom panel, the same membrane was hybridized with the 600 bp actin fragment PCR amplified from *T. virens* wt cDNA as a control for even loading.

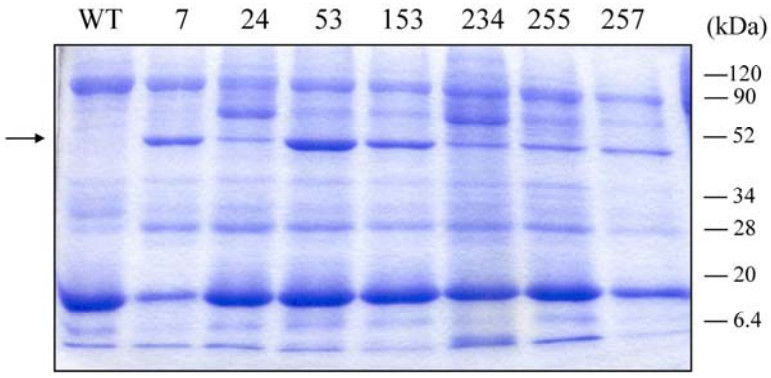

Figure 4.10. Tv-bgn3 Production in Over-Expression Strains**.**

Protein profile of culture filtrates from *T. virens* wt and over-expression strains. Coomassie stained pattern of the SDS-PAGE analysis. 8 µg of total protein were loaded per lane. Lanes, from left to right: *T. virens* (wt); over-expression strains strains (GOE7, GOE24, GOE53, GOE153, GOE234, GOE255, GOE257); Molecular weights, in kiloDaltons, are indicated on the right. Tv-bgn3 protein is indicated by an arrow.

Growth Assays of Transformants

There were no phenotypic changes in cultures of GKO, or GOE transformants when compared to Gv29-8 with respect to the production of aerial hyphae or pigmentation during sporulation. *T. virens* WT, three GKOs (GKO111, GKO112, GKO212) and three GOEs (GOE7, GOE53, GOE153) transformants were selected for growth analysis. Growth area was compared after one and two days of growth in VMS, PDA, or WA plates. Based on analysis of variance test (ANOVA; $P<0.05$) there was no significant differences in growth among WT, GKOs, and GOEs strains in any of the media tested (Figure 4.11). However, there was a difference in growth among WT and selected dGOEs (dGOE1, dGOE3, dGOE38, dGOE41) with WT growing significantly faster than dGOEs in all three media (Figure 4.12). In addition it appeared that dGOE38 was phenotypically slightly different than WT and other transformants with respect to the culture appearance and sporulation (Figure 4.13).

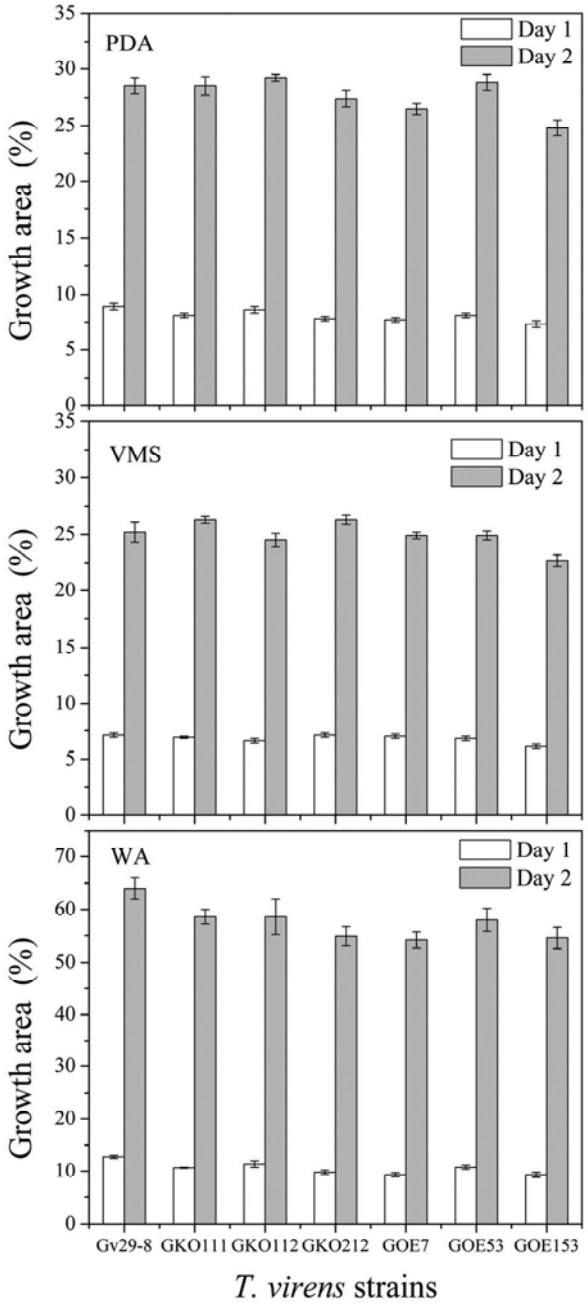

Figure 4.11 Growth Analysis of WT, GKOs and GOEs.

The surface area of growth of WT, GKOs and GOEs in WA, PDA and VMS plates recorded for two days. The data are represented as the ratio of fungal growth area to the total area of plate. Each bar represents mean growth area of four replicates from two independent experiments with the standard error.

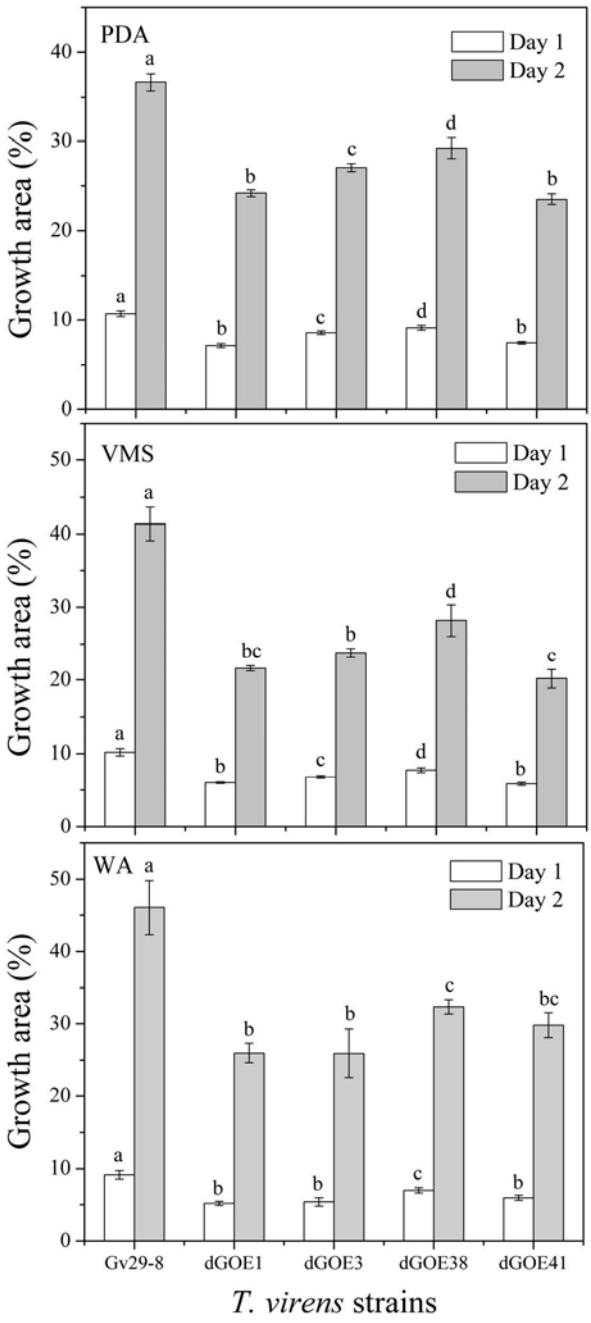

Figure 4.12. Growth Analysis of WT, and dGOEs.

The surface area of growth of WT and dGOEs in WA, PDA and VMS plates recorded for two days. The data are represented as the ratio of fungal growth area to the total area of plate. Each bar represents mean growth area of four replicates from two independent experiments with the standard error. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at the significance level of 5%.

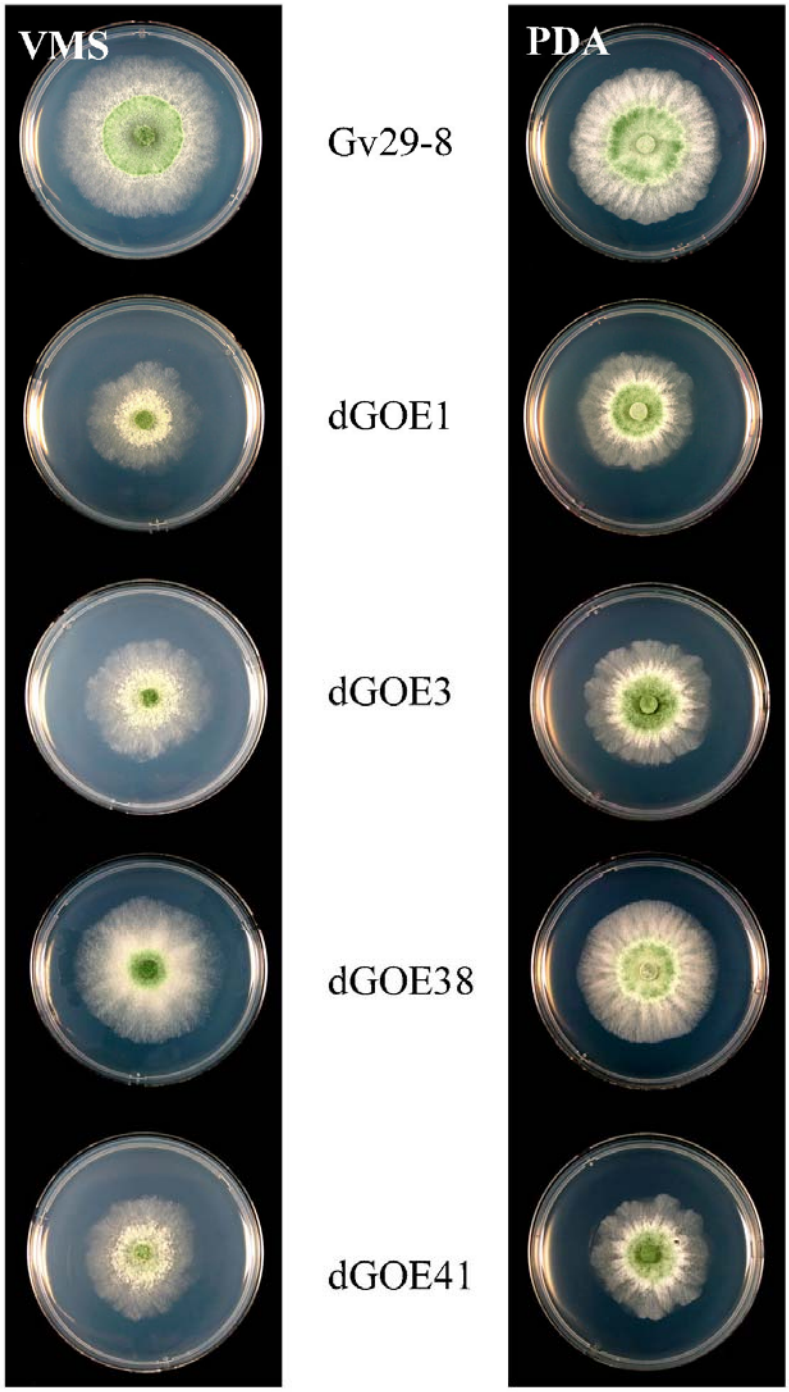

Figure 4.13. Reduced Hyphal Growth of dGOEs.

Cultures of WT and dGOEs were prepared by inoculation of a single plug of mycelium at the center of a Petri dish containing VMS (left panel) or PDA (right panel) and incubated for two days at 27^{o}C in dark.

Western Analysis and β-1,6-Glucanase in Plant-Fungal Interaction

Previously using an aseptic hydroponic system (Chapter III), we demonstrated the presence of various extracellular proteins being secreted during plant-fungal interaction. To examine if β-1,6-glucanase is detected during this interaction, concentrated proteins secreted into hydroponic growth medium of Gv29-8 growing in the presence/absence of cotton or maize seedlings for 48 h were analyzed by Western analysis. Polyclonal antibody was raised in rabbits against Tv-bgn3 protein fraction electro-eluted from SDS-PAGE and confirmed by N-terminal sequencing. Protein extracts obtained from 5-days-old culture filtrates of the *TV-BGN3* over-expressor strain GOE53 was included as a positive control. No Tv-bgn3 protein could be detected in hydroponic media of the wild-type growing with/without the cotton or maize seedlings (Figure 4.14).

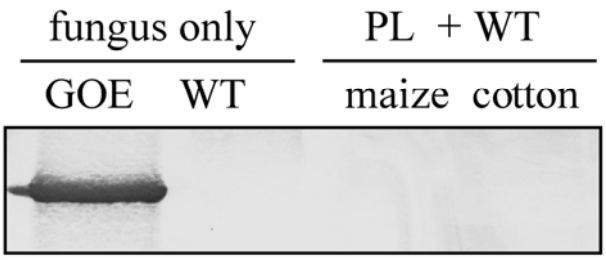

Figure 4.14. Immunoblot Analyses for Detection of Tv-bgn3 Secreted in the Hydroponic Growth Medium.

Equal volumes of concentrated proteins equivalent to 300 mL medium from the hydroponic system when *T. virens* Gv29.8 (WT) was growing in the presence/absence of cotton or maize seedlings for 48 h were loaded on a 15 % SDS-PAGE and electroblotted to a nitrocellulose membrane. Culture filtrates of an over-expression strain GOE53 was included as positive control (GOE).

Growth Inhibition of Pathogenic Fungi by *TV-BGN3* Single- and Double Over-expression Strains

Growth inhibition of *Pythium ultimum* or *Rhizoctonia solani* hyphae by extracellular proteins of *T. virens* wild-type and transformants was compared. One representative from each group of transformants which displayed the highest enzymatic activity and produced the most Tv-bgn3 protein for single- or double-over-expression strains and the least for the deletion transformants was selected for this study. After inoculation of the pathogen onto the medium containing protein extracts of WT and transformants, the incubation was considered completed when the hyphae of the pathogen reached the edges of the well. Hyphal extension was recorded and the fungal growth area calculated for each treatment (for example, see Figure 4.15A). The data are represented as the ratio of the growth area of pathogen hyphae in *Trichoderma* treated medium to the water treated medium.

The measurements for *P. ultimum* were taken 7 h after inoculation, recording a 1.12 mm/hr growth rate. Even with such a high growth rate, *P. ultimum* was inhibited with extracellular protein extracts of *T. virens*. The inhibition resulted from the fraction of proteins with molecular weight higher than 10 kDa; as that was the size of the membrane used for protein concentration. This excluded additional toxic effects of culture filtrates due to the presence of antibiotic compounds of smaller molecular weight known to be produced by *T. virens.* When 1x concentration, representing the original concentration of culture filtrates was used, proteins of dGOE significantly inhibited the growth of *P.*

ultimum compared to the wild type. Wild type, GKO, and GOE were not significantly different in their inhibition ability at this protein concentration (Figure 4.15B, left panel). A similar trend was observed with 0.5x concentration of proteins, but with less inhibition for the *Trichoderma* strains as compared to untreated control (data not shown). However, when 2x concentrations were used, the level of inhibition of transformants was much more prominent. The effect of the deletion of the β-1,6-glucanase was clear as the inhibition ability of GKO strain was significantly lower than wild-type. Remarkably, the inhibition activity of dGOE was 8 fold higher than wild-type. The over-expression strain also displayed significantly higher inhibition activity than the wild-type (1.6 fold) (Figure 4.15B, right panel).

The measurements for *R. solani* were taken 17 h after inoculation, yielding an average growth rate of 0.59 mm/hr, almost twice slower than *P. ultimum*. Regardless of the concentration of proteins applied (1x or 2x), the inhibition pattern of wild-type and transformants was similar. Overall, the highest level of inhibition by *Trichoderma* strains was at 2x concentration of proteins. Proteins of dGOE significantly inhibited the growth of *R. solani* at 1x or 2x as compared to the wild type. Wild type, GKO, and GOE did not significantly differ in inhibition ability. In both cases the inhibition activity of dGOE was approximately 1.6 fold higher than wild-type. (Figure 4.16).

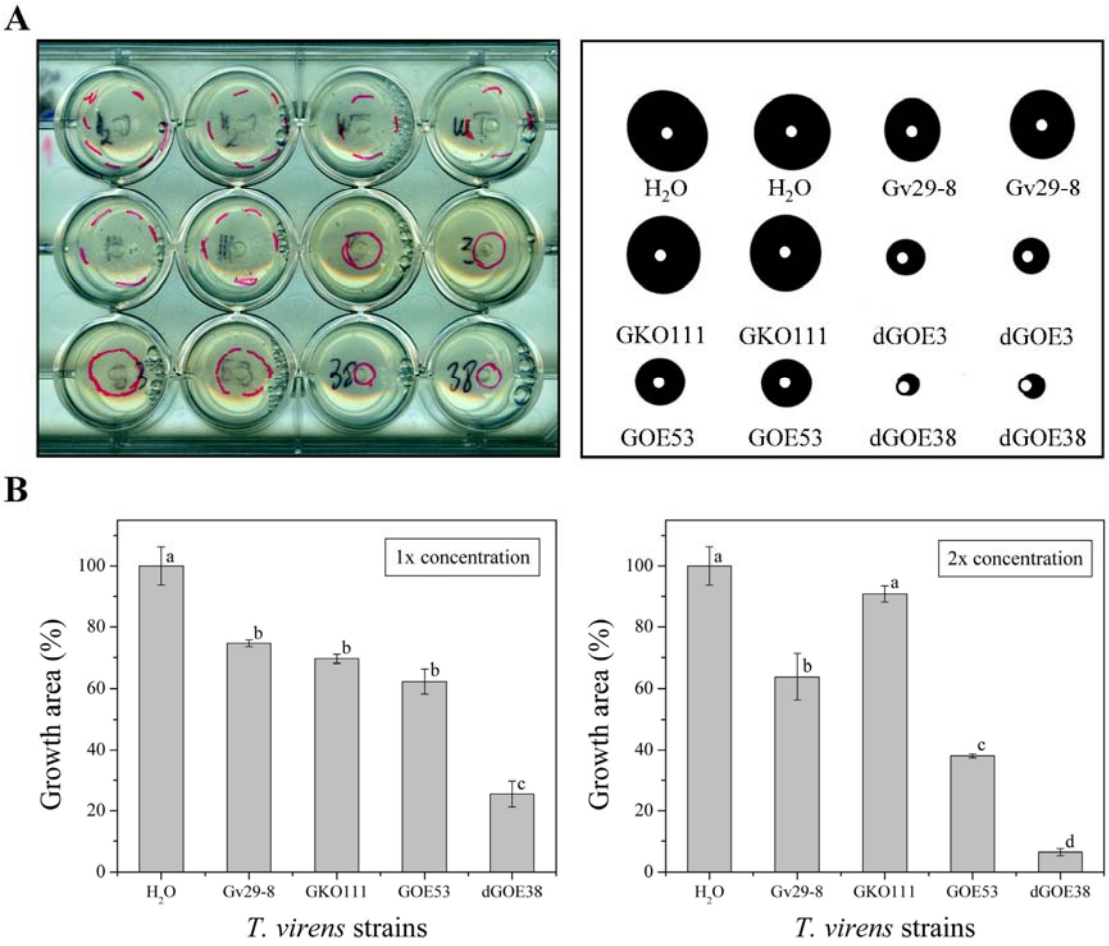

Figure 4.15. Growth Inhibition of *P. ultimum* by Protein Extracts Obtained from WT, GKO, GOE and dGOE Strains of *T. virens*.

(A) The image of growth inhibition of *P. ultimum* by 2x protein extracts, as an example for processing of data for the growth inhibition assay. After incubation period, the pathogen hyphal extension was recorded (left panel) and the area of growth calculated (right panel).
(B) *P. ultimum* growth inhibition at two concentrations of proteins. The data are represented as the ratio of the *Pythium* growth area in *Trichoderma* treatments to the *Pythium* growth area in water treatment. Each bar represents mean growth area of two replicates with the standard error. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at the significance level of 5%. Left panel, 1x concentrations, representing original culture filtrate concentration; right panel, 2 x concentration of proteins.

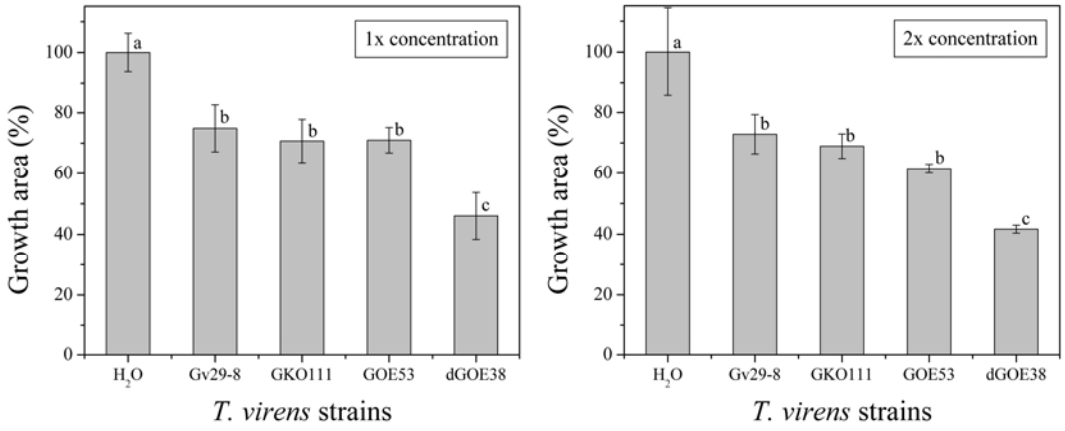

Figure 4.16. Growth Inhibition of *R. solani* by Protein Extracts Obtained from WT, GKO111, GOE53 and dGOE38 Strains of *T. virens*.

R. solani growth inhibition by 1x concentrations, representing original culture filtrate concentration (left panel) and 2 x concentration of proteins (right panel). The data are represented as the ratio of the *Rhizoctonia* growth area in *Trichoderma* treatments to the *Rhizoctonia* growth area in water treatment. Each bar represents mean growth area of two replicates with the standard error. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at the significance level of 5%.

DISCUSSION

Tv-bgn3 Is Likely Processed by Kex2 Proteinase for Activation During Secretion

Tv-bgn3 from *T. virens* encodes an extracellular β-1,6-glucanase. The mature protein starts with a stretch of 40 amino acids before the first residue as determined by N-terminal sequencing. The first 17 residues form a signal peptide promoting secretion of the enzyme into its immediate environment, a factor considered to be vital in the mycoparasitic ability of mycopathogens (Kim et al., 2002). However, additional cleavage of the polypeptide is required to activate the protein after secretion (Markaryan et al., 1996). The segment comprising residues 18-40 ends with the KR sequences, a recognition site for a subtilisin-like endoproteinase (Kex2) known to processes secreted proteins in different organisms (Thomas et al., 1991; Fuller et al., 1989; Tao et al., 1990; Goller et al., 1998). A Kex2 recognition structure has been detected in β-1,6-glucanase (Bgn16.2) from *T. harzianum*, but also in other hydrolytic enzymes from *Trichoderma*, such as chitinase CHIT42 (Garcia et al., 1994), protease Prb1 (Geremia et al., 1993) and protease Tvsp1 (Pozo et al., 2004). Thus, the mature protein resulting from cleavage after KR sequence contains 389 amino acids, and predicted molecular mass of 43.6 kDa which corresponds with size observed in SDS-PAGE. This was additionally confirmed in the Western blot analysis as the polyclonal antibodies obtained against the Tv-bgn3 recognized the protein of that size (Figure 4.14).

Tv-bgn3 Is a Member of Glycosyl Hydrolase Family 5

Tv-bgn3 is highly similar to β-1,6-glucanases from other filamentous fungi including biological control agents, endophytes of grasses and mycoparasites. However, sequence alignments identified similarity of Tv-gbn3 to the glycosyl hydrolase family 5, which mostly include exo-β-1,3-glucanases or endo-β-1,4-glucanases (Henrissat and Davies, 1997). The process by which polysaccharide hydrolases cleave at the glycosidic linkage is complex. From chemical modification studies, crystallographic data, and site-direct mutagenesis, the crucial residues of the enzymatic active site responsible for the catalytic breakdown of the glycosidic linkage have been identified (Chen et al., 1993; Cutfield et al., 1999; Mackenzie et al., 1997). Six of the eight amino acid residues in the active site of the *Candida albicans* exo-β-1,3-glucanases, determined from crystallography (Cutfield et al., 1999) were found to be conserved in *T. virens* β-1,6-glucanase and its homologues.

***TV-BGN3* Expression Is Induced by Fungal Cell Walls**

Mycoparasitism, in its simplest form, is the acquisition of carbon from another fungus, proposed to be regulated by catabolite repression in *Trichoderma* species (Lorito et al., 1996a; Steyaert at al., 2004). Since the soil is generally considered as a nutritionally sparse environment, when carbon deprives, alternative pathways are tuned on in response to environmental stimuli, such as the presence of another fungus. Genes

involved in mycoparasitism from *Trichoderma* species have been shown to be differentially regulated in a carbon/nitrogen-dependent-fashion (Lorito et al., 1996a; Baek, et al., 1999; Olmedo-Monfil et al., 2002; Mach and Zeilinger, 2003; Pozo et al., 2004). In this study, gene expression analysis demonstrated the induction of TV-*BGN3* by the presence of fungal cell walls, a condition often regarded as a simulation of mycoparasitism. The increase of *TV-BGN3* transcripts seems to correspond to the inducer stimulus, and not to the starvation condition, as incubation in media lacking carbon or nitrogen resulted in very low levels of transcription. Gene expression pattern is coherent with the presence of regulatory motifs in the *TV-BGN3* promoter region. No cis-acting elements involved in carbon and nitrogen regulation were found, yet several MYRE elements proposed to be involved in mycoparasitism (Cortes et al., 1998; Steyaert at al., 2004) were identified. However, the fact that *TV-BGN3* expression was repressed by glucose (VMG), as only faint bands could be observed, and the lack of potential CreA sequences suggests carbon regulation may take place by system different from CreA, as it has been suggested in other fungi (Espeso et al., 1993; Ronne, 1995).

Recently, Montero et al. (2005) described similar pattern of expression for another β-1,6-glucanase (Bgn16.3) purified from *T. harzianum* when media containing fungal cell walls was the inducer. Bgn16.3 was not detected under chitin induction, the condition most frequently used to isolate extracellular enzymes from *Trichoderma* (de la Cruz et al., 1995), but did specifically and greatly accumulate in the presence of fungal cell walls suggesting the involvement of this enzyme in mycoparasitism (Montero et al., 2005). The presence of several other putative regulatory sequences in the promoter region of

TV-BGN3, such as cellulose and stress response elements, suggests a complex transcriptional regulation and deserves further experimental analysis. The relatively moderate level of induction of *TV-BGN3* expression by cell walls is probably due to the composition of *R. solani* cell walls, being predominantly composed of chitin and β-1,3-glucans. Much higher expression is expected in the presence of more inducible cell wall fractions of *Pythium* or *Saccharomyces*, as they contain a higher fraction of β-1,6-glucans than *R. solani* cell walls.

Tv-bgn3 Seems Not to Be Involved in Plant-Fungal Interaction

Fungal elicitors, such as oligomers of chitin or glucan, could be released from fungal cell walls by the hydrolytic activity of their own extracellular enzymes during plant-fungal interactions, thus indirectly playing a role in the induction of plant defense responses (Lamb et al., 1989). Furthermore, a recent study identified a β-1,6-glucanase being secreted into apoplast of the host grass, *Poa ampla*, by its endophyte fungus *Neotyphodium* sp. (Moy et al., 2002). Multiple functions of such an enzyme have been proposed including the interesting hypothesis that β-1,6-glucanases could function in the degradation of the cell walls of other fungi that may be encountered within the host plants (Moy et al., 2002). The target of such enzymes is less likely to be plant, as plant cell walls do not contain β-1,6-glucans (Varner and Lin, 1989). Interestingly, the mycelia of *Neotyphodium* sp. can be found epiphytically on some endophyte grass species, functioning as a defensive net preventing colonization by other organisms (Moy

et al., 2002). Investigating the expression of Tv-bgn3 following colonization of roots by *T. virens*, a Western blot analysis of secreted proteins from cotton/maize *T. virens* interactions was performed. No Tv-bgn3 product was detected in either plant-fungal interaction. Analysis of plant apoplast, cytoplasm or cell wall proteins from plant colonized by *T. virens* needs to be performed to conclude that Tv-bgn3 is not involved in the plant-fungal interactions.

Growth/Survival of Genetically Modified Strains

Even though β-1,6-glucanases have been identified and purified from many fungi (Schep at al., 1984; Lora et al., 1995; de la Cruz et al., 1995; Oyama et al., 2002; Montero et al., 2005), their physiological function has not been conclusively established in any species. As being secreted hydrolytic enzymes, β-1,6-glucanases may have a role in fungal metabolism by degrading glucan polymers in their environment and providing nutritional carbon sources. They may also play a role in fungal development including hyphal growth and branching, sporulation, or autolysis after exhaustion of external carbon source (reviewed in Pitson et al., 1993). In our study, neither deletion nor over-expression of *TV-BGN3* seems to affect cell functions such as viability, growth, or conditiation. Therefore Tv-bgn3 is probably not a major enzyme involved in fungal development. However, when two glucanase genes encoding β-1,3- and β-1,6-glucanase, were constitutively co-expressed in *T. virens*, the growth of transformants was markedly reduced compared to the wild-type in first several days of growth. After 7-8 days,

growth and appearance of the culture were not distinguishable (data not shown). The observed reduction in growth may be due to highly increased levels of enzymes, and misbalance of processes of synthesis and degradation in early stages of fungal growth. The reduction in growth did not seem to affect the protein production, as these dGOEs produced much higher levels of total protein than wild-type (data not shown). Having obtained antibodies for both glucanases, further cytological studies will be conduced to better understand this observation.

Increased Inhibition of Pathogen Growth by Synergistic Activity of Tv-bgn3 and Tv-bgn2

The involvement of cell wall degrading enzymes in mycoparasitic processes by *Trichoderma* species has been widely accepted. Direct evidence for the role of chitinases, proteases and β-1,3-glucanases in mycoparasitism by *Trichoderma* species has been demonstrated by several authors (Lorito et al., 1994; El-Katatany et al., 2001; Pozo et al., 2004). In contrast, the information of β-1,6-glucanases in these processes is very limited (Lora, 1995; Amey et al., 2003). In our study, the possible involvement of Tv-bgn3 in mycoparasitism has been indicated by gene expression analysis, as *TV-BGN3* transcripts were found to be induced by presence of the fungal cell walls (Figure 4.5). To further test this hypothesis, genetically modified strains lacking, over-expressing or co-expressing *TV-BGN3* in *T. virens* were used in growth inhibition assays against plant pathogens.

As the activity of hydrolytic enzymes can be enhanced in the presence of gliotoxin or peptaibols produced by *Trichoderma* (Lorito et al., 1994, 1996b; Di Petro et al., 1993), these compounds were eliminated from the proteins extracts to test primarily the inhibition activity of secreted enzymes. When *R. solani* was used as a substrate for β-1,6-glucananse, the only significant inhibition was observed in treatments containing β-1,3- and β-1,6-glucananse. These data corroborate a previous report that a *T. harzianum* mutant producing higher levels of chitinase, β-1,3- and β-1,6-glucananse, displayed better biocontrol properties than the wild-type (Rey et al., 2001). In plants, deployment of a similar strategy to generate transgenic plants constitutively expressing or co-expressing hydrolytic enzymes resulted in improved antifungal protection (Zhu et al., 1994; Lorito and Scala, 1999; Emani et al., 2003). In addition to overproduction, synergistic effects of extracellular enzymes may also contribute to higher levels of enzymatic activity and increased degradation of cell walls (de la Cruz et al., 1995). This is in agreement with the fact that the protein extracts that were tested, indeed, did contain other extracellular enzymes, just in much lower amounts (Figure 4.10).

The effect of protein extracts on the pathogen growth was more prominent with *P. ultimum*. This was somewhat anticipated as *P. ultimum* cell walls are composed of β-1,3- and β-1,6-glucanans. However, we did not expect such a remarkable inhibition of *P. ultimum* growth when β-1,3- and β-1,6-glucananse were constitutively co-expressed (Figure 4.15B, right panel). The performance of an over-expression strain was improved when the concentration of the protein was increased (Figure 4.15B, right panel). In addition, in the same experiment, the effect of β-1,6-glucananse deletion from the

genome was notable as the disruptant displayed significantly reduced capability to inhibit growth of *P. ultimum* as compared to the wild-type. Lack of significant difference in growth of *P. ultimum* between the untreated control and the treatment with proteins from the disruptant strain suggests a very important role of β-1,6-glucananse in mycoparasitic activity of *T. virens*. Similar findings were obtained when the gene encoding β-1,6-glucananse from *V. fungicola* was disrupted; the mycoparasitic ability on *A. bisporus* was markedly reduced (Amey et al., 2003). Taken together, inhibition of growth of both *R. solani* and *P. ultimum* in all experiments was greatest when over-expression of β-1,3-and β-1,6-glucananse was combined. To further verify these findings, pathogenicity assays will be conducted to determine if these dGOE strains enhance the level of the plant protection against *P. ultimum*.

The data presented in this study establish that the over-expression of *TV-BGN3* in a wild-type background is sufficient to promote the over-expression of this gene. This is not always the case as the over-expression *BGN16.2* (β-1,6-glucananse) from *T. harzianum* did not result in an increase of extracellular protein probably due to degradation by acidic proteases and instability at low pH (Delgado-Jarana et al., 2000). Furthermore, the successful constitutive co-expression of two genes in *T.virens* was demonstrated. Targeted gene disruption provided the opportunity to study the importance of this Tv-bgn3 in *T. virens* biocontrol activity. Since this is the first deletion of β-1,6-glucananse in any of the *Trichoderma* species, conducting further studies may unravel complex regulation and the role this gene may have in the fungal physiology and ecology. Successful overproduction of two genes afforded new opportunities to

investigate construction of *T. virens* strains expressing 'multigene' combinations to achieve greater protection against a broader range of plant pathogens.

METHODS

Fungal and Plant Materials

Two strains of *T. virens* were used in this study: wild-type strain, Gv29-8, and an arginine auxotrophic strain, Tv10.4, recipient for fungal transformation (Baek and Kenerley, 1998). The strains were routinely maintained on potato dextrose agar (PDA, Difco). For screening of transformants, Vogel's minimal medium supplemented with 1.5 % sucrose (VMS) and PDA were used. The isolate of the seedling pathogen, *Pythium ultimum*, was kindly provided by Dr. Howell (USDA-ARS SPARC, College Station, TX).

DNA Manipulation

T. virens genomic DNA was isolated as previously described (Xu et al., 1996). Southern hybridization analyses were carried out according to Sambrook et al. (1989). Probes were random labeled using Random Primer DNA Labeling Kit (Takara, Madison, WI) and hybridizations were performed overnight at 42 ^{o}C with Ultrahyb as hybridization buffer (Ambion, Austin, TX). QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was

used for plasmid DNA purification. Nucleotide sequencing was performed by a primer-walking strategy (Sambrook et al., 1989). All sequencing reactions were performed at the Gene Technologies Laboratory (Texas A&M University). DNA sequences were analyzed by DNA Strider 1.2 (Marck, 1988), and Sequencher 4.1 (GCC, Ann Arbor, MI).

Southern Hybridization with Genomic DNA

Isolated genomic DNA of *T. virens* was digested with following restriction enzymes: *Hin*dIII, *Eco*RI, *Eco*RV, *Xba*I, *Bam*HI, *Apa*I, *Xho*I, *Sal*I, *Cla*I, *Spe*I, *Sac*I, *Kpn*I, *Bsph*I, *Pst*I, *Sma*I, *Not*I, and *Mlu*I (Takara, Madison, WI). The products were separated by agarose gel electrophoresis, transferred to Hybond-N^+ nylon membrane (Amersham Biosciences, UK), and hybridized overnight at 42 ^oC using Ultrahyb (Ambion). The 1.35 kb ORF of the β-1,6-glucanase cDNA clone was used as a probe in a standard Southern blot procedure (Sambrook et al., 1989).

***TV-BGN3* Gene Expression Analysis**

To assess nutritional regulation of *TV-BGN3*, Vogel's minimal medium without a carbon source (VM) or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *R. solani* (VMR) was used (as described in Chapter II). Regulation of *TV-*

BGN3 expression by nitrogen was examined in VM with glucose or *R. solani* cell walls as a carbon source in the absence of nitrogen source (VMG-N and VMR-N).

Analysis of β-1,6-glucanase expression under different nutritional conditions was assessed by RT-PCR as described in previous chapter. The *TV-BGN3* gene specific primers were forward, 5'- ATTACAGGCGAGTGGAGCAT-3' (Sd6), and reverse 5'-GCGTTCGTTGGGATGTAGTT-3' (Sd5). These primers amplified a 200 bp PCR product from wt genomic DNA. PCR amplification of *TV-BGN3* fragments comprised of 30 cycles (each cycle: 30sec at 94 ^{o}C, 20sec at 57C, and 20sec at 72 ^{o}C). Actin PCR amplification with ActF-ActR (indicated in previous chapters) consisted of 25 cycles (each cycle: 30sec at 94 ^{o}C, 30sec at 58C, and 40sec at 72 ^{o}C). PCR products were electrophoresed on 2 % agarose gels, and band intensities compared within each experiment after ethidium bromide staining.

Construction of β-1,6-Glucanase Deletion-, Over-expression-, and Double Over-expression (β-1,6-Glucanase and β-1,3-Glucanase 2) Transformants

After probing a *T. virens* bacterial artificial chromosomal (BAC) library (Grzegorski, 2001) with the 1.35 kb ORF of the β-1,6-glucanase cDNA clone, one of the positive clones was further digested with several restriction enzymes and the fragments subcloned into pBluescript II SK (+/-) vector. The following vectors were obtained for use in subsequent experiments: pSZD4 (a *Cla*I subclone), pSZD5 (an *Eco*RI subclone),

pSZD6 (a *Kpn*I subclone), pSZD7 (a PstI subclone), and pSZD8 and pSZD9 (the *Sac*I subclones).

The β-1,6-glucanase disruption vector (pSZD12) was constructed by replacing a 2 kb fragment containing the *TV-BGN3* ORF with a 3.0 kb *SmaI/EcoRV* fragment of the *T. virens ARG2* gene (Baek and Kenerley, 1998). The resulting vector contained 1.2 kb and 3.8 kb fragments from pSZD4 flanking the selectable *ARG2* gene. The 8 kb *Apa*I/*Not*I deletion cassette was isolated from pSZD12 and used for fungal transformation. An over-expression vector (pJCC6) was constructed (Chou and Kenerley, unpublished) by placing the 1.35 kb ORF of the *TV-BGN3* cDNA clone between the promoter and the terminator regions of the *T. virens GPD* (glyceraldehydes-3-phosphate dehydrogenase) gene (Xu et al., 1996). This vector includes a 3.0 kb *SmaI/EcoRV* fragment containing the *ARG2* gene as a selectable marker. For fungal transformation, circular *TV-BGN3* over-expression vector was used in our standard transformation protocol (Baek and Kenerley, 1998).

For double-over-expression of two glucanase genes, β-1,6-glucanase (*TV-BGN3*) and β-1,3-glucanase 2 (*TV-BGN2*), a second vector (pDXG13) was constructed (Grzegorski and Kenerley, unpublished). Plasmid pDXG13 was constructed by placing the 2.4 kb ORF of the *TV-BGN2* genomic clone between the promoter and the terminator regions of the *T. virens GPD* gene (Xu et al., 1996). This vector includes a 2.6 kb *Kpn*I fragment containing the *ARG2* gene as a selectable marker. To obtain double over-expression strains, Tv10.4 was co-transformed with circular forms of pJCC6 and pDXG13.

Transformation and Screening of Transformants

Preparation of protoplast and PEG-mediated transformation of Tv10.4 with selection for arginine prototrophy was performed as previously described in Baek and Kenerley (1998). Stable prototrophic transformants were selected by consecutive transfer of single colonies to VMS, PDA, and VMS.

Screening of deletion strains was first performed by PCR. For deletion transformants, β-1,6-glucanase specific primers, Sd6 and Sd5, were used in an attempt to amplify the 200 bp PCR product from the wild-type genomic DNA. The strains in which PCR resulted giving no product were further selected for Southern blotting analysis.

Screening the potential transformants for over-expression of *TV-BGN3*, or both *TV-BGN3* and *TV-BGN2*, was initially performed by enzymatic activity assays of culture filtrates. Culture filtrates were obtained by inoculating 12-well MULTIWELL^{TM} tissue-culture plates (Becton Dickinson and Co., Franklin Lakes, NJ) containing 3 ml per well of VMM (1.5% mannose) with a conidial suspension of the appropriate fungal strain. Plates were incubated on a rotary shaker with the slight agitation for 2 days at 23 ^{o}C. Culture filtrates were collected by filtration through a 10 μm NITEX nylon cloth (TETKO Inc., Depew, NY). Activity of β-1,6-glucanase and β-1,3-glucanase in the culture filtrates was determined by liberation of reducing sugars from pustulan (*Unbilicaria papullosa*, Calbiochem, San Diego, CA) or laminarin (*Laminaria digitata*, Sigma, Woodlands, TX) as the substrates, respectively (de la Cruz et al., 1995; Nelson,

1957). Strains with the highest enzymatic activity were selected for Southern blotting analysis.

Northern Analysis of Transformants

Conidia of 7-day-old *T. virens* wt and transformants cultured on PDA was used to inoculate VMS liquid media (for deletion transformants) to a final concentration of 10^6 spores /ml. After 2 days of growth at 23 ^oC with shaking at 130 rpm, the mycelia were harvested on a on 10 µm NITEX nylon cloth and rinsed thoroughly with sterile water. Then, the mycelia were transferred to fresh VM supplemented with 0.3% fungal cell walls from *R. solani* and incubated for 18h under the same conditions. Total RNA was extracted following the protocol of Jones et al, 1985. 5 µg of total RNA per sample was denaturated, resolved in 1.5 % agarose formaldehyde gel, transferred to a Hydrobond-N^+ nylon membrane (Amersham Biosciences, UK), and hybridized overnight at 42 ^oC using Ultrahyb (Ambion). A 538 bp *Sal*I/*Pst*I fragment from pJCC6 was used as *TV-BGN3* probe. The 600 bp actin fragment PCR amplified from Gv29-8 cDNA (primers indicated in previous section) was used as control for even loading.

SDS-PAGE Analysis of Transformants

To examine the production of Tv-bgn2 in *T. virens* over-expression transformants, the protein extracts and SDS-PAGE analyses were performed as described in Chapter III

(section **SDS-PAGE and Western analysis of transformants)**. For deletion strains, protein extracts of culture filtrates from strains growing in the presence of *R. solani* cell wall (as described in **Northern analysis of transformants)** were quantified by Bradford, electrophoresed on SDS-PAGE gel following Coomassie staining.

N-terminal Sequencing and Antibody Production

Protein extracts were obtained by precipitation of 5-days-old culture filtrates of *T. virens* (see section **SDS-PAGE and Western analysis of transformants,** Chapter III) and analyzed by SDS-PAGE. The identified Tv-bgn3 protein band was excised and electro-eluted in the Electroeluter 422 (Bio-Rad, CA). This fraction was used to produce a polyclonal antibody in rabbits (Sigma, Woodlands, TX). For confirmation of the signal peptide, N-terminal sequencing of the protein was performed by automated Edman chemistry on a Hewlett Packard G1005A Protein Sequencher (Protein Chemistry Laboratory, Texas A&M University).

Computational protein analyses were performed using the ExPASy proteome server at the Swiss Institute of Bioinformatics (http://us.expasy.org/), and EMBOSS at European Bioinformatics Institute (http://www.ebi.ac.uk/emboss). Multiple sequence alignments were performed using ClustalW at Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

Growth Assays of Transformants

Cultures of selected transformants were compared with the wild-type strain for radial growth. Agar plugs from actively growing colonies were inoculated in the center of VMS, PDA, or WA (water agar) plates, and the hyphal extension was recorded at 24 and 48h of growth at 27^{o}C. After each day, the border of the hyphal extension was traced with the red permanent marker. Each plate was photographed with a digital camera against a dark background from a constant distance. The surface area of growth for each day was determined by developing a Matlab code capable of independently processing large number of consecutively numbered images. Each treatment contained four repetitions and each experiment was repeated at least twice.

Western Analysis and β-1,6-Glucanase in Plant-Fungal Interaction

Protein extracts tested for presence of β-1,6-glucanase were obtained as previously described in Chapter III. Briefly, proteins secreted into hydroponic growth medium when *T. virens* WT was growing in the presence/absence of cotton or maize seedlings for 48 h were precipitated or concentrated, separated by 15 % SDS-PAGE, and electroblotted to a nitrocellulose membrane (Osmonics Inc., Gloucester, MA). Tv-bgn3 protein was detected using TV-BGN3 protein polyclonal antibodies (dilution 1: 1000) in a standard Western blot procedure (Sambrook et al., 1989).

Fungal Growth Inhibition

Growth inhibition of *P. ultimum* and *R. solani* by extracellular proteins of *T. virens* wild-type and transformants was examined in 12-well MULTIWELL^{TM} tissue-culture plates (Becton Dickinson and Co., Franklin Lakes, NJ). The wells contained 1 mL of PDA or PDA supplemented with different concentrations of extracellular proteins of *T. virens* strains that had been grown in 100 mL of VMS medium for four days previously inoculated with the 10^6/ of conidia per mL (final concentration). Extracellular proteins were obtained by filtering culture filtrates through a 10 μm NITEX nylon cloth and through a 0.45 μm filter (Fisher Sci., Hampton, NH). Fifty mL of culture filtrate of each treatment was concentrated 10 kDa cutoff Millipore Amicon Ultra centrifugal filter devices (Bedford, MA). Prior the concentration, culture filtrates were not treated with a protease inhibitor cocktail. Following concentration, proteins were brought to 2x, 1x, and 0.5x concentration by the addition of the appropriate volume of sterile water. The 1x concentration represented the concentration of the original culture filtrate. Proteins were added to the cooled autoclaved PDA just before pouring medium into the wells. Agar plugs (5 mm diameter) of actively growing cultures of *P. ultimum* or *R. solani* were placed on the middle of the wells and incubated at 27 C. When the hyphae in any of the treatments reached the edges of the well, the incubation was considered complete, growth area of the fungus marked and the data processed using Matlab program.

GeneBank accession numbers for the sequences shown in Figure 4.3 are as follows: AF395757 (*Tv*), X79197.1(*Tz*), XM_388441 (*Gz*), AY695381 (*Asp*), AF535131 (*Nsp*) and AY184497 (*Vf*). Gene accession number for *TV-BGN2* is AF395756.

CHAPTER V

CONCLUSION

The soil-borne filamentous fungus *Trichoderma virens* is a biocontrol agent with a well known ability to produce antibiotics, parasitize pathogenic fungi and induce systemic resistance in plants. As *T.virens* exhibits all the mechanisms that have been indicated in biocontrol of plant pathogens by fungal agents and a major sequencing project of its genome is underway, this fungus represents a model system to further define these mechanisms and associated signaling pathways at the molecular level. In this research, two of the most investigated mechanisms of biocontrol, mycoparasitism and induction of plant resistance, were studied.

A small protein (Sm1) with the ability to elicit resistance in plants was identified in culture filtrates of *T. virens.* Sm1 has been purified to homogeneity in a native form by anion-exchange and gel filtration chromatography, and its molecular mass determined by MALDI/TOF. *SM1* was cloned and shown to encode a protein of 138 amino acids with a predicted molecular mass of 14.4 kDa, an 18 amino acid signal peptide and a pI of 5.76. Mass spectrometry of purified protein revealed a molecular weight of 12.6 kDa, which was in agreement with the predicted molecular mass of the mature protein. Based on similarity searches (BLAST), Sm1 was found to be a member of the cerato-platanin family. This family consists of small (~150 amino acids) secreted proteins produced by plant and human fungal pathogens that are mainly associated with toxicity and infection processes. Since several members of the cerato-platanin family are associated with

toxicity, it was first determined whether Sm1 in its native form displayed toxic activity towards other microbes or plants. Antibiotic or toxic effects were not observed against any of the diverse collection of bacteria or fungi tested. Additionally, no necrosis was found in any of the different plants treated with Sm1 (cotton, rice, tobacco, and peanut). Sm1 was also found to lack any glucanase, chitinase or protease activity.

Instead, the infiltration of Sm1 into the leaves of rice or cotton caused elevated production of H_2O_2 and autofluorescence; the early events in plant-pathogen/elicitor interactions. Native, purified Sm1 was shown to induce the expression of pathogenesis related (*PR*) genes (chitinase and β-1,3-glucanase), genes encoding major two enzymes of sesquiterpenoid phytoalexin pathway (HMG-CoA reductase and (+)-δ-cadinene synthase), class III peroxidase, and 9S-lipoxygenase both locally and systemically in cotton (*Gossypium hirsutum*). Expression analysis revealed that *SM1* is expressed throughout fungal development and is transcriptionally regulated by nutrient conditions and host plant presence. Using an axenic hydroponic system allowing co-culture of *T. virens* and cotton, it was shown that *SM1* expression and protein secretion is significantly higher in the presence of the plant. To correlate these findings, the expression of plant defense genes when cotton seedlings were grown in the presence of *T. virens* was examined in an aseptic hydroponic system. This interaction resulted in induced expression of *PR* genes locally in roots of the cotton seedlings. These results indicate that Sm1 is involved in plant-*Trichoderma* recognition and the induction of resistance by activation of plant defense mechanisms (Chapter II).

Following the cloning of *SM1*, strains disrupted in or over-expressing SM1 were successfully generated following the transformation system of Baek et al. (1999)(Chapter III). Gene replacement and over-expression transformants were confirmed by Southern, Northern and SDS-PAGE analysis. Targeted gene disruption revealed that *SM1* was not involved in fungal development. However, obtaining *SM1* disrupted strains provided an opportunity to further study the role Sm1 in fungal metabolism and ecology. Using the axenic hydroponic system for the co-culture of *T. virens* and seedlings, the host defense responses of maize (monocot) and cotton (dicot) were shown to be mediated by the fungus. *T. virens* was found to systemically induce expression of a similar set of genes in cotton cotyledon tissue as when roots were treated with Sm1 (Chapter II). This is the first report of systemic induced responses in cotton mediated by *T. virens*. Clearly, the next step is to determine if this induction is sufficient to provide protection against foliar pathogens.

T. virens-maize interaction was examined at the molecular level for the first time. The major defense related gene in this interaction, *PAL*, a gene commonly associated with both SA and JA/ET resistance pathways, was found to be upregulated by *T. virens* wild-type and over-expression strains and downregulated by the disrupted strain. The clear pattern of expression in both roots and leaves may indicate that *T. virens* mediates defense responses in maize through this pathway. In addition, this finding supported the hypothesis that ability of *T. virens to* communicate with the plant is affected by the lack of Sm1. Proteomics studies provided evidence that there are many more secreted proteins in the pant-*Trichoderm*a interaction than when the fungus is growing without

the presence of the plant (Chapter III). This may be result of mutualistic interactions with these proteins acting as putative host determinants or other signaling molecules. The hydroponics system provides an excellent experimental design to study gene expression as well as profiling proteins produced by the fungus and the host. Another component (a plant pathogen) may be added to this system to simulate more complex natural settings. Hydroponic systems may be used for comparative studies of symbiotic versus pathogenic systems to possible identify novel virulence proteins (Chapter III).

Information provided by *T. virens* mediated responses in cotton and maize is of great interest as most of the work on this topic has been conducted with the non-agricultural *Arabidopsis* model system. Providing evidence that the induction of host resistance in two agriculturally important crops is microbially mediated should have a significant impact on the management of plant pathogens globally. Evidence that Sm1 is an effective elicitor secreted by *T. virens* (Chapter II and III) affords new opportunities to investigate alternative approaches for biocontrol. The generation of transgenic plants expressing *SM1* or combining two different mechanisms of protection by constructing *Trichoderma* strains that constitutively over-express Sm1 and cell wall degrading enzymes presents new applications in biological control.

Finally, the results presented in Chapter IV provided evidence for a very important role of β-1,6-glucananse in mycoparasitic activity of *T. virens*. Gene expression analysis revealed induction of expression of *TV-BGN3* by the fungal cell walls. Strains disrupted in *TV-BGN3* displayed reduced capability to inhibit growth of *P. ultimum* as compared to the wild-type. On the contrary, strains over-expressed in *TV-BGN3*, individually, or

co-expressed with *TV-BGN2* (β-1,3-glucananse) from *T. virens* showed a significantly increased capability to inhibit growth of *P. ultimum* hyphae. Additionally, the double-over-expressing strain was significantly more effective that the wild-type against the *R. solani* as well. Furthermore, the successful constitutive co-expression of two genes in *T.virens* was demonstrated. Targeted gene disruption provided the opportunity to study the importance of Tv-bgn3 in *T. virens* biocontrol activity. Since this is the first deletion of β-1,6-glucananse in any of the *Trichoderma* species, conducting further studies may unravel complex regulation and the role this gene plays in fungal physiology and ecology. Successful overproduction of two genes afforded new opportunities to investigate construction of *T. virens* strains expressing 'multigene' combinations to achieve greater protection against a broader range of plant pathogens.

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