Chapter 10
Molecular Mechanisms of Biocontrol by *Trichoderma* spp.

P.K. Mukherjee(✉), C.S. Nautiyal, and A.N. Mukhopadhyay(✉)

10.1 Introduction

Trichoderma spp. are ubiquitous soil fungi. By virtue of their ability to decompose organic matter, they are free-living in soil as saprophytes. However, these species also have the capability to live on other fungi, and the ability to colonize plant roots and rhizosphere. *Trichoderma* spp. produce a range of hydrolytic enzymes that make them useful in industry (Mach and Zeilinger 2003). These fungi are capable of parasitizing some plant pathogenic fungi that makes them useful as biofungicides (Mukhopadhyay et al. 1992; Chet et al. 1998; Mukhopadhyay and Mukherjee 1996; Harman and Bjorkmann 1998; Hjeljord and Tronsmo 1988) (Fig. 10.1).

Trichoderma spp. produce various kinds of secondary metabolites in abundance, including antibacterial and antifungal antibiotics (Sivasithamparam and Ghisalberti 1998). Some of the species/strains are reported to be plant growth promoters and inducers of systemic resistance in plants (Harman et al. 2004). Faster metabolic rates, anti-microbial metabolites, and physiological conformation are key factors which chiefly contribute to antagonism of these fungi. Mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defence system are typical biocontrol actions of these fungi. On the other hand, *Trichoderma* spp. have also been used in a wide range of commercial enzyme productions, namely, cellulases, hemicellulases, proteases, and ß-1,3-glucanase. Information on the classification of the genus, *Trichoderma*, mechanisms of antagonism and role in plant growth promotion has been well documented. All these qualities have made *Trichoderma* spp. popular in industry as sources of enzymes, and in agriculture as biofungicides/growth promoters. Even though several commercial formulations based on *Trichoderma* are available in the world

P.K. Mukherjee
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India

A.N. Mukhopadhyay
Sangini, 151 Akansha, Udhay-II, Raibareilly Road, Lucknow, India
e-mail: mukhopadhyayamar@indiatimes.com

C.S. Nautiyal, P. Dion (eds.) *Molecular Mechanisms of Plant and Microbe Coexistence.* Soil Biology 15, DOI: 10.1007/978-3-540-75575-3

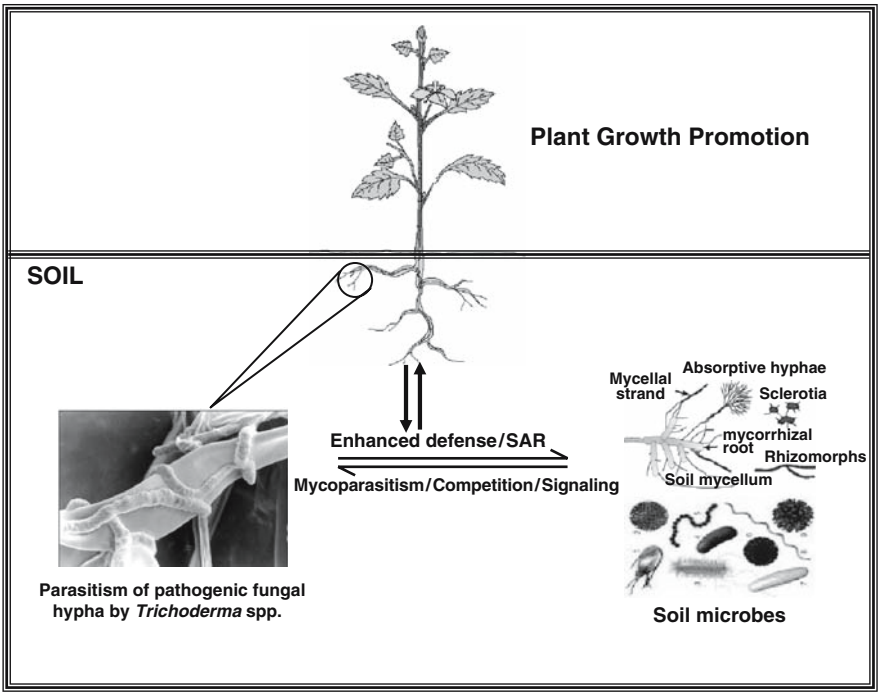

Fig. 10.1 Parasitization of *Rhizoctonia solani* hypha by *Trichoderma virens*. **A** chemotropical attraction of T, virens towards *R. solani* and formation of appressorium. **B** Parasitization of *R. solani* hypha by *T. virens*. Note intensive coiling of the *R. solani* by *T. virens*. **C** Rupture of R. solani hypha after intensive coiling. **D** Lysis of *R. solani* after parasitization by *T. virens*

market for use as biofungicides, their efficacies, in most cases, are not comparable with those of chemical pesticides. This is expected, because *Trichoderma* spp. are living entities, the activity and survival of which are dependent on biotic and abiotic environmental factors. This limitation has been overcome, to some extent, by combining *Trichoderma* spp. with chemical fungicides in the form of an integrated plant disease management (Mukhopadhyay et al. 1992). However, the growing demand for a ban on many chemical fungicides is likely to make the issue complicated, unless we have strains of *Trichoderma* with improved biocontrol potential, as well as survival under ability adverse environmental conditions. The genus *Trichoderma* is able to colonize every different niches because of its metabolic versatility and to its tolerance to stress conditions. These properties make *Trichoderma* a widespread biocontrol agent for management of plant diseases. Studies concerning this phenomenon have mainly focused on characterization of actively attacking processes (i.e. lytic enzyme production), but defense mechanisms by which *Trichoderma* tolerate biotic and abiotic stresses have been poorly addressed. One of the most interesting aspects of the science of biocontrol is the study of the mechanisms employed by the biocontrol agents to effect disease control (Howell 2003). Understanding the mechanisms of biocontrol at the molecular level would be useful to improve the

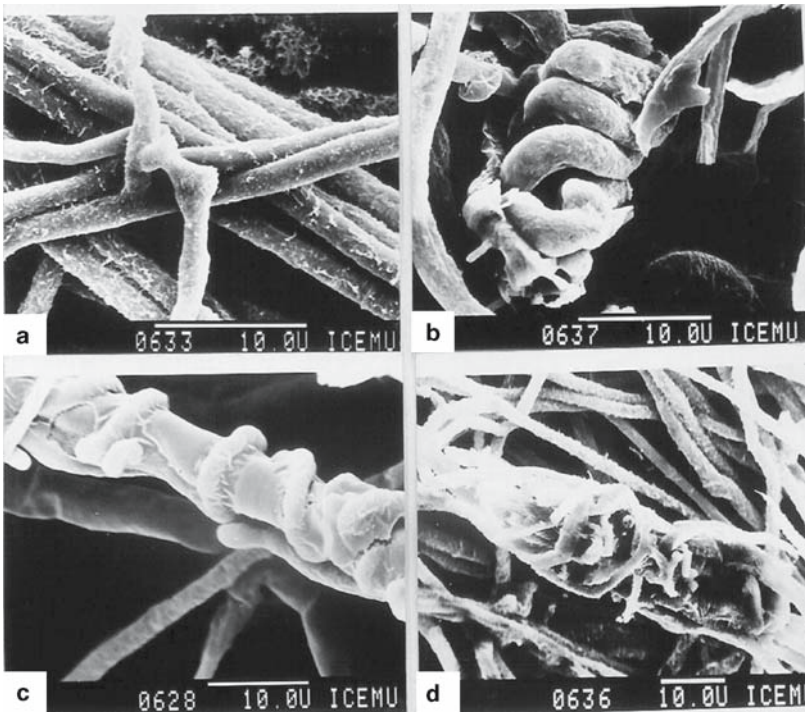

Fig. 10.2 Mechanisms of plant growth promotion by *Trichoderma* spp

potential of *Trichoderma* spp. as biocontrol agents. This is an emerging field; however, some outstanding work has been done in the recent past on understanding how *Trichoderma* spp. work against the pathogens, both directly and indirectly, at the molecular level (Fig. 10.2). This review is intended to consider recent developments, which we consider are just the beginning, in understanding the molecular mechanisms of biocontrol by *Trichoderma* spp.

10.2 Mechanisms of Biocontrol: An Overview

Classically, three principal mechanisms of action of *Trichoderma* spp. have been recognized – mycoparasitism (parasitism of one fungus by another fungus), antibiosis (production of antimicrobial metabolites, and thus inhibiting other fungi) and the universal phenomenon of competition for food, space or oxygen. In a typical mycoparasitic interaction, the parasite (e.g., *Trichoderma*) receives the chemical stimulus released by the host (e.g., *Rhizoctonia solani*) and gets chemotropically attracted towards the host. This is followed by coiling of the host hyphae, running adpressed to the host, production of appressoria-like structures, penetration of the host and derivation of nutrients, and finally, lysis of the host.

The work from the Ilan Chet group at the Hebrew University of Jerusalem, Israel, established the role of recognition by a biomimetic experiment where nylon fibres coated with lectins were coiled by *Trichoderma harzianum*, but not the fibres that have not been coated (Inbar and Chet 1992). This experiment underlined the possible role of signal interplay in this novel host-parasite interaction. It has been rather ironic that the role of parasitism of the surviving structures in biocontrol has been largely neglected. Since mycoparasitism and antibiosis are easy to assay, plenty of work, mostly in vitro, has been done describing how *Trichoderma* spp. kill other fungi through mycoparasitism and what the enzymes are which are secreted during the act of mycoparasitism. However, direct evidence for the exact role of mycoparasitism in in vivo biocontrol is rare. Howell (1987) generated a mutant of *T. virens* deficient in mycoparasitism (hyphal coiling); the mutant was as effective as the parental strain in control of *R. solani* in cotton. He thus questioned the role of mycoparasitism in biocontrol in this system. Mukherjee et al. (1995b), by comparison of an isolate of both *T. harzianum* and *T. virens*, postulated that parasitism of the sclerotia, rather than hyphal coiling or antibiosis, is the principal mechanism of biocontrol of *S. rolfsii* and *R. solani*, when *Trichoderma* is applied to soil. Similarly, the role of antibiosis has been established through the analysis of mutants deficient for the production of a particular antibiotic substance. Based on the biosynthesis of either gliotoxin (Q) or gliovirin (P), Howell et al. (1993) classified *T.* virens strains into two groups; "Q" groups were effective against *R. solani* and "P" groups against *Pythium ultimum*. Later, using gliotoxin or gliovirin-deficient mutants, gliotoxin was implicated to be involved in biocontrol of *Pythium* damping-off (Wilhite et al. 1994), but not for *R. solani* in cotton (Howell and Stipanovic 1995). In other studies (Howell et al. 2000; Howell 2002), a mutant of *T. virens* deficient for both mycoparasitism and gliotoxin biosynthesis still retained the biocontrol potential against *P. ultimum* and *R. solani*. Taken together, these experiments failed to establish whether mycoparasitism or antibiosis is the principal mechanism that effects biocontrol. The phenomenon of competition is universal and it is difficult to assay for its role in biocontrol. Nevertheless, pre-colonization of the spermosphere/rhizosphere, and thus pre-emptying the possibility of a subsequent colonization by the pathogen could be a strong factor responsible for bringing down the infection level. The rapid colonization of the dead/necrotic tissues by *Trichoderma*, thus preventing further spread, has been demonstrated by Mukherjee et al. (1995a) in the case of foliar application of *T. viride* against *B. cinerea* in chickpea. It is very likely that more than one mechanism is involved, and the biocontrol could be the outcome of host-pathogen-antagonist interactions under a given set of biotic and abiotic environmental conditions. In addition to direct effects on the plant pathogen, *Trichoderma* spp. can colonize plant roots and induce systemic resistance against root and foliar pathogens; they have been described as opportunistic, avirulent plant symbionts (Harman et al. 2004). This is a relatively recent discovery and the mechanisms will be discussed in later sections. Some other mechanisms that deserve special mention, but will not be discussed in detail, are the protection of rice plants against sheath blight by degradation of a host-specific phytotoxin (RS toxin) through the production of an extracellular α-glucosidase

(Shanmugam et al. 2001), inhibition of 5′-hydroxyaverantin dehydrogenase, an enzyme involved in aflatoxin biosynthesis by *T. harzianum* (Sakuno et al. 2000), suppression of fumonisin B1 production by *Fusarium moniliforme* by *T. viride* (Yates et al. 1999), inhibition of cell wall synthesis of *B. cinerea* by *T. harzianum* through the production of the peptaibols trichorzianin TA and TB (Lorito et al. 1996), and enhancing the production of nematicidal compounds by *Pseudomonas fluorescens* against *Meloidogyne incognita*, by *T. harzianum* (Siddiqui and Shaukat 2004).

10.3 Role of Hydrolytic Enzymes

The key factor to the ecological success of this genus is the combination of very active mycoparasitic mechanisms plus effective defense strategies induced in plants. The production and regulation of hydrolytic enzymes, particularly the chitinases, glucanases and proteases, have been studied very widely (reviewed by Viterbo et al. 2002b). However, direct evidence on their role in mycoparasitism/biocontrol by selective inactivation or overexpression of a particular enzyme is relatively scarce. *Trichoderma* spp. produce several types of chitinases, both endo and exo, that exhibit mycolytic activities. Many new chitinases are being discovered, and a genome-wide search would reveal more. In this section, we will focus on some aspects of regulation of these enzymes, and discuss direct evidence on the role of these proteins in mycoparasitism/biocontrol. In general, the hydrolytic enzymes are induced by the specific substrates (like chitin, glucan, fungal cell wall), and repressed by glucose. The first report on the systematic study of regulation of an endochitinase-encoding gene ech42 during mycoparasitism came in 1994 (Carsolio et al. 1994); it was shown that this gene is expressed during mycoparasitic interactions, on chitin and by exposure to light. In a significant finding, Lorito et al. (1996) observed that mycoparasitic interaction relieves binding of the Cre1 catabolite repressor protein to promoter sequences of the ech42 gene in *T. harzianum*. The proteinase encoding gene prb1 and the endochitinase-encoding gene ech42 in dual culture with *R. solani* get induced before physical contact, indicating that the induction is contact-independent, and is triggered by a diffusible factor, which was subsequently identified to be soluble chitooligosaccharides (Cortes et al. 1998, Zeilinger et al. 1999). In contrast, chit33 is induced only during the stage of overgrowth on *R. solani* (de las Mercedes Dana et al. 2001). Like chit33, chit36 in *T. asperellum* is also expressed before contact with the host fungus, and predicted to be triggered by a diffusible factor (Viterbo et al. 2002a). Expression of ech42 gene of *T. atroviride* under carbon starvation is antagonized via a BrlA-like *cis*-acting element (Brunner et al. 2003). The regulation of expression of two major chitinase genes (ech42 and nag1, encoding CHIT73) of *T. atroviride* is triggered by different regulatory signals (Mach et al. 1999). The expression of a gene encoding an antifungal glucan 1,3-β-glucosidase is repressed by glucose and induced by laminarin and other glucans (Donzelli et al. 2001). The basic, antifungal exo-α-1,3-glucanase from the

biocontrol fungus *T. harzianum* is induced by fungal cell walls and autoclaved mycelium (Ait-Lahsen et al. 2001). A gene encoding an α-1,3-glucanase is induced during mycoparasitic interactions with B. cinerea. (Sanz et al. 2005). BGN16.3, a novel acidic β-1,6-glucanase from *T. harzianum*, is induced by fungal cell walls, indicating a possible role in biocontrol (Montero et al. 2005). Olmedo-Monfil et al. (2002) showed that the expression of prb1 gene is subject to nitrogen catabolite repression, and is induced by *Rhizoctonia solani* cell walls and osmotic stress. Overexpression of the proteinase-encoding gene *prb1* in *T. harzianum* improved the biocontrol activity against *Rhizoctonia solani* (Flores et al. 1997). The direct evidences on the role of hydrolytic enzymes in biocontrol came from the gene knockout studies, where a gene is selectively deleted through homologous recombination or inactivated by antisense/RNAi. Disruption of ech42 in *T. harzianum* resulted in almost no endochitinase42 activity, whereas strains carrying multicopies of this gene exhibited up to a 42-fold increase in enzyme activity (Carsolio et al. 1999). However, no significant difference in disease control ability of the wild type, or strains with no ech42 gene or harboring multiple copies was observed against *S. rolfsii* and *R. solani*. These results indicated that the 42-kDa endochitinase may not play a significant role in biocontrol in situ in this system. Woo et al. (1999) disrupted ech42 in *T. harzianum* P1 (*T. atroviride*) and showed reduced biocontrol activity against *B. cinerea* on bean leaves. However, interestingly, the biocontrol activities of the disruptants was enhanced against *R. solani*, and remained unaltered against *P. ultimum*. In *T. virens*, biocontrol of knockout and over-expression strains against *R. solani* in cotton were significantly decreased and enhanced, compared with the wild type strain, indicating that the CHIT42 is involved in biocontrol in this host-parasite interaction (Baek et al. 1999). This is interesting because the same enzyme is involved in biocontrol of same pathogen in *T. virens*, but not in *T. harzianum*. Disruption of nag-1, encoding a 73-kDa *N*-acetyl-β-D-glucosaminidase resulted in 30% reduced ability of *T. atroviride* to protect bean seedlings against infection by *R. solani*. An interesting observation in this experiment was that nag1 is essential for induction of ech42, and hence, the reduced biocontrol in the disruptant could have been due to reduced expression of ech42 or nag1 or both (Brunner et al. 2003). Through gene deletion and overexpression, Pozo et al. (2004) proved that a serine protease TVSP1 plays role in biocontrol of *R. solani* by *T. virens*. Recently, using similar genetic approach, Djonovic et al. (2006b) showed that a β-1,6-glucanase is involved in mycoparasitism and biocontrol of *P. ultimum* by *T. virens*, while the cellulose formation of *T. reesei* was found to be dispensable for the biocontrol of *P. ultimum* on zucchini plants (Seidl et al. 2006b).

10.4 Antibiosis

Compared to the bacterial antagonists, molecular evidences on the role of antibiosis in biocontrol in *Trichoderma* is scanty, and hardly any molecular biology data are available. The secondary metabolism in fungi is a very actively researched area, as

many of the useful and toxic metabolites are produced by fungi, like *Gibberella* and *Aspergillus*. Extensive researches in this field led to the identification of gene clusters, and the biosynthetic pathways elucidated through gene deletion analysis (Keller et al. 2005). In contrast, there are only a few reports on the identification of genes responsible for the antifungal metabolite production in *Trichoderma* spp. *T. virens* produces many antifungal peptide metabolites. Wilhite et al. (2001) cloned a 5-kb partial cDNA encoding a putative peptide synthase (Psy1). The disruption of psy1 indicated a role in siderophore production in *T. virens*. However, the disrupted strains exhibited normal biocontrol properties against *R. solani* and *P. ultimum*, indicating that the iron competition may not play an important role in biocontrol in this system. Wiest et al. (2002) identified a 62.8-kb continuous open reading frame encoding a peptaibol synthetase from *T. virens*; the mutation of the gene eliminated the production of all the peptaibol isoforms, thus confirming that this gene is responsible for the synthesis of the peptaibols. A putative peptide synthetase gene has recently been identified in *T. harzianum* (Vizcaino et al. 2005). *T. virens* produces four major metabolites – gliotoxin, gliovirin, viridin and viridiol (Howell et al. 1993). A strain of *T. virens* (IMI 304061) has been found to produce plenty of viridin and its derivative viridiol in culture, while a mutant M7 did not produce these metabolites. Using this mutant, and suppression subtractive hybridization (SSH), Mukherjee M et al. (2006) identified several genes known to be involved in secondary metabolism in fungi. By sequencing a cosmid clone, a gene cluster was identified that consisted of cytochrome P450s and a cyclase. Based on the expression pattern and by comparison of the gene organization vis-à-vis other fungi, it was predicted to be involved in viridin synthesis. However, a gene knockout study would be required to confirm the role of this cluster in secondary metabolite production, as well as antagonistic properties.

One potential problem, which may affect the acceptance of *Trichoderma* spp. as useful biocontrol agents, is the possibility of activity against non-target species. Because Pr1 from insect pathogens has similar properties to prb1 from *Trichoderma* spp. it is possible that the proteinases may play a key role in both entomopathogenicity and antifungal action. Shakeri and Foster (2007) have recently reported on two strains of *Trichoderma harzianum*, 101645, an insect pathogen and 206040, used for biological control of fungal plant pathogens, which were investigated for the production of serine protease, chitinase and antibiotic activity in relation to entomopathogenicity. Both strains produced serine protease with a *M*r of 31 kDa and chitinase with a *M*r of 44 kDa. Enzymes from both strains had similar characteristics and were produced during the growth phase. Both strains also produced peptaibols active against fungi in late growth and stationary phases which differed in their amino-alcohol content. The peptaibols were insecticidal when fed to larvae of *Tenebrio molitor* or when applied to the cuticle together with the serine protease. The results suggest that the virulence factors involved in biocontrol are the same as those for insect pathogenicity. This may affect the use of *Trichoderma* spp. for biocontrol as there may be effects on non-target insect species.

10.5 Induced Resistance

The observation that *Trichoderma* spp. colonize plant roots and induce systemic resistance against a wide range of fungal, bacterial and viral pathogens can be considered a breakthrough in biocontrol research (reviewed by Harman et al. 2004). Inoculation of roots of cucumber seedlings with conidia of *T. harzianum* T-203 (*T. asperellum*) in an aseptic hydroponic system resulted in induction of defense responses (Yedidia et al. 1999). Electron microscopy of ultra-thin sections from *Trichoderma* treated roots revealed penetration of the mycoparasite into the roots, restricted mainly to the epidermis and outer cortex. *Trichoderma* colonization resulted in strengthening of the epidermal and cortical cell walls and deposition of newly formed barriers, these typical host reactions being found even beyond the sites of potential fungal penetration. The inoculation of *Trichoderma* initiated increased peroxidase and chitinase activities, both in roots and leaves. Later on, the authors showed that inoculation of cucumber roots with *Trichoderma* induced an array of PR proteins (Yedidia et al. 2000). Inoculation of cucumber roots with *T. asperellum* reduced the inoculum load of *Pseudomonas syringe* pv *lachrymans* to the extent of 80%, when challenge inoculated on leaves (Yedidia et al. 2003), thus providing direct evidence on induced defense-mediated protection of crop plants in response to *Trichoderma* inoculation. The protection afforded by the biocontrol agent was associated with the accumulation of mRNA of two defense-related genes: the phenylpropanoid pathway gene encoding phenylalanine ammonia lyase (PAL) and the lipooxygenase pathway gene encoding hydroxyperoxidase lyase (HPL). Recently, using the gene knockout approach, a hydrophobin TasHyd1 has been demonstrated to be involved in root colonization by *T. asperellum* (Viterbo and Chet 2006). In a significant finding, Shoresh et al. (2006) identified a MAPK (TIPK – *Trichoderma* induced MAPK) in cucumber, antisense – mediated silencing of this gene made plants susceptible even after inoculation of roots with *T. asperellum*. It was thus proved that *Trichoderma* exerts its positive effects on plants through the activation of a MAPK gene involved in signaling the pathway of defense response. In studies with *T. virens*, Howell et al. (2000) demonstrated that seed treatment of cotton with the antagonist or application of the culture filtrate to seedling radicles induced synthesis of much higher concentrations of the terpenoids deoxyhemigossypol, hemigossypol and gossypol in developing roots than those found in untreated controls. All these compounds were toxic to *R. solani*. Biocontrol activity was highly correlated with induction of terpenoid synthesis in cotton roots by *Trichoderma. T. virens* also induced significantly higher levels of peroxidase activity. Subsequently, Hanson and Howell (2004) identified an 18-kDa protein (a serine proteinase) from *T. virens* that stimulated terpenoid and peroxidase activity in cotton radicles. A definite role of phytoalexin induction in biocontrol has recently been demonstrated by Howell and Puckhaber (2005), who showed that the "P" strains of *T. virens* failed to stimulate phytoalexin synthesis in cotton and were ineffective as biocontrol, while the "Q" strains that stimulated phytoalexin biosynthesis were effective. This difference was attributed to the ability of "Q" strains to produce the

18-kDa elicitor protein. Recently, three groups independently identified a homologue of SnodProt proteins, variously named as SnodProt1 (GV Sible and PK Mukherjee, unpublished; GenBank Acc. no. DQ494198), Sm1 (Djonovic et al. 2006a) from *T. virens*, and Epl1 (Seidl et al. 2006a) from *T. atroviride*. Purified Sm1 protein triggered the production of reactive oxygen species in rice and cotton and induced expression of defense-related genes both locally and systemically in cotton (Djonovic et al. 2006a). Pre-treatment of cotton cotyledons with this protein also produced high levels of protection to the foliar pathogen *Colletotrichum* sp. These results indicated that Sm1, is involved in the induction of resistance by *Trichoderma* spp. through the activation of plant defense mechanisms.

Recently Olson and Benson (2007) have studied three root-colonizing fungi, binucleate *Rhizoctonia* (BNR) isolates BNR621 and P9023 and *T. hamatum* isolate 382 (T382), for suppression of Botrytis blight in geraniums by induction of host systemic resistance. Resistance to Botrytis blight was observed in geraniums transplanted into potting mix amended with formulations of P9023 and T382 two weeks prior to inoculation with *Botrytis cinerea* when grown under environments either highly or less conducive to disease development. Restriction of lesion development may play a role in the suppression of Botrytis blight in geraniums. This may be the first to demonstrate induced systemic resistance by BNR fungi to a foliar pathogen and support additional research into use of T382 in an integrated management program for *B. cinerea* (Olson and Benson 2007). Research on the specific effects of induced systemic resistance should be continued with additional pathogens since there is some indication of pathogen specificity in the method of suppression.

10.6 Signal Transduction and Biocontrol

Signal transduction through the G-protein/cAMP and MAP kinase pathways have long been known to be involved in the parasitism of plants by pathogenic fungi (Xu 2000; Lengeler et al. 2000). Since eukaryotic signaling mechanisms are well conserved, it is interesting to examine the role of these signaling elements in mycoparasitism, and hence biocontrol. The first direct evidence on the role of a G-protein came from Rocha-Ramirez et al. (2002). Antisense-mediated gene silencing of Tga1 attenuated mycoparasitism of *T. atroviride* against *R. solani*. On the other hand, transgenic strains carrying multicopies of the gene overgrew *R. solani* colonies at a faster rate. Using gene knockout, Reithner et al. (2005) demonstrated that Tga1 modulates chitinase formation and secondary metabolism in *T. atroviride*. The deletion of another G protein, Tga3 resulted in loss of mycoparasitism in *T. atroviride* (Zeilinger et al. 2005). Mukherjee et al. (2004) studied the role of the G-proteins TgaA and TgaB in *T. virens*. Deletion of these genes individually had no effect on hyphal coiling of R. solani, but TgaA was involved in the parasitism of sclerotia of *S. rolfsii*. Deletion of the MAPK TmkA in *T. virens* resulted in attenuation of sclerotial parasitism of *S. rolfsii* and *R. solani*, while the hyphal parasitism was unaltered (Mukherjee et al. 2003). The TmkA mutants also had reduced ability to

induce resistance in cucumber seedlings, even though there was no effect on root colonization (Viterbo et al. 2005). The mutants also had reduced biocontrol of *S. rolfsii* in greenhouse tests. In contrast, however, Mendoza-Mendoza et al. (2003) reported improvement in biocontrol potential of *T. virens* through inactivation of the MAP kinase Tvk1. Whether this apparent contradiction is due to strain differences needs to be examined carefully. Recently, a *T. harzianum* stress-response MAPK ThHOG1 has been identified to be involved in osmotic and oxidative stress response (Delgado-Jarana et al. 2006). Recently, Mukherjee et al. (2007) has cloned the adenylate cyclase-encoding gene *tac1* of *T. virens* and obtained knockout mutants through homologous recombination. The mutants grew extremely slowly, failed to germinate in water, were impaired in mycoparasitism and produced lower amounts of secondary metabolites. This study proved that the cAMP signaling is involved in growth, germination and biocontrol properties in *T. virens*. Using suppression subtractive hybridization, the genes regulated by signaling genes like the TmkA/Tvk1 in *T. virens* have been identified (Mukherjee M et al. 2006; Mendoza-Mendoza et al. 2007). These target genes include some novel genes like the mrsp1 (MAPK Repressed Secreted Protein) with an expansin-like domain that could be involved in Trichoderma-plant root interactions (Mukherjee PK et al. 2006). Recently Reithner et al. (2007) have examined the function of the tmk1 gene encoding a MAPK during fungal growth, mycoparasitic interaction, and biocontrol was examined in *T. atroviride*. Dtmk1 mutants exhibited altered radial growth and conidiation, and displayed de-regulated infection structure formation in the absence of a host-derived signal. In confrontation assays, tmk1 deletion caused reduced mycoparasitic activity although attachment to *R. solani* and *B. cinerea* hyphae was comparable to the parental strain. Under chitinase-inducing conditions, nag1 and ech42 transcript levels and extracellular chitinase activities were elevated in a Dtmk1 mutant, whereas upon direct confrontation with *R. solani* or *B. cinerea* a host-specific regulation of ech42 transcription was found and nag1 gene transcription was no more inducible over an elevated basal level. Dtmk1 mutants exhibited higher antifungal activity caused by low molecular weight substances, which was reflected by an over-production of 6-pentyl-a-pyrone and peptaibol antibiotics. In biocontrol assays, a Dtmk1 mutant displayed a higher ability to protect bean plants against *R. solani* (Reithner et al. 2007). These findings strongly suggest the presence of further, still unknown, mycoparasitism related factors which are missing in our Dtmk1 mutants and which are therefore affected by a signaling pathway involving Tmk1.

10.7 The Genomics and Proteomics

With the advent of genomics, it is not long before the whole genome sequences of many *Trichoderma* spp. would be available, thus allowing the analysis of gene expression and gene functions on a genome-wide scale, rather than looking at the individual genes. The first step in characterizing genes potentially involved in

biocontrol is to isolate and sequence them. Which strategy is most appropriate depends on previous results and on the objectives of the study. The targeted strategy should be privileged if the aim is to acquire further genetic knowledge on a well-studied mechanism of action. Differential gene expression techniques are very useful in original research aiming to isolate new genes potentially related to biocontrol properties when there is no a priori reason to suspect their involvement. Large-scale sequencing techniques, finally, support a broader endeavor: to improve genetic knowledge on a strain by sequencing numerous genes. Putative biocontrol genes may emerge from this endeavor. The genome sequencing of *T. virens* and *T. atroviride* is already under way, and is expected to be published soon (Charles Kenerley and Christian Kubicek, personal communication). In the meantime, quite a few ESTs database are available that identify hundreds of genes in various *Trichoderma* spp. Liu and Yang (2005) identified genes with biocontrol functions in *T. harzianum* mycelium using an ESTs approach – out of the 3298 clones sequenced, 673 represented novel genes. Suarez et al. (2005) identified a fungal cell wall induced aspartic protease from *T. virens* using the SSH approach. Carpenter et al. (2005) identified 19 novel genes in *T. hamatum* that are induced during mycoparasitism on *Sclerotinia sclerotiorum*. These included monooxygenases, metallopeptidases, gluconate dehydrogenase and endonuclease and a proton ATPase. Seidl et al. (2005) did a genome-wide search for chitinase genes in the *T. reesei* genome and identified three distinct subgroups of family 18 chitinases. Recently, an ESTs of 502 unique gene sequences of *T. virens* IMI 304061 have been deposited at the GenBank (PD Sherkhane, GV Sible and PK Mukherjee, unpublished) – this database includes many genes known to be involved in biocontrol and stress response. One of the earliest attempts to identify proteins from *T. harzianum* using the proteomic approach was by Grinyer et al. (2004a,b) who identified 25 proteins using 2D gel electrophoresis and LC MS/MS, either from mitochondria or whole cells. Using a proteomic approach, Grinyer et al. (2005) identified several novel proteins that were produced in response to mycoparasitism by *T. atroviride* on *B. cinerea* and *R. solani*. These included several hydrolytic enzymes also. Using a novel proteomic approach, Marra et al. (2006) studied the three-way interaction between *T. atroviride*, plant pathogens (*B. cinerea* and *R. solani*) and bean plant, in order to identify the proteins expressed in the various combination of host-pathogen-mycoparasite. This approach resulted in the identification of numerous differential proteins.

Trichoderma mycoparasitic activity depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall. Suarez et al. (2005) have analysed the extracellular proteome secreted by *T. harzianum* CECT 2413 in the presence of different fungal cell walls. This allowed them to overcome the problems associated with the lack of genome sequence data for the identification of non-conserved *Trichoderma* spp. proteins. Optimized 2DE protein profiles were compared to that obtained on chitin and the most abundant protein induced by fungal cell walls was identified as the novel pepsin-like aspartic protease P6281 by a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), liquid chromatography mass spectrometry (LC–MS/MS) and in

silico analysis of the available EST library. Significant differences were detected in 2DE maps, depending on the use of specific cell walls or chitin. A combination of MALDITOF and liquid chromatography mass spectrometry allowed the identification of a novel aspartic protease (P6281: MW 33 and pI 4.3) highly induced by fungal cell walls. A broad EST library from *T. harzianum* CECT 2413 was used to obtain the full-length sequence. The protein showed 44% identity with the polyporopepsin (EC 3.4.23.29) from the basidiomycete Irpex lacteus. Lower identity percentages were found with other pepsin-like proteases from filamentous fungi (<31%) and animals (<29%). Northern blot and promoter sequence analyses support the implication of the protease P6281 in mycoparasitism (Suarez et al. 2005).

In the future, numerous genes of various biocontrol agents will be identified by means of open strategies and with the help of advanced gene isolation and sequencing methods. The isolated genes will have to be characterized, and characterization will remain a bottleneck requiring much more time and effort than gene identification and sequencing.

10.8 The Transgenic Approach

Trans-kingdom transfer of genes for biocontrol from *Trichoderma* to plants to enhance disease resistance was, for the first time, demonstrated by Lorito et al. (1998). An endochitinase-encoding gene ech42, expressed in tobacco and potato provided near total protection against *Alternaria alternata*, *A. solani*, *B. cinerea* and *R. solani*. The high degree of broad-spectrum resistance was attributed to high fungitoxicity of *Trichoderma* chitinase, relative to the plant endogenous chitinases. This was followed by several reports on transfer of *Trichoderma* genes to plants. Bolar et al. (2001) produced transgenic apple resistant to *Venturia inaequalis* by expression of both an endochitinase and an exochitinase, and observed synergistic interaction. *T. harzianum* endochitinase, transferred to broccoli produced transgenic plants resistant to *Alternaria* leaf spot (Mora and Earle 2001). Liu and Yang (2005) produced transgenic rice resistant to blast and sheath blight by expressing ech42, nag70 and gluc78, in different combinations. A *T. virens* endochitinase gene ech42 transferred to cotton enhanced resistance against *A. alternata* and *R. solani* (Emani et al. 2003). Noël et al. (2005) introduced an endochitinase gene (ech42) from the biocontrol fungus *T. harzianum* into black spruce (*Picea mariana*) and hybrid poplar (*Populus nigraXP. maximowiczii*) by Agrobacterium-mediated transformation. Fifteen transgenic black spruce lines and six poplar lines were obtained. Northern hybridization analysis showed an increased accumulation of the transcript encoding the recombinant endochitinase gene in all the transgenic plants tested. Endochitinase activity 55–115 times the level of the control was detected in transformed poplar leaves. Embryogenic tissue of transgenic black spruce showed endochitinase activity two to eight times that of the non-transgenic line, despite stronger basal endogenous activity. In vitro assays using inoculated leaf disks demonstrated that the transgenic poplars had increased resistance to the leaf rust

pathogen *Melampsora medusae*. Seedlings of transgenic spruce lines showed an increased resistance to the spruce root pathogen *Cylindrocladium floridanum* in vitro. These results suggest that constitutive expression of the ech42 gene from *T. harzianum* could be exploited to enhance resistance to fungal pathogens in important forest tree species. Thus tree genetic engineering with endochitinase genes could provide an alternative to the use of fungicides and help reduce tree growth losses caused by phytopathogenic fungi. In a very recent report, expression of *T. harzianum* endochitinases to tobacco was shown to enhance resistance not only against pathogens, but also against abiotic stress, presumably through the release of some elicitors (de las Mercedes Dana et al. 2006). Contrary to these reports, expression of *T. atroviride* ech42 in transgenic alfalfa did not yield resistance against *Phoma medicaginis* var *medicaginis*, even though there was a 50- to 2650-fold greater chitinase activity in transgenic plants (Samac et al. 2004). The protection provided by expression of mycoparasitism-related genes in plants thus may not be universal.

Promoter analysis can be used to confirm molecular models of gene regulation deriving from studies carried out under various in vitro conditions. Published studies of the promoter regions of genes involved in biocontrol have focused on either promoter sequences or regulatory proteins. Some investigators have studied the promoter sequence of a gene in order to confirm the involvement of previously identified motifs in the regulation of its transcription under biocontrol conditions. Electromobility Shift Assays (EMSAs), in vivo footprinting, and/or promoter deletion analysis (Peterbauer et al. 2002a) are the techniques used. Regulatory proteins can influence gene transcription either directly (by binding to the promoter sequence or indirectly via signal transmission). The molecular tools are also used to inactivate genes coding for regulatory proteins. Peterbauer et al. (2002b) found that inactivation of the seb1 gene does not modify transcription of the nag1, chit33, and ech42 genes. They also showed that other proteins can bind to the 5′-AGGGG-3′ promoter motifs of nag1 and ech42 in the disrupted strain. In in vitro studies, Mukherjee et al. (2003) examined how inactivating two mitogen activated protein kinases (MAPKs) affect the mycoparasitic properties of *T. virens*. In many fungal species, MAPK proteins participate in cascade signals involved, e.g., in plant parasitism. The role of two G-protein α-subunits, TgaA and TgaB, in biocontrol by *T. virens* has been studied by Mukherjee et al. (2004). G-proteins play an important role in intracellular signaling. They amplify receptor responses and influence the amplitude and duration of cellular signals. Using null-TgaA and null-TgaB strains, these authors showed that TgaA is involved in the biocontrol activity against *S. rolfsii*, but that neither TgaA nor TgaB is required for its activity against *R. solani*. The authors conclude that the involvement of G-proteins in biocontrol by *T. virens* depends on the plant pathogen with which the biocontrol agent is in contact. Zeilinger et al. (2005) have shown that the tga3 gene of *T. atroviride*, also coding for a G-protein α-subunit, is involved in this biocontrol agent's vegetative growth and mycoparasitic activity.

Zhou et al. (2007) have recently used restriction enzyme mediated integration (REMI) technique to construct mutants with improved cyanide-degradation ability

from biocontrol fungus *T. koningii* strain T30. This successful insertional mutagenesis of the cyanide-biodegrading agent, *Trichodema* spp., led to the creation of mutants with deficient and enhanced cyanide-degrading properties. Liu et al. (2007) transformed three genes encoding for fungal cell wall degrading enzymes (CWDE), ech42, nag70 and gluc78 from the biocontrol fungus *T. atroviride* into rice mediated by *Agrobacterium tumefaciens* singly and in all possible combinations. These results indicated that expression of several genes in one T-DNA region interfered with each other and expression of exogenous gene in recipient plant was a complex behavior. It has been suggested that target gene must avoid being lost in transgenic process so as to be sure of expressing in transgenic plants and on the other hand, gene breaking and segregation in transgenic process can be used to delete selective gene so as to enhance transgenic security. This approach and the biological materials thus obtained could find a variety of applications in the discovery and manipulation of genes and gene products from *Trichoderma*.

10.9 Conclusion

Trichoderma spp. are a group of very useful fungi with many commercial applications in agriculture and industry. The popularity of *Trichoderma*-based biofungicides is growing by the day and hence, *Trichoderma*-based formulations have become an integral part of the crop management practices. However, an often-inconsistent performance vis-à-vis chemical fungicides is the major limitation associated with biological control. In order to improve the performance of *Trichoderma*-based formulations, we need to improve the strains for increasing its disease control potential (i.e., should be more effective than the existing strains against a particular target pathogen – this could be possible by generating strains with higher degree of mycoparasitism, competition, antibiosis and induced resistance), spectrum of activity (i.e., a single strain should be effective against a wide range of plant pathogens), as well as its survival ability (i.e., the ability to survive and perform under adverse environmental conditions). All these could be achieved if the physiology and genetics of these species are fully understood. However, until recently, this field of research remained largely neglected, which is apparent from the small proportion of the literature published on the mechanisms of biocontrol compared to the huge amount of work done on the biocontrol studies in laboratory, greenhouse and fields. Molecular techniques have been used to study the genetic basis of biological mechanisms and to identify partial or complete molecular pathways regulating gene expression. This is also true in the field of biocontrol. Whatever the technique used, it is paramount to choose an appropriate experimental model, as this will determine the reliability and validity scope of any conclusions drawn from an experiment. Molecular techniques have shed light on the antagonistic properties of numerous biological control agents, but they have also underlined the complexity of genetic regulation. They are now essential to studying the mechanisms of action of biocontrol agents and must be

included in comprehensive studies that should also include microbiological, biochemical, and microscopic approaches.

It is a matter of relief that this trend is changing with quite a few classical works published in recent years on this aspect that have contributed greatly to our understanding the system at the molecular level. For example, the work on the negative regulation of conidiation (conidia are important in survival of *Trichoderma* spp., and also form the major biomass of the formulation products) by a MAP kinase, positive regulation of secondary metabolism (antibiotics production) by the adenylate cyclase Tac1 of *T. virens,* and the identification of the elicitor protein Sm1 (when applied to seedlings, it offered protection against infection) could be cited as major recent contributions, at the basic level, that would directly help in genetically improving *Trichoderma* spp. In addition, several new genes have been identified in the *Trichoderma* ESTs database, like many heat shock proteins/pH response proteins, regulators of heat shock response and genes involved in signal transduction, that could directly be used to genetically transform *Trichoderma* spp. for improving the biocontrol/survival potential. Recently, Montero-Barrientos et al. (2007) demonstrated such a possibility – they have successfully imparted thermotolerance in *T. harzianum* by heterologous expression of a small heat shock protein gene *hsp23* from *T. virens*. Massart and Jijakli (2007) have reviewed the techniques used in such studies, with their potential and limitations. It should provide a guide for researchers wanting to study the molecular basis of the biocontrol in diverse biocontrol agents.

The ability of a biocontrol agent to respond quickly and adequately to an environmental signal such as the presence of a potential host is a key factor in the development of mycoparasitism or in the metabolization of plant nutrients. The study of regulatory proteins is thus essential to an in-depth understanding of the genetic basis of biocontrol properties. It notably highlights relationships between the environment and biocontrol gene expression. The induction or repression of gene expression in response to environmental signals may occur through various pathways. So far research has focused on G-proteins and MAPK pathways. Other candidate genes, like the Abc transporters or the OPT protein family, should be studied for their possible involvement in biocontrol. Once the whole genome sequences are available, we will know about the genetic blue-print of these organisms, and, by comparison with many other fungal genome sequences that are already available, it would be possible to have an idea of what makes *Trichoderma* spp. effective as biocontrol agents. By functional genomics approaches, it would also be possible to identify the functions of the genes that are unique to *Trichoderma* in structure and function. In addition to direct genetic manipulation, once we identify the genes responsible for biocontrol, it would also be possible to discover/design drugs that could act as stimulants for genes/gene products, thus improving their bio-efficacy. Finally, we should bear in mind that biological control is an outcome of very complex interactions between plant, pathogen, antagonist and the environment, and there is unlikely to be a quick-fix, single-step solution to the major problems associated with biocontrol, especially the low efficiency and inconsistency. A consorted approach taking into account all the relevant parameters,

including improvement of the antagonist for better inhibition of the pathogen, strengthening of the host plant, and improved survival potential of antagonists, would be helpful in bringing the biocontrol at par or even more effective than the chemical pesticides in terms of applicability under the field conditions.

References

Ait-Lahsen H, Soler A, Rev M, de la Cruz J, Monte E, Llobell A (2001) An antifungal exo-alpha-1,3-glucanase from the biocontrol fungus *Trichoderma harzianum*. Appl Env Microbiol 67:5833–5839

Baek JM, Howell CR, Kenerley CM (1999) The role of an extracellular chitinase from *Trichoderma* virens Gv29-8 in the biocontrol of *Rhizoctonia solani*. Curr Genet 35:41–50

Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride (T harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants Transgenic Res 10:533–543

Brunner K, Montero M, Mach RL, Peterbauer CK, Kubicek CP (2003) Expression of the ech42 (endochitinase) gene of *Trichoderma atroviride* under carbon starvation is antagonized via a BrlA-like cis-acting element. FEMS Microbiol Lett 218:259–264

Carpenter MA, Stewart A, Ridgway HJ (2005) Identification of novel *Trichoderma hamatum* genes expressed during mycoparasitism using subtractive hybridization. FEMS Microbiol Lett 251:105–112

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I, Herrera-Estrella A (1994) Characterization of ech42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci 10903–10907

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I, Herrera-Estrella A (1999) Role of the *Trichoderma harzianum* endochitinase gene, ech42, in mycoparasitism. Appl Environ Microbiol 65:929–935

Chet I, Benhamou N, Haran S (1998) Mycoparasitism and lytic enzymes. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 2. Enzymes, biological control and commercial applications. Taylor and Francis London, United Kingdom, pp 153–171

Cortes C, Gutierrez A, Olmedo V, Inbar J, Chet I, Herrera-Estrella A (1998) The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol Gen Genet 260:218–225

de las Mercedes Dana M, Limon MC, Mejias R, Mach RL, Benitez T, Pintor-Toro JA, Kubicek CP (2001) Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. Curr Genet 38:335–342

de las Mercedes Dana M, Pintor-Toro JA, Cubero B (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. Plant Physiol 142:722–730

Delgado-Jarana J, Sousa S, Gonzalez F, Rey M, Llobell A (2006) ThHog1 controls the hyperosmotic stress response in *Trichoderma harzianu*. Microbiol 152:1687–1700

Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM (2006a) Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma* virens induces plant defense responses and systemic resistance Mol Plant Microbe Interact. 19:838–853

Djonovic S, Pozo MJ, Kenerley CM (2006b) Tvbgn3, a beta-1,6-glucanase from the biocontrol fungus *Trichoderma virens*, is involved in mycoparasitism and control of *Pythium ultimum*. Appl Environ Microbiol 72:7661–7670

Donzelli BG, Lorito M, Scala F, Harman GE (2001) Cloning, sequence and structure of a gene encoding an antifungal glucan 1,3-beta-glucosidase from *Trichoderma atroviride (T harzianum*). Gene 277:199–208

Emani C, Garcia JM, Lopata-Finch E, Pozo MJ, Uribe P, Kim DJ, Sunilkumar G, Cook DR, Kenerley CM, Rathore KS (2003) Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*. Plant Biotechnol J 1:321–336

Flores A, Chet I, Herrera-Estrella A (1997) Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1 Curr Genet 31:30–37

Grinyer J, McKay M, Nevalainen H, Helbert BR (2004a) Fungal proteomics: initial mapping of biological control strain *Trichoderma harzianum*. Curr Genet 45:163–169

Grinyer J, McKay M, Helbert BR, Nevalainen H (2004b) Fungal proteomics: mapping of the mitochondrial proteins of a *Trichoderma harzianum* strain applied for biological control. Curr Genet 45:170–175

Grinyer J, Hunt S, McKay M, Herbert BR, Nevalainen H (2005) Proteomic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*. Curr Genet 47:381–388

Hanson LE, Howell CR (2004) Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. Phytopathol 94:171–176

Harman GE, Bjorkmann T (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 2. Enzymes, biological control and commercial applications Taylor and Francis London, UK, pp 229–265

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56

Hjeljord L, Tronsmo A (1998) *Trichoderma* and *Gliocladium* in biological control: an overview. In: GE Harman, CP Kubicek (eds) *Trichoderma* and *Gliocladium*, vol 2. Enzymes, biological control and commercial applications Taylor and Francis London, UK, pp 129–155

Howell CR (1987) Relevance of mycoparasitism in the biological control of *Rhizoctonia solani* by *Gliocladium virens*. Phytopathology 77:992–994

Howell CR (2002) Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp and its biological control with *Trichoderma* spp. Phytopathology 92:177–180

Howell CR (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis 87:4–10

Howell CR, Puckhaber LS (2005) A study of the characteristics of P and Q strains of *Trichoderma virens* to account for differences in biological control efficacy against cotton seedling diseases. Biol Control 33:217–222

Howell CR, Stipanovic RD (1995) Mechanisms in the biocontrol of Rhizoctonia solani- induced cotton seedling disease by Gliocladium virens: antibiosis. Phytopathology 85:469–472

Howell CR, Stipanovic RD, Lumsden RD (1993) Antibiotic production by strains of *Gliocladium virens* and its relation to the biocontrol of cotton seedling diseases. Biocontrol Sci Technol 3:435–441

Howell CR, Hanson LE, Stipanovic RD, Puckhaber LS (2000) Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology 90:248–252

Inbar J, Chet I (1992) Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibres. J Bacteriol 174:1055–1059

Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism- from biochemistry to genomics. Nat Rev Microbiol 3:937–947

Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev 64:746–785

Liu M, Zhu J, Sun Z, Xu T (2007) Possible suppression of exogenous β-1,3-glucanase gene gluc78 on rice transformation and growth. Plant Sci 172:888–896

Liu PG, Yang Q (2005) Identification of genes with a biocontrol function in *Trichoderma harzianum* mycelium using the expressed sequence tag approach. Res Microbiol 156:416–423

Lorito M, Mach RL, Sposato P, Strauss J, Peterbauer CK, Kubicek CP (1996) Mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter

sequences of the ech42 (endochitinase-encoding) gene in *Trichoderma harzianum*. Proc Natl Acad Sci USA 93:14868–14872

Lorito M, Woo SL, Garcia I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95:7860–7865

Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. Appl Microbiol Biotechnol 60:515–522

Mach RL, Peterbauer CK, Payer K, Jaksits S, Woo SL, Zeilinger S, Kullnig CM, Lorito M, Kubicek CP (1999) Expression of two major chitinase genes of *Trichoderma atroviride* (*T harzianum* P1) is triggered by different regulatory signals. Appl Environ Microbiol 65:1858–1863

Marra R, Ambrosino P, Carbone V, Vinale F, Woo SL, Ruocco M, Ciliento R, Lanzuise S, Ferraioli S, Soriente I, Gigante S, Turra D, Fogliano V, Scala F, Lorito M (2006) Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr Genet 50:307–321

Massart S, Jijakli HM (2007) Use of molecular techniques to elucidate the mechanisms of action of fungal biocontrol agents: a review. J Microbiol Met 69:229–241

Mendoza-Mendoza A, Pozo MJ, Grzegorski D, Martinez P, Garcia JM, Olmedo-Monfil V, Cortes C, Kenerley C, Herrera-Estrella A (2003) Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. Proc Natl Acad Sci USA 100:15965–15970

Mendoza-Mendoza A, Rosales-Saavedra T, Cortes C, Castellanos-Juarez V, Martinez P, Herrera-Estrella A (2007) The MAP kinase TVK1 regulates conidiation, hydrophobicity and the expression of genes encoding cell wall proteins in the fungus *Trichoderma virens*. Microbiology 153:2137–2147

Montero M, Sanz L, Rey M, Monte E, Llobell A (2005) BGN163, a novel acidic beta-1-6-glucanase from mycoparasitic fungus *Trichoderma harzianum*. CECT 2413 FEBS J 272:3441–3448

Montero-Barrientos M, Cardoza RE, Gutierrez S, Monte E, Hermosa R (2007) The heterologous overexpression of hsp23, a small heat-shock protein gene from *Trichoderma virens*, confers thermotolerance to *T harzianum*. Curr Genet 52:45–53

Mora A, Earle ED (2001) Combination of *Trichoderma harzianum* endochitinase and a membrane-affecting fungicide on control of *Alternaria* leaf spot in transgenic broccoli plants. Appl Microbiol Biotechnol 55:306–310

Mukherjee M, Horwitz BA, Sherkhane PD, Hadar R, Mukherjee PK (2006) A secondary metabolite biosynthesis cluster in *Trichoderma virens*: evidence from analysis of genes underexpressed in a mutant defective in morphogenesis and antibiotic production. Curr Genet 50:193–202

Mukherjee M, Mukherjee PK, Kale SP (2007) cAMP signaling is involved in growth, germination, mycoparasitism and secondary metabolism in *Trichoderma virens*. Microbiology 153:1734–1742

Mukherjee PK, Haware MP, Jayanthi S (1995a) Preliminary investigations in integrated biocontrol of *Botrytis* gray mold of chickpea. Ind Phytotpath 48:141–149

Mukherjee PK, Mukhopadhyay AN, Sarmah D, Shreshtha SM (1995b) Comparative antagonistic properties of *Gliocladium virens* and *Trichoderma harzianum* on *Sclerotium rolfsii* and *Rhizoctonia solani* – its relevance to understanding the mechanisms of biocontrol. J Phytopathol 143:275–279

Mukherjee PK, Latha J, Hadar R, Horwitz BA (2003) TmkA, a mitogen activated protein kinase of *Trichoderma virens*, is involved in biocontrol properties and repression of conidiation in the dark. Eukaryot Cell 2:446–455

Mukherjee PK, Latha J, Hadar R, Horwitz A (2004) Role of two G-protein alpha subunits, TgaA and TgaB, in the antagonism of *Trichoderma virens* against plant pathogens. Appl Environ Microbiol 70:542–549

Mukherjee PK, Hadar R, Pardovitz-Kedmi E, Trushina N, Horwitz BA (2006) *MRSP1*, encoding a novel *Trichoderma* secreted protein, is negatively regulated by MAPK. Biochem Biophys Res Commun 350:716–722

Mukhopadhyay AN, Mukherjee PK (1996) Fungi as fungicides. Int J Trop Plant Dis 14:1–17
Mukhopadhyay AN, Shrestha SM, Mukherjee PK (1992) Biological seed treatment for control of soilborne plant pathogens FAO. Plant Prot Bull 40:21–30
Noël A, Levasseur C, Le V-Q, Séguin A (2005) Enhanced resistance to fungal pathogens in forest trees by genetic transformation of black spruce and hybrid poplar with a *Trichoderma harzianum* endochitinase gene. Physiol Mol Plant Pathol 67:92–99
Olmedo-Monfil V, Mendoza-Mendoza A, Gomez I, Cortes C, Hererra-Estrella A (2002) Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene prb1 in *Trichoderma atroviride*. Mol Genet Genom 267:703–712
Olson HA, Benson DM (2007) Induced systemic resistance and the role of binucleate *Rhizoctonia* and *Trichoderma hamatum* 382 in biocontrol of *Botrytis blight* in geranium. Biol Control 42:233–241
Peterbauer C, Brunner K, Mach RL, Kubicek CP (2002a) Identification of the N-acetyl-D-glucosamine-inducible element in the promoter of the *Trichoderma atroviride* bag1 gene encoding N-acetyl-glucosamidase. Mol Genet Genomics 267:162–170
Peterbauer C, Litscher D, Kubicek CP (2002b) The *Trichoderma atroviride* seb1 (stress response element binding) gene encodes an AGGGG-binding protein which is involved in the response to high osmolarity stress. Mol Genet Genomics 268:223–231
Pozo MJ, Baek JM, Garcia JM, Kenerley CM (2004) Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens* Fungal Genet Biol 41:336–348
Reithner B, Brunner K, Schumacher R, Peissl I, Seidl V, Krska R, Zeilinger S (2005) The G protein alpha ?subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites Fungal Genet Biol 42:749–760
Reithner B, Brunner K, Schumacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the Trichoderma atroviride mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44:1123–1133; doi:101016/jfgb200704001
Rocha-Ramirez V, Omero C, Chet I, Horwitz BA, Herrera-Estrella A (2002) *Trichoderma atroviride* G-protein alpha subunit gene *tga1* is involved in mycoparasitic coiling and conidiation. Eukaryot Cell 1:594–605
Sakuno E, Yabe K, Hamasaki T, Nakajima H (2000) A new inhibitor of 5′-hydroxyaverantin dehydrogenase, an enzyme involved in aflatoxin biosynthesis, from *Trichoderma hamatum* J Nat Prod 63:1677–1678
Samac DA, Tesfaye M, Dornbusch M, Saruul P, Temple SJ (2004) A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). Transgenic Res 13:349–361
Sanz L, Montero M, Redondo J, Llobell A, Monte E (2005) Expression of an alpha-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J 272:493–499
Seidl V, Huemer B, Seiboth B, Kubicek CP (2005) A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. FEBS J 272:5923–5939
Seidl V, Marchetti M, Schandl R, Allmaier G, Kubicek CP (2006a) Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. FEBS J 273:4346–4359
Seidl V, Schmoll M, Scherm B, Balmas V, Seiboth B, Migheli Q, Kubicek CP (2006b) Antagonism of *Pythium* blight of zucchini by *Hypocrea jecorina* does not require cellulase gene expression but is improved by carbon catabolite derepression. FEMS Microbiol Lett 257:145–151
Shakeri J, Foster HA (2007) Proteolytic activity and antibiotic production by *Trichoderma harzianum* in relation to pathogenicity to insects. Enz Micro Technol 40(4):961–968
Shanmugam V, Sriram S, Babu S, Nandakumar R, Raguchander T, Balasubramanian P, Samiyappan R (2001) Purification and characterization of an extracellular alpha-glucosidase protein from *Trichoderma viride* which degrades a phytotoxin associated with sheath blight disease in rice. J Appl Microbiol 90:320–329
Shoresh M, Gal-On A, Leibman D, Chet I (2006) Characterization of a mitogen-activated protein kinase gene from cucumber required for *Trichoderma*-conferred plant resistance. Plant Physiol 142:1169–1179

Siddiqui IA, Shaukat SS (2004) *Trichoderma harzianum* enhances the production of nematicidal compounds in vitro and improves biocontrol of *Meloidogyne javanica* by *Pseudomonas fluorescens* in tomato. Lett Appl Microbiol 38:169–175

Sivasithamparam K, Ghisalbarti E (1998) Secondary metabolism. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 1. Basic biology, taxonomy and genetics Taylor and Francis London, UK, pp 139–191

Suarez MB, Sanz L, Chamorro MI, Rey M, Gonzalez FJ, Llobell A, Monte E (2005) Proteomic analysis of secreted proteins from *Trichoderma harzianum*. Identification of a fungal cell wall-induced aspartic protease. Fungal Genet Biol 42:924–934

Viterbo A, Chet I (2006) TasHyd1, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum* is involved in plant root colonization. Mol Plant Path 7:249–258

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, Chet I (2002a) Expression regulation of the endochitinase chit36 from *Trichoderma asperellum (T harzianum* T203). Curr Genet 42:114–122

Viterbo A, Ramot O, Chemir I, Chet I (2002b) Significance of lytic enzymes from *Trichoderma* spp in the biocontrol of fungal plant pathogens. Anton Leewenhoek 81:549–556

Viterbo A, Harel M, Horwitz BA, Chet I, Mukherjee PK (2005) *Trichoderma* mitogen-activated protein kinase signaling is involved in induction of plant systemic resistance. Appl Environ Microbiol 71:6241–6246

Vizcaino JA, Sanz L, Cardoza RE, Monte E, Gutierrez S (2005) Detection of putative peptide synthetase genes in *Trichoderma* species: application of this method to the cloning of a gene from *T harzianum* CECT 2413. FEMS Microbiol Lett 244:139–148

Wiest A, Grzegorski D, Xu BW, Goulard C, Rebuffat S, Ebbole DJ, Bodo B, Kenerley C (2002) Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J Biol Chem 277:20862–20868

Wilhite SE, Lumsden RD, Straney DC (1994) Mutational analysis of gliotoxin production by the biocontrol fungus *Gliocladium virens* in relation to suppression of Pythium damping-off Phytopathology 84:816–821

Wilhite SE, Lumsden RD, Straney DC (2001) Peptide synthetase gene in *Trichoderma virens*. Appl Environ Microbiol 67:5055–5062

Woo SL, Donzelli B, Scala F, Mach R, Harman GE, Kubicek CP, Del Sorbo G, Lorito M (1999) Disruption of the ech42 (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol Plant Microbe Interact 12:419–429

Xu JR (2000) Map kinases in fungal pathogens Fungal Genet Biol 31:137–152

Yates IE, Meredith F, Smart W, Bacon CW, Jaworski AJ (1999) *Trichoderma viride* suppresses fumonisin B1 production by *Fusarium moniliforme*. J Food Prot 62:1326–1332

Yedidia I, Benhamou N, Chet I (1999) Induction of defense responses in cucumber plants (Cucumis sativus L) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65:1061–1070

Yedidia I, Benhamou N, Kapulnik Y, Chet I (2000) Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol Biochem 38:863–873

Yedidia I, Shoresh M, Kerem Z, Benhamou N, Kapulnik Y, Chet I (2003) Concomitant induction of systemic resistance to *Pseudomonas syringae* pv *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl Env Microbiol 69:7343–7353

Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP (1999) Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 26:131–140

Zeilinger S, Reithner B, Scala V, Piessl I, Lorito M, Mach R (2005) Signal transduction by Tga3, a novel G protein alpha subunit of *Trichoderma atroviride*. Appl Environ Microbiol 71:1591–1597

Zhou X, Xu S, Liu L, Chen J (2007) Degradation of cyanide by *Trichoderma* mutants constructed by restriction enzyme mediated integration (REMI). Bio Res Technol 98:2958–2962