DISSERTATION

Trichoderma atroviride – Investigations on Regulation of Expression of Biocontrol Related Genes and Strain Improvement

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Deutschsprachige Zusammenfassung

Filamentöse Pilze der Gattung *Trichoderma* kommen in beinahe allen Bodentypen und Habitaten mit totem organischen Material vor. Ein breites Spektrum an lytischen Enzymen zum Abbau komplexer organischer Stoffe ermöglicht *Trichoderma* eine saprophytische Lebensweise und die Nutzung einer Vielzahl verschiedenartiger Kohlenstoff- und Stickstoffquellen.
Mit steigender Besorgnis über die Belastung von Boden und Grundwasser durch chemische Pestizide wurden Stimmen nach biologischen Alternativprodukten laut, und dies lenkte die Aufmerksamkeit zunehmend auf die Gattung *Trichoderma*. Manche *Trichoderma* spp. sind mykoparasitische Pilze, die pflanzenpathogene Pilze in deren Wachstum unterdrücken und kontrollieren können und sich damit hervorragend als Biokontrollorganismen eignen. Eine wesentliche Rolle im Antagonismus gegen andere Pilze übernehmen zellwandlytische Enzyme; im Besonderen Chitinasen erscheinen dabei von vorrangiger Bedeutung. Bisher sind zahlreiche chitinolytische Enzyme aus *Trichoderma* spp. bekannt, dennoch wurde erst eine sehr begrenzte Anzahl an Chitinase-kodierenden Genen isoliert und charakterisiert. In der vorliegenden Arbeit wird der molekulare Mechanismus der Regulation des Endochitinase-kodierenden Gens *ech42* (kodierend für die Endochitinase 42) aus *T. atroviride* untersucht. Ferner wird die Bedeutung des N-Acetylglukosaminidase-kodierenden Gens *nag1* für die Expression anderer chitinolytischer Enzyme von *T. atroviride* gezeigt. Letztlich wird ein transgener *Trichoderma* Stamm auf verbesserte Biokontrolleigenschaften getestet.

1. *ech42* Genexpression in *T. atroviride* unter Kohlenstoffmangelbedingungen. Bisher wurden nur wenige molekulare Untersuchungen zur Regulation des *ech42* Promotors durchgeführt, so zeigten z.B. Lorito *et al.* (1996) die Beteiligung von Cre1 (Carbon catabolite repressor protein) an der Regulation von *ech42* unter Biokontrollbedingungen. Für eine nähere Charakterisierung der Vorgänge an diesem Promotor unter Kohlenstoffmangelbedingungen wurden Reporterstämme konstruiert, die das *Aspergillus niger goxA*-Gen, das für Glukoseoxidase kodiert ,unter Kontrolle verschiedener regulierender Elemente des *ech42*-Promoters tragen. Dadurch konnte ein Bereich, der für die Induktion von *ech42* von Bedeutung ist, grob eingeschränkt werden. Zur genaueren Charakterisierung einzelner Motive der *ech42*-regulatorischen Sequenz wurden „Electrophoretic mobility shift assays" (EMSAs) durchgeführt, wodurch ein neuer, unbekannter DNA-bindender Faktor gefunden werden konnte.

2. Beteiligung von *nag1* an der Induktion von Chitinase-kodierenden Genen und deren Bedeutung für die Biokontrolle. Eine *Δnag1*-Disruptante sollte über die Beteiligung einer N-Acetylglukosaminidase an der Induktion des chitinolytischen Enzymsystems von *T. atroviride* Auskunft geben. Morphologisch war dieser *nag1*-negative Stamm vom Wildtyp nicht zu unterscheiden, dennoch sanken alle Chitinaseaktivitäten bei Induktion durch kolloidales Chitin drastisch. Wurde die Δ*nag1*-Disruptante mit einem funktionstüchtigen *nag1* Gen rücktransformiert, zeigte sie wieder dem Wildtyp vergleichbare Chitinaseaktivitäten. Trotz des Rückganges der Chitinaseaktivitäten *in vitro*, zeigte die Disruptante in Dual-Kultur Experimenten gegen *Rhizoctonia solani* und *Sclerotinia sclerotiorum* keine verminderten antagonistischen Fähigkeiten. Versuche zur Untersuchung der Biokontrolleigenschaften *in situ* offenbarten jedoch eine etwa 30 prozentige Reduktion der Protektion des Saatgutes gegen *R. solani* und *S. sclerotinia*.

3. Verbesserung der Biokontrolleigenschaften von *T. atroviride* durch Expression des für Glukoseoxidase kodierenden Gens *goxA* von *Aspergillus niger* unter Kontrolle des *nag1* Promoters. Zahlreiche Versuche zur Stammverbesserung von *T. atroviride* wurden bereits unternommen. Eine Überexpression von zellwandlytischen Enzymen führte aber nur zu einer geringfügigen Verbesserung der Biokontrolleigenschaften *in situ*. Die antagonistischen Fähigkeiten von *Talaromyces flavus* basieren vorwiegend auf der Sekretion von Glucoseoxidase, die Glukose zu Gluconsäure und Wasserstoffperoxid oxidiert. Um diese verschiedenen Ansätze für erfolgreiche Biokontrolle zu kombinieren, wurde ein transgener, Glucoseoxidase- produzierender *T. atroviride* Stamm getestet, in dem das für Glukoseoxidase kodierende Gen *goxA* aus *A. niger* unter Kontrolle des biokontrollabhängig regulierten *nag1* Promotors steht. *In vitro* Experimente zeigten eine deutlich Verbesserung der antagonistischen Eigenschaften des transgenen Stammes, *in situ* wurde eine verbesserte Kontrolle von pflanzenpathogenen Pilzen vorwiegend unter hohem Pathogendruck beobachtet.

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Chapter 1

General Introduction

1. General Introduction

1.1 The genus *Trichoderma*

Trichoderma is found worldwide in soil and other habitats containing dead organic material and only rarely this genus is associated with diseases of living plants (Domsch et. al. 1980). A broad variety of lytic enzymes allows saprophytic fungi the utilisation of complex carbon and nitrogen sources (Danielson et. al. 1973).
The genus *Trichoderma* has been known since the beginning of the 19th century. Although the link to the teleomorphs *Hypocrea* was discovered already in 1865 by the Tulasne brothers, an accurate taxonomy of *Trichoderma* remained obscure until recent decades. Rifai (1969) was the first to define nine different spezies aggregates to distinguish the isolates and established further teleomorph connections in cooperation with Webster (Rifai and Webster 1966, Webster and Rifai 1968). Bisset (1984, 1991a,b,c, 1992) carried out the most detailed morphological investigations of anamorphes and now distinguishes 21 taxa in sect. *Pachybasium* and seven in *Longibrachiatum*. No examinations of comparable accuracy have been carried out for the remaining sections.
In the recent 20 years, a new era of fungal systematics was initiated by the use of new molecular techniques. In particular analysis of ITS sequences of ribosomal DNA and fingerprinting are used as an effective taxonomic tool for resolving the systematics of *Trichoderma*. The most comprehensive molecular based studies on the genus *Trichoderma* have recently been published by Kullnig *et al.* (2002). Combined analysis based on sequences of the ITS 1, ITS2, D1 and D2 regions of the 28S rDNA, the small subunit of the mitochondrial rDNA, the fifth and part of the sixth exon of the gene encoding translation elongation factor 2, and a fragment of the endochitinase-encoding gene *ech42* showed that the genus *Trichoderma* is part of a monophyletic branch within the *Hypocreaceae*. The authors distinguished four clades: clade A comprises species of Bissett's (1991) sect. *Trichoderma* but also *T. hamatum, T. pubescens, T. asperellum*, and *T. strigosum*, clade B, on the other hand, contains a large and taxonomically heterogenous mixture of species, clade C comprises all the species contained in section *Longibrachiatum*, and clade D contains only *T. aureoviride* which is genetically most distant to all other species.

1.2. Biological control of plant pathogenic fungi by mycoparasitic species of *Trichoderma*

As plant pathogenic fungi are a severe threat to only a few different crops growing on a big area in modern monoculture–agriculture, farmers depend on the application of chemical fungicides to fight these pathogens. However, in recent years consumers are increasingly concerned about chemical pesticides as they tend to persist in soil and in food. Furthermore, pathogens become more and more resistant to available chemical products and consequently alternative methods for disease control have to be found. Reduction or even replacement of chemicals could be achieved through the application of biologically based fungicides.
The ability of *Trichoderma* to suppress plant pathogenic fungi was discovered years ago by Chet and Baker (1981). Certain soils were found to be highly supressive to the soil borne plant pathogen *Rhizoctonia solani*. Typical disease symtoms caused by *Rhizoctonia* did not occur, or occurred only in reduced strength in particular soil samples. Further investigations showed a high density of *Trichoderma* spores in these suppressive soils and inoculation of non-supressive soil with *Trichoderma* resulted in reduced disease symptoms of plants (Chet

and Baker, 1981). These promising antagonistic features provide *Trichoderma* as a superior alternative to, or powerful supplement of chemical fungizides. On the other hand, like all other living organisms *Trichoderma* is highly dependent on environmental factors. The species vary in their optimal temperature for growth and even the antagonistic activity is influenced by temperature (Köhl and Schlösser 1989, Tronsmo and Dennis 1978). In greenhouse experiments Elad *et al.* (1993) found that humidity effected the biocontrol ability even more than temperature did and the best disease control was achieved at 100% relative humidity. Caused by the sensitivity to abiotic factors and additionally by nutrient availability, the application of *Trichoderma* in field experiments shows variable results. Therefore, the basic mechanisms of biocontrol and the possibility of strain improvement remains to be further investigated.

1.2.1. Pathogen specifity

Trichoderma strains are usually effective only against specific pathogens, resulting in the problem that their spectrum of potential hosts could be too narrow for acceptable results in field applications. In fact, it was shown that one particular *Trichoderma* isolate could be highly effective against one specific isolate of a pathogen and has only minimal effects against other isolates of the same pathogen (Bell et. al. 1982). Therefore the authors suggested to combine different *Trichoderma* isolates to obtain satisfying results. Contrary to Bell (1982), Harman and co-workers (1989) demonstrated that *T. harzianum* strain 1295-22 provided good control against a broad range of pathogens, including *Pythium, Rhizoctonia, Fusarium, Sclerotium* and *Botrytis*. On the other hand, undesired green mould epidemics on mushrooms and damages of Shiitake have often been reported (Badham et. al. 1991, Tokimoto et. al. 1985) but Williams *et al.* (2003) demonstrated that only a few isolates of *T. harzianum* can harm *Agaricus* severely and the authors presented evidence that the antagonism of *Trichoderma* against mushrooms is caused mainly due to its competitive saprophytic abilities and not to aggressive mycoparasitism.

1.2.2. Mycorrhizae, effects of *Trichoderma* on plants, and rhizosphere competence

Mycorrhizal fungi promote plant growth in different ways, e. g. by increasing the water and mineral uptake of plant roots and the alteration of root exudates affects other microorganisms in the rhizosphere (Linderman et. al. 1988). Concerning the effect of *Trichoderma* species to mycorrhizal fungi, contradictionary results were found. *T. harzianum* and *T. konigii* were reported to significantly reduce the growth of the mycorrhizal fungus *Glomus mossae* (Wyss et. al. 1992, McAllister et. al. 1994a,b) and a *T. harzianum* isolate even penetrated and grew within the cells of *Glomus intraradices* (Rousseau et. al. 1996). *In vitro* investigations with *T. aureoviride* indicated enhanced spore germination of *G. mossae* and furthermore, synergistic effect on plant growth promotion could be observed when *T. aureoviride* and *G. mossae* were applied together (Calvet et. al. 1993).

Oppositional conclusions are drawn concerning plant growth promotion or inhibition caused by different *Trichoderma* isolates. For many years *Trichoderma* is reported to improve plant growth (Lindsay and Baker 1967). The enhancement of plant growth is not restricted to *Trichoderma* strains, as *Fusarium, Rhizopus, Chaetomium* and *Penicillium* isolates are also reported to increase the growth rates of plants (Lindsey and Baker 1967, Shivanna et. al. 1996). Experiments using soil treated with *T. harzianum* conidial suspensions resulted in enhanced germination of seeds, more rapid flowering and increased height and weight of

plants (Chang et. al. 1986, Harman et. al. 1989). Naseby *et al.* (2000) tested five strains of *Trichoderma* for their ability to promote plant growth in presence and in absence of *Pythium*. Only two strains, namely *T. harzianum* T4 and N47 showed significant beneficial characteristics in improvement of plant growth and *Pythium* control. Inbar *et al.* (1994) used *T. harzianum* T-203 in a commercial greenhouse production system for vegetables and achieved increased seedling height and dry weight at the time of marketing. It is not yet clear how *Trichoderma* acts to promote plant growth. The *T. harzianum* isolate T-203 was able to penetrate and live in plant roots similar to mycorrhizal fungi. (Kleinfeld and Chet 1992). Electron microscopic observations of *T. harzianum*-colonized cucumber roots showed that the fungal hyphae penetrate into the root, restricted to the epidermis and outer cortex (Yeddida et. al. 1998). Strengthening of epidermica and cortical cells was observed and additionally, the peroxidase and chitinase activity of the plant increased significantly in roots and leaves, providing strong evidence for the induction of the plants systemic acquired resistance (Yeddida et. al. 1998). Windham *et al.* (1986) suggested that *Trichoderma* secretes an until now unknown growth-promoting diffusable factor as seeds of maize, tomato and tobacco exhibited enhanced growth rates even when separated from homogenized mycelia of *T. harzianum* and *T. koningii* by a cellophane membran. Furthermore, *Trichoderma* can activate the induced resistance in plants, a plant defense system to protect not only the roots but the whole plant against pathogen attack. *T. viride, T. hamatum* and *T. harzianum* produce a 22 kDa xylanase that induces plant defense responses like PR-protein synthesis (Lotan and Fluhr 1990) and ethylene biosynthesis (Fuchs 1989).
In contrast, *Trichoderma* is known to secrete not only antifungal substances but also herbicidal compounds. Gliotoxin and viridin inhibited the germination and root growth of mustard seeds at concentrations of only 1 ppm in petri dishes but were not inhibitory to wheat or clover (Wright et. al. 1951). Another compound with considerable herbicidal activity is viridiol. Viridiol secreted by *Gliocladium virens* is known to be effective against a broad range of plant species and viridiol producing strains are even suggested to be necrotic pathogens (Jones et. al. 1987, 1988). Pulverized preparations of *G. virens* were successfully applied to suppress pigweed germination in infested soil (Howell et. al. 1984).
Another important factor to provide protection of seeds and roots by *Trichoderma* is the rhizosphere competence. Rhizosphere competence means the ability of an organism to establish itself in the rhizosphere of plants and it is influenced by the environment and the competition with other microorganisms in the soil (Harman, 1992). Most *Trichoderma* wild type strains have very limited ability to colonize the rhizosphere. Chemical mutation of *T. harzianum, T. koningii, T. polysporum* and *T. viride* and selection for benomyl-resistance resulted in improved rhizosphere competence. While wild type strains failed to colonize soil beyond 3 cm away from the root, these mutants colonized the entire system, up to 8 cm from the root tips (Ahmad and baker 1987). Another strain with improved rhizospere competence was produced by protoplast fusion of *T. harzianum* strains T12 and T95. The resulting strain T1295-22 was able to colonize maize roots to a depth of 22 cm and cotton roots to a depth of 16 cm (Sivan and Harman 1991) and increased root and shoot growth of corn in greenhouse studies to an average of 66% over untreated controls (Björkman et. al. 1998).

1.2.3. Antagonistic mechanisms of *Trichoderma*

Three different mechanisms, namely antibiosis, mycoparasitism (including secretion of cell wall lytic enzymes) and competition for nutrients are involved in the antagonistic interaction between *Trichoderma* and its host. However, a particular host – mycoparasite interaction can fall into more than one of these categories as, e. g. the production of toxic metabolites is

affected by available nutrients (Howell et. al. 1994) and antibiotics can act synergistically with cell wall lytic enzymes (Schirmböck et. al. 1994).

Competition

Competition occurs when two or more organisms demand more of the same resources than is immediately available. Competition between a plant pathogen and a biocontrol oganism may result in reduced growth of the pathogen due to the limitation of nutrients. Two different forms of competition have to be distinguished: (i) interference competition involves mechanisms by which one organism limits another organism's access to substrates. (ii) exploitation competition means competition for nutrients without directly limiting other organisms (Lockwood et. al. 1992, Wicklow et. al. 1992).

Antibiosis

Trichoderma strains produce a variety of secondary metabolites with inhibitory effect to other microorganisms. The first substance to be found, isolated and characterized was gliotoxin (Weindling et. al. 1936). Other antifungal metabolites produced by *Trichoderma* strains are viridin and gliovirin (Howell et. al. 1983). 6-n-pentyl-2H-pyran-2-one (PPT) is characteristic for some particular isolates (Claydon et. al. 1987). Serveral substances like alamethicine (Brewer et. al. 1987), trichorzianine (Rebuffat et. al. 1989), trichocellines (Wada et. al. 1994), atroviridines (Seung-Uk et. al. 2000) and trichosetin (Marfori et. al. 2002a,b), that exhibit antifungal activity *in vitro*, have been isolated and characterized. *Trichoderma* species groups differ in their ability to produce antibiotic substances. Further, the inhibitory effect of the different antifungal metabolites is highly dependent on the plant pathogens (Claydon 1987, Dennis and Webster 1971a,b, Howell et. al. 1995).
It is suggested that the inhibitory effect of *Trichoderma* is not only due to the antibiotics alone but caused by the synergistic activity of antifungal metabolites and hydrolytic enzymes.

Mycoparasitism

The term mycoparasitism describes the direct attack of the host fungus by the *Trichoderma* hyphae followed by the nutrient utilisation by the parasite.
Chet *et al.* (1981, 1990) demonstrated that *Trichoderma* can detect its potential host from a distance and grows directed towards it. As all experiments showed high specifity of particular *Trichoderma* isolates to only a limited number of hosts, it is considered that the molecular recognition of the partners is essential prior to antagonistic activities. Elad *et al.* (1983) demonstrated the role of lectins in the recognition of the plant pathogen *R. solani by T. harzianum. R. solani* appears to contain a particular lectin that specifically interacts with a suitable binding site on the *Trichoderma* cell wall. Another lectin was isolated from the plant pathogen *Sclerotium rolfsii* that absorbs only to *T. hamatum* T-244, the antagonist of *S. rolfsii* (Chet et. al. 1990). Inbar and Chet (1994) purified a second *Sclerotium* lectin and developed a biomimetic system in which the purified lectin was bound to nylon fibers with a diameter similar to *S. rolfsii* hyphae. *Trichoderma* showed typical mycoparasitic behaviour like coiling and appressoria formation when confronted with these fibers but not when confronted with uncoated or protein coated fibers.

Following recognition, *Trichoderma* hyphae attach to the host hyphae, coil around them and form hook-like stuctures (Elad et. al. 1983, Harman et. al. 1981). This typical attachment is the last step before the lysis of the host.

Secretion of lytic enzymes

Degradation of fungal cell walls by *Trichoderma* is mainly due to chitinase, protease and glucanase activity (Elad et. al. 1982). After attachment and coiling *Trichoderma* hyphae were removed from the hyphae of plant pathogens and left lysed sites and penetration holes on the hosts. High concentrations of β-glucan, chitotriose and N-acetyl-D-glucosamine could be detected at the interaction sites (Elad et. al 1983). The connection between cell wall lytic enzymes and the ability to parasitize a particular host was examined by Elad *et al.* (1984). It was found that particular *Trichoderma* strains that were able to attack a pathogen, secreted significantly more glucanases and chitinases than strains that were incapable of attack. Most experiments dealing with the regulation of cell wall lytic enzymes were performed in liquid cultures with different inducers and a few reports describe the regulation of the expression of lytic enzymes during mycoparasitism in plate confrontation experiments (Inbar et. al. 1995, Flores et. al. 1996, Carsolio et. al. 1994, Haran et. al 1996, Zeilinger et. al. 1999, Viterbo et. al. 2002). However, they do not always reflect the conditions present in the complex environment in the rhizosphere.

1.3. Applications of *Trichoderma*

The ability of *Trichoderma* to control plant disease and to promote plant growth has been known for a long time (Weindling et. al. 1932, 1934, 1941, Lindsay et. al. 1967). In spite of promising results, many years passed before biocontrol products based on *Trichoderma* became commercially available.

Two different kinds of protection must be provided by a suitable biocontrol organism:

I) Seed protection. As plants are most vulnerable to pathogen attack for a short time after sowing, an antagonist needs to be effective for a period of 7 to 14 days after planting. In several reports *Trichoderma* has been tested for this purpose (Jensen et. al. 1995, Harman et. al. 1991, Howell et. al. 1991, Jin et. al. 1991). *Pythium* is the primary seed rotting fungus and starts to infect seeds within 4 – 6 hours after they are planted. As *Trichoderma* spores germinate more slowly, the antagonistic fungus is at a temporal disadvantage (Harman et. al. 1994). Since timing is critical, methods were developed to compensate this handicap. Either antibiotic producing strains can be used, since the biocontrol preparation contains effective levels of the antibiotic (Howell et. al. 1991, 1983) or an additional Agro-Lig layer is applied to the *Trichoderma* coated seeds. This treatment delayed infection by *Pythium* for several hours (Taylor et. al. 1991).

II) The second kind of protection is the long term protection of subterranean parts of a plant. Rhizosphere competent biocontrol fungi can grow near the root surface but they differ in their ability to grow fast enough to inhabit the root tip. *T. harzianum* 1295-22 is used commercially (sold as T22) for long term protection, since this strain exhibits superior rhizosphere competence.

In greenhouses, the application of biocontrol agents is less complex than in fields. As sterile composts are used, there is no microflora that competes with antagonistc fungi (Harman 1998). Additionally, environmental factors like moisture and temperature are usually well controlled. For greenhouse applications there are several different products commercially

available including composts fortified with *T. hamatum* 382 and granulates containing a gliotoxin-producing *T. virens* isolate or *T. harzianum* 1295-22, respectively.

1.4. References

Ahmad J.S., Baker R. (1987). Rhizospere competence of *Trichoderma harzianum*. Phytopathology 77:182-189.

Badham E. (1991). Growth and competition between *Lentinus* and *Trichoderma harzianum* on sawdust substrates. Mycologia 83:455-463.

Bell D.K., Wells. H.D. Markham C.H. (1982). In vitro antagonism of *Trichoderma* species against six fungal plant pathogens. Phytopathology 73:379-382.

Bisset J. (1984). A revision of the genus *Trichoderma* I. Section *Longibrachiatum*, new section. Can. J. Bot. 62:924-931.

Bisset J. (1991a). A revision of the genus *Trichoderma* II. Infragerneric classification. Can. J. Bot. 69:2357-2373.

Bisset J. (1991b). A revision of the genus *Trichoderma* III. Section *Pachybasium*. Can. J. Bot. 69:2373-2417.

Bisset J. (1991c). A revision of the genus *Trichoderma* IV. Additional notes on section *Longibrachiatum*. Can. J. Bot. 69:2418-2420.

Bisset. J. (1992). *Trichoderma atroviride*. Can. J. Bot. 70:639-641.

Björkman T., Blanchard L.M., Harman G.E. (1998). Growth enhancement of shrunken 2-sweet corn by *Trichoderma harzianum* 1295-22. J. Am. Soc. Hort. 123:35-40.

Brewer D., Mason F.G., Taylor L. (1987). The production of alamethicins by *Trichoderma* spp. Can. J. Mircrobiol. 33:619-625.

Calvet C., Pera J., Barea J.M. (1993). Growth response of marigold to inoculation with *Glomus mossae*, *Trichoderma aureoviride* and *Pythium ultimum* in a peat-berlite mixture. Plant and Soil 148:1-6.

Chang Y.C. Chang Y.C., Baker R. (1986). Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. Plant Dis. 70:145-148.

Chet I., Harman G.E., Baker R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium ultimum*. Microb. Ecol. 7:29-38.

Chet I., Baker R. (1981). Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. Phytopathology 71:286-290

Claydon N., Allon M., Hanson J.R., Avent A.G. (1987). Antifungal alkyl-pyrones of *Trichoderma harzianum*. Trans. Br. Mycol. Soc. 88:503-513.

Danielson R.M., Davey C.B. (1973). Carbon and nitrogen nutrition of *Trichoderma*. Soil Biol. Biochem. 5:505-515.

Dennis C., Webster J. (1971). Antagonistic properties of species groups of *Trichoderma* I. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. 57:25-39.

Dennis C., Webster J. (1971). Antagonistic properties of species groups of *Trichoderma* II. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. 57:41-48.

Domsch K.H., Gams W., Anderson T.H. (1980). Compendium of soil fungi, Vol. 1. Academic Press, London.

Elad Y, Chet I., Henis Y. (1982), degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can J. Microbiol. 28:719-25

Elad Y., Chet I., Boyle P., Henis Y. (1983). Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii* – SEM studies and fluorescence microscopy. Phytopathology 73:85-88.

Elad Y., Barak R., Chet I. (1984). Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*. Soil Biol. Biochem. 16:381-386.

Elad Y., Kirshner B., (1993) survival in the phylloplane of an introduced biocontrol agent (*Trichoderma harzianum*) and populations of the plant pathogen *Botrytis cinerea* as modified by abiotic conditions. Phytoparasitica 21:303-313.

Fuchs Y., Saxena A., Gamble H.R., Anderson J.D. (1989). Ethylene biosynthesis induction protein from cellulysin is an endoxylanase. Plant physiol. 89:138-142.

Haran S., Schickler H., Oppenheim A., Chet I. (1996). Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. Phytopathology 86:980-985.

Harman G.E., Chet I., Baker R. (1981). Factors effecting *Trichoderma hamatum* applied to seeds as a biocontrol agent. Phytopathology 71:569-572

Harman G.E. Jin X., Stasz T.E., Peruzzotti G. Leopold A.C., Taylor A.G. (1991). Production of conidial biomass of *Trichoderma harzianum* for biological control. Biol. Control 1: 23-28.

Harman G.E. (1991). Seed treatments for biological control of plant disease. Proc. AAS Symposium. Crop Protect. 10:166-171.

Harman G.E. (1992). Development and benefits of rhizosphere competent for biological control of plant pathogens. J. Plant Nutr. 15:835-843.

Harman G.E., Nelson E.B. (1994). Mechanisms of protection of seed and seedlings by biological seed treatments: implications for practical disease control. In T. Martin (ed), Seed Treatment: Progress and Prospects. British Cropp Protection Council.

Howell C.R., Stipanovic R.D. (1983). Gliovirin, a new antibiotic from *Gliocladium virens*, and its role in the biological control of *Pythium ultimum*. Can. J. Microbiol. 29:321-324.

Howell C.R., Stipanovic R.D. (1984). Phytotoxicity to crop plants and herbicidal effects on weeds of viridiol produced by *Gliocladium virens*. Phytopathology 74:1346-1349.

Howell C.R., Stipanovic R.D. (1995). Mechanisms in the biocontrol of *Rhizoctonia solani*-induced cotton seedlings by *Gliocladium virens*: antibiosis. Phytopathology 85:469-472.

Howell C.R. (1991). Biological control of *Pythium* damping.off of cotton with seed coating preparation of *Gliocladium virens*. Phytopathology 81:738-741

Inbar J, Chet I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. J Bacteriol. Feb;174(3):1055-9.

Inbar J., Chet I. (1994). A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and its role in mycoparasitism. Microbiology 141:2823-2829.

Jensen D.F., Wolffhechel H. (1995). The use of fungi, particularly Trichoderma spp. and Gliocladium spp. to control root rot and damping.off diseases. Biocontrol Agents: Benefits ad Risks. Cambridge University Press.

Jin X., Hayes C.K. Harman G.E. (1991). Principles in the development of biological control systems employing *Trichoderma* spp. against soil borne plant pathogenic fungi. Symposium on industrial Mycology, Am. Mycol. Soc., Brock-Springer series in Contemporary Biosciences.

Jones R.W., Lanini W.T., Hancock J.G. (1988). Plant growth response to the phytotoxin viridiol produced by the fungus *Gliocladium virens*. Weed. Sci. 36: 683-687.

Kleifeld O., Chet I. (1992). *Trichoderma harzianum* – interactions with plants and effect on growth response. Plant Soil. 144:267-272.

Köhl J., Schlösser E. (1989). Decay of sclerotia of *Botrytis cinerea* by *Trichoderma* spp. at low temperatures. J. Phytopathol. 125:320-326.

Kullnig-Gradinger CM, Szakacs G, Kubicek, C.P. (2002) Phylogeny and evolution of the genus Trichoderma: a multigene approach. Mycol Res, 106: 757 – 767

Linderman R.G. (1988). Mycorrhizal interactions with the rhizosphere microflora. Phytopathology 78: 366-371.

Lindsay D.L., Baker R. (1967). Effect of certain fungi on dwarf tomatoes grown under gnobiotic conditions. Phytopathology 57:1262-1263.

Lockwood J.L. (1992). Exploitation competition. In: The fungal Community. Its organisation and role in the ecosystem. Marcel Dekker, NY.

Lotan T., Fluhr R. (1990). Xylanase, a novel elicitor of pathogenesis related proteins in tobacco, uses a non-ethylene pathway for induction. Plant Physiol. 93:811-817.

Marfori EC, Kajiyama S, Fukusaki E, Kobayashi A. Phytotoxicity of the tetramic acid metabolite trichosetin. Phytochemistry. 2003 Mar;62(5):715-21.

Marfori EC, Kajiyama S, Fukusaki E, Kobayashi A. Trichosetin, a novel tetramic acid antibiotic produced in dual culture of *Trichoderma harzianum* and *Catharanthus roseus* Callus. Z Naturforsch [C]. 2002 May-Jun;57(5-6):465-70.

McAllister C.B., Garcia-Romera I., Godeas A., Ocampo J.A. (1994a). Interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mossae*. Soil Biol. Biochem. 26:1363-1367.

McAllister C.B., Garcia-Romera I., Godeas A., Ocampo J.A. (1994b). *In vitro* interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mossae*. Soil Biol. Biochem. 26:1369-1374.

Naseby DC, Pascual JA, Lynch JM. (2000). Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* polulations, soil microbial communities and soil enzyme activities. J Appl Microbiol. Jan;88(1):161-9.

Rebuffat S., El Hanjji M., Henning P., Davoust P., Bodo B. (1989). Isolation, sequence and conformation of seven trichorzianines B from Trichoderma harzianum. Int. J. Peptide Protein Res. 34 :200-210.

Rifai M.A. (1969). A revision of the genus *Trichoderma*. Mycol Pap. 116:1-56

Rifai M.A. and Webster J. (1966). Culture studies on *Hypocrea* and *Trichoderma*. III. *H. pulifera*. Trans. Br. Mycol. Soc. 51:511-514

Rousseau A., Benhamou N., Chet. I., Piche Y. (1996). Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*. Phytopathology 86:443-442.

Schirmbock, M., M. Lorito, Y. L. Wang, C. K. Hayes, I. Arisan-Atac, F. Scala, G. E. Harman, and C. P. Kubicek. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl Environ Microbiol 60:4364-70

Seung-Uk O., Sang-Jun L., Jung-Han K., Ick-Dong Y. (1999). Structural elucidation of new antibiotic peptides, atrovirins A, B and C from *Trichoderma harzianum*. Terahedron Letters 41:61-64.

Shivanna M.B., Meera M.S., Kageyama K., Hyakumachi M. (1996). Growth promotion ability of zoysiagrass rhizosphere fungi in consecutive plantings of wheat and soybean. Mycoscience 37: 163-168.

Sivan A., Harman G.E. (1991). Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum*. J. Gen. Microbiol. 137:23-29.

Taylor A.G., Min T.G., Harman G.E., Jin X. (1991). Liquid coating formulation for the application of biological seed treatments of *Trichoderma harzianum*. Biol. Control 1:16-22.

Tokimoto K. (1985). Physiological studies on antagonism between *Lentinus edodes* and *Trichoderma* spp. in bedlogs of the former. Rep. Tottori. Mycol. Inst. 23:1-54.

Tronsmo A. and Dennis C. (1978). Effect of temperature an antagonistic properties of *Trichoderma* species. Trans Br. Mycol. Soc. 71:469-474.

Viterbo A, Haran S, Friesem D, Ramot O, Chet I. 2001. Antifungal activity of a novel endochitinase gene (*chit36*) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200, 169-174

Wada S., Nishimura T., Iida A., Toyama N., Fujita T. (1994). Primari structures of antibiotic peptides, Trichocellins A and B from *Trichoderma viride*. Tetrahedron Letters 35(19):3095-3098.

Webster J. and Rifai M.A. (1968). Culture studies on *Hypocrea* and *Trichoderma*. IV. *H. lacteal* and *H. pulvinata.*

Weindling R. (1932). *Trichoderma ligniorum* as a parasite of other soil fungi. Phytopathology 22:837-845.

Weindling R. (1934). Studies on a lethal principle effective in the parasitic action of *Trichoderma*. Phytopathology 24:1153-1179.

Weindling R. (1941). Experimental consideration of the mold toxins of *Gliocladium* and *Trichoderma*. Phytopathology 31: 991-1003.

Wicklow D.T. (1992). Interference competition. In: The fungal Community. Its organisation and role in the ecosystem. Marcel Dekker, NY.

Williams J, Clarkson JM, Mills PR, Cooper RM. (2003). Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. Appl Environ Microbiol. Jul;69(7):4192-9.

Windham M.T., Elad Y., Baker R. (1986). A mechanism for increased plant growth induced by *Trichoderma* spp. Phytopathology 76:518-521.

Wright J.M. (1951). Phytotoxis effects of some antibiotics. Ann. Bot. 15: 493-499.

Wyss P., Boller T., Wiemken A. (1992). Testing the effect of biocontrol agents on the formation of vesicular arbuscular mycorrhiza. Plant and soil 147: 159-162.

Yedidia, I. I., N. Benhamou, and I. I. Chet. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) By the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65:1061-70.

Zeilinger, S., C. Galhaup, K. Payer, S. L. Woo, R. L. Mach, C. Fekete, M. Lorito, and C. P. Kubicek. 1999. Chitinase gene expression during mycoparasitic interaction of Trichoderma harzianum with its host. Fungal Genet Biol 26:131-40

Chapter 2

Aim of the work

As this thesis deals with two distantly related topics, it is divided into two main parts. The first section comprises investigations on two chitinases of *Trichoderma atroviride*, including examinations on the 5' regulatory region of *ech42* and the impact of the deletion of the Nag1 encoding gene *nag1* deletion on chitinase gene expression and biocontrol. The second section focuses on strain improvement of *T. atroviride* by heterologous expression of the *Aspergillus niger goxA* gene under control of the biocontrol related *nag1* promoter.

2.1. Investigations on regulation of chitinase gene expression in *T. atroviride*

***ech42* gene expression under carbon starvation conditions in *Trichoderma atroviride*.** *In silico* analysis of the *ech42* regulatory region in *T. atroviride* revealed some putative binding elements for transcriptional regulators known from other fungi, namely three binding sites for the carbon catabolite repressor CreA (SYRGRG) (Kulmburg *et. al.* 1993), four sites corresponding to the *Saccharomyces cerevisiae* Msn2/4 binding motif (AGGGG) (Marchler *et. al.* 1993), and finally three putative binding sites for the BrlA protein to *Aspergillus* (MRAGGGR) (Chang *et. al.* 1992). The carbon catabolite repressor Cre1 is known to bind to promoters of various fungal genes and down-regulates their expression (Dowzer et. al. 1991). Lorito *et al.* (1996) demonstrated, that a DNA-binding protein of *T. atroviride* contacts Cre1 binding sites in the *ech42* promoter *in vitro* and that these sites are released from protein-DNA binding under mycoparasitism-induced conditions. The DNA-binding motif for Msn2/4 is found in stress responding genes in yeast. This AGGGG-element is also present in the 5' regulatory region of *chit33*, a *T. harzianum* endochitinase-encoding gene inducible by various stress conditions and regulated by carbon and nitrogen levels comparable to *ech42* in *T. atroviride*. Furthermore, three copies of the BrlA-binding motif can be found in the upstream noncoding region of *ech42*. BrlA is responsible for conidiophore development in *Aspergillus* and thereby causes the switch from vegetative growth to asexual conidiation (Adams *et. al.* 1999). Glucose oxidase activity can be detected on the surface of spores from an *ech42*::*goxA* reporter strain (Brunner, unpublished findings) , whereof can be deduced, that endochitinase activity presumably is presented the conidia surface, too. Additionally, like *ech42* the BrlA-encoding gene is regulated by carbon and nitrogen limitation.
As these preliminary investigations point to the importance of the above described motifs in regulation of *ech42* gene transcription, a deeper insight into the underlying molecular mechanisms should be obtained. Therefore, reporter strains containing the *A. niger* glucose oxidase-encoding gene *goxA* under control of native and mutated *ech42* upstream regulatory sequences will be used to provide a first insight. Regions with a major impact on reporter gene induction are to be further characterized by electrophoretic mobility shift assays to identify whether or not the above mentioned motifs are contacted by DNA-binding proteins *in vitro*.

Involvement of *nag1* from *T. atroviride* in chitinase gene induction and biocontrol. In contrast to *T. harzianum* and *T. virens* which are known to express two N-acetylglucosaminidases to date, only one has been found in *T. atroviride*. The corresponding gene (*nag1*) encoding for this 73 kDa exochitinase has been isolated and characterised by Peterbauer *et al.* (1996). Disruption of *nag1*, thereby eliminating the entire N-acetylglucosaminidase activity, should provide more information about the involvement of this gene in biocontrol of plant pathogenic fungi by *T. atroviride*. The Δ*nag1*-disruptant strain has previously been constructed and characterized at its molecular level by Clemens Peterbauer.

This strain had to be tested in comparison with the wild type *T. atroviride* P1 concerning general morphology, growth rate on different media, and sporulation. Furthermore, the chitinolytic activity on substrates inducing endochitinase, chitobiosidase and N-acetylglucosaminidase have to be examined to elucidate the role of Nag1 in induction of other chitinases. Additionally, this N-acetylglucosaminidase-negative strain has to be tested for its biocontrol ability *in vitro* and *in situ*.

2.2. Improvement of the biocontrol activity of *Trichoderma atroviride* P1 by expression of multiple copies of the *Aspergillus niger* glucose oxidase gene *goxA* under a chitinase (*nag1*) promoter

Trichoderma is known to be one of the most efficient biocontrol organisms and is already used for commercial greenhouse and field applications. However, until now the biological control of plant pathogens cannot really compete with chemical fungicides as the antagonistic action of *Trichoderma* is often limited to only a few pathogens. Previous attempts of strain improvement by overexpression of chitinase-encoding genes, their fusion to strong promoters (Limon *et. al.* 1999) or the overexpression of proteases (Flores *et. al.* 1997) did not result in strongly enhanced biocontrol abilities *in situ*.
Talaromyces flavus is another antagonistic fungus against fungal plant pathogens, generally used for biological control control of verticillium wilt (Marois *et. al.* 1982). The antifungal principle of *Talaromyces* is maily based on the secretion of glucose oxidase (Fravel *et. al.* 1991). This secreted enzyme releases hydrogen peroxide in presence of glucose (e.g. present in root exudates) which is highly toxic to several microorganisms. *Trichoderma* is known to tolerate high concentrations of hydrogen peroxide (Klessing *et. al.* 1994), therefore being an ideal organism for the heterologous expression of glucose oxidase. Previously, the glucose oxidase-encoding gene of *A. niger* was fused to the biocontrol-related *nag1* promoter as a reporter system (Mach *et al.*, 1996). A recombinant *T. atoviride* strain bearing 12-14 copies of this fusion construct (ref mach 1999) has been selected for further investigations. The aims of the study were: (i) to demonstrate glucose oxidase expression under antagonistic conditions *in vitro* (plate confrontations assay), (ii) to use culture filtrates in spore germination inhibition experiments to test the coaction of *Trichoderma*'s own antifungal system and the heterologous expressed enzyme and (iii) to get further information about the antagonistic effect of this new strain *in situ* by plant protection experiments for confirming its putative enhanced biocontrol activity.

2.3. References

Adams, T. H. and Wieser, J.K. (1999) Asexual sporulation: conidiation. In: Molecular Fungal Biology (Oliver, R.P. and Schweizer, M., Eds.) pp. 185 – 207. Cambridge University Press, NY.

Dowzer CE, Kelly JM. (1991) Analysis of the creA gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol Cell Biol. Nov;11(11):5701-9.

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. Jan;31(1):30-7.

Fravel, D. R., Roberts D. P. 1991. *in situ* Evidence for the Role of Glucose Oxidase in the Biocontrol of Verticillium Wilt by *Talaromyces flavus*. Biocontrol Science and Technology 1:91-99.

Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J., and Felenbok, B. (1993) Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol. Microbiol. 7, 847 - 857.

Limon, M. C., Pintor-Toro J. A., Benitez T. 1999. Increased Antifungal Activity of Trichoderma harzianum Transformants That Overexpress a 33-kDa Chitinase. Phytopathology 89:254-261.

Lorito, M., Mach, R.L., Sposato, P., Strauss, J., Peterbauer, C.K., and Kubicek, C.P. (1996) Mycoparasitic interaction relieves binding of Cre1 carbon catabolite repressor protein to promoter sequence of *ech-42* (endochitinase-encoding) gene of *Trichoderma harzianum*. Proc. Natl. Acad. Sci. USA 93, 14868-14872

Marchler, G., Schüller, C., Adam, G. and Ruis, H. (1993) A *Saccharomyces cerevisiae* AUS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 12, 1997 - 2003

Marois J. J., J. S. A., Dunn M. T., Papavizas G. C. 1982. Biological Control of Verticillium wilt of eggplant in the field. Plant Dis. 66:1166-1168.

Peterbauer C, Lorito M, Hayes CK, Harman GE, Kubicek CP. 1996. Molecular cloning and expression of *nag1* (N-acetyl-ß-D-glucosaminidase-encoding) gene from *Trichoderma harzianum* P1. Curr Genet 29, 812-820

Strauss J, Mach RL, Zeilinger S, Hartler G, Stoffler G, Wolschek M, Kubicek CP. (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. FEBS Lett. Nov 27;376(1-2):103-7.

Chapter 3

Investigations on chitinase-encoding genes of *Trichoderma atroviride*

3.1. Introduction

3.1.1. Chitinolytic enzymes

Chitin, the β-1,4-linked homopolymer of N-acetyl-D-glucosamine, is supposed to be one of the most abundant polymers in the biosphere. As the degradation of chitin is involved in many biological processes, chitinolytic enzymes are found in almost all kingdoms, e. g. protista, bacteria, fungi, plants, invertebrates and vertebrates. Enzymatic degradation of chitin plays a crucial role in many biological processes, such as morphogenesis including autolysis, cell separation, spore swelling, germination, sporangium formation and response to mechanical injuries (Gooday *et. al.* 1990, Sahai *et. al.* 1993). Additionally, the hydrolysis of chitin is involved in nutrition, plant-fungus and fungus-fungus interaction (Lorito 1998). Numerous strains of the genus *Trichoderma* are well known producers of chitinolytic enzymes and as the secretion of chitinolytic enzymes is closely related to mycoparasitism, they are among the most effective organisms for a biological control of plant pathogenic fungi (Chet *et. al.* 1987, Harman *et. al.* 1990, 1993, Lo *et. al.* 1996, Samuels *et. al.* 1996).
All enzymes capable of catalyzing the degradation of chitin or chitooligomers are defined as chitinolytic enzymes and are grouped into three classes according to the released end products. The nomenclature according to the recommendations by Webb (1992, Enzyme nomenclature) is used.

Endochitinase (EC 3.2.1.14) cleaves the chitin chain at randomly distributed internal sites and releases chitooligomers of various lengths. It leaves soluble low molecular mass endproducts, mainly diacetylchitobiose.

Chitin 1,4-β-chitobiosidase releases only diacetylchitobiose progressively from the non-reducing end of the polymer. This enzyme accepts chitin as well as chitooligomers as a substrate.

β-N-acetylhexosaminidase (EC 3.2.1.52) cleaves chitin and chitooligomers from the non-reducing end and releases only the momomer N-acetyl-D-glucosamine. It is the only enzyme with the ability to cleave diacetylchitobiose.

3.1.2. Chitinases secreted by *Trichoderma*

Numerous chitinolytic enzymes have been found in *T. harzianum, T. virens, T. aureoviride, T. atroviride, T. koningii, T. longibrachiatum, T. viride, T. asperellum, T. inhamatum, T. pseudokonigii, T. longipilis, T. minutisporum, T. hamatum* and *T. reesei*. As the chitinolytic system of *T. harzianum* is by far the best characterized, it will serve as representative for a tabular overview (see table 1). Additionally, since *T. atroviride* was used throughout the present work, its chitinases are added in table 1, too.

Enzyme	Strain	Reference	Enzymatic activity
CHIT102	*T. harzianum* TM	Haran, 1995	N-acetylhexosaminidase
CHIT72/73	*T. atroviride* P1	Lorito, 1994	N-acetylhexosaminidase
	T. harzianum TM	Haran, 1995	
	T. harzianum IMI298372	Ridout, 1993	
CHIT64	*T. harzianum* 39.1	Ulhoa, 1991	N-acetylhexosaminidase
CHIT28	*T. harzianum* Z198	Deane, 1995	N-acetylhexosaminidase
CHIT52	*T. harzianum* TM	Haran, 1995	Endochitinase
CHIT42	*T. harzianum* 39.1	Ulhoa, 1992	Endochitinase
	T. harzianum CECT2413	de la Cruz, 1993	
	T. atroviride P1	Harman, 1993	
	T. harzianum TM	Haran, 1995, Lorito 1993	
CHIT37	*T. harzianum* CECT2413	de la Cruz, 1993	Endochitinase
CHIT33	*T. harzianum* CECT2413	de la Cruz, 1993	Endochitinase
CHIT31	*T. harzianum* TM	Haran, 1995	Endochitinase
CHIT40	*T. atroviride* P1	Harman, 1993	Chitobiosidase

Table 1. Exemplary overview of chitinolytic enzymes found in different *T. harzianum* and *T. atroviride* strains.

It has to be mentioned that all the chitinases of *T. harzianum* are not produced by one single strain but were found in five different strains.

3.1.3. Mechanisms of chitinase gene induction in *Trichoderma*

When *Trichoderma* is grown on fungal mycelia, cell walls, or chitin as sole carbon source, chitinases are secreted at high levels. However, the induction of distinct chitinases seems to be rather complex and not all chitinases follow the same mechanism of induction.
Although it is known that chitinases are repressed by glucose and sucrose (Carsolio *et. al.* 1994, de la Cruz *et. al.* 1993, Garcia *et. al.* 1994, Peterbauer *et. al.* 1996), knowledge on their precise induction is still limited.
The production of N-acetyl-D-glucosaminidases is induced by N-acetylglucosamine, chitooligomers and fungal cell walls in *T. atroviride*, whereas *ech42* expression is not triggered by any of the oligomers but induced by cell walls, influenced by carbon and nitrogen starvation, and physiological stress (Mach *et. al.* 1999, Donzelli *et. al.* 2001). Similar to *ech42* from *T. atroviride*, the expression of *chit36*Y from *T. asperellum* is regulated by physiological stress, carbon and nitrogen limitation, and is induced by N-acetylglucosamine (Viterbo *et. al.* 2002). There is evidence that at least the genes coding for CHIT72 and CHIT42 are induced in a concentration-dependent way by chitooligomers (Peterbauer *et. al.* 1996, de la Cruz *et. al.* 1993). Furthermore, it is supposed that CHIT102, CHIT42 and CHIT33 are produced in small amounts even on glucose as sole carbon source (Bruce *et. al.* 1996, Garcia *et. al.* 1994, Haran *et. al.* 1995, Inbar *et. al.* 1995, Limon *et. al.* 1995 Margolles-Clark *et. al.* 1996b). Probably these enzymes are secreted to hydrolyse chitin of fungal hosts in the soil with the aim to produce soluble chitooligomers for localization of the host.

Inbar and Chet (1992, 1995) used lectin-coated nylon fibers to induce the typical mycoparasitic behavior of *Trichoderma* and thereby detected the production of a chitinase, although there was no chitin present in this test system. *Pythium ultimum* does not contain any chitin, nevertheless the chitinolytic system of *T. harzianum* and *T. hamatum* is induced during growth on media containing *Pythium* cell walls. Interestingly, only one chitinase was found in *T. hamatum* in these experiments, whereas *T. harzianum* produced four chitinases in the presence of *Pythium* cell walls under the same growth conditions (Inglis *et. al.* 2001).
As most experiments concerning induction of chitinase-encoding genes were performed in liquid cultures with varying inducers, they do not reflect the complex situation occurring during mycoparasitism. Only a few reports describe the regulation of lytic enzymes during mycoparasitism *in situ*. Carsolio (1994) demonstrated that the expression of the *T. atroviride* endochitinase-encoding gene *ech42* is induced in dual culture assays with *R. solani* as host. Cortes *et al.* (1998) found that *prb1* and *ech42* are induced by a diffusable factor released from *R. solani*. This factor is heat and proteinase resistant and has a molecular weight of less than 30 kDa. *T. atroviride* reptorter strains containing fusions of the *gfp* reporter gene to the *nag1* or *ech42* 5'-regulatory sequences were used to investigate the different induction mechanisms of these two chitinase-encoding genes during mycoparasitism. Whereas the *ech42* promoter was already active before contact of the *Trichoderma* hyphae with *R. solani*, the *nag1* promoter was induced only after contact with the host. The use of a dialysis membrane separating the two fungi demonstrated that the diffusable factor responsible for pre-contact induction of *ech42* has a molecular weight of less than 12 kDa (Zeilinger *et. al.* 1999). Experiments with a strain of *T. asperellum* bearing the *gfp*-reporter gene indicated, that direct contact of the antagonist and the host hyphae is not necessary to induce a gene encoding for a 36 kDa endochitinase. Apparently, a diffusible factor, capable to pass through a dialysis membrane, neither heat nor proteinase sensitive, is released by *R. solani* (Viterbo *et. al.* 2002). Kullnig *et al.* (2000) carried out experiments using sequential plate confrontation experiments to further characterize this unknown factor. The authors provided evidence that a macromolecule released by *Trichoderma* must act on *Rhizoctonia* to release this chitinase gene-inducing diffusable factor.
In contrast to the *T. asperellum* endochitinase gene, the *gfp* reporter gene under control of the *T. harzianum chit33* promoter is induced only after contact with *R. solani* (de la Mercedes Dana, 2001). The differential expression of chitinases during mycoparasitic interaction with *S. rolfsii* was studied by Inbar and Chet (1995) showing that first a 102 kDa N-acetylglucosaminidase (CHIT102) is expressed. This first chitinase activity decreases concomitantly with the appearance of a 73 kDa N-acetylglucosaminidase (CHIT73) at later stages of the antagonistic action. These results could be detected only when living hyphae of *S. rolfsii* were present, whereas autoclaved mycelium of *Sclerotium* did not induce these chitinase-encoding genes. The activity of CHIT102 is also observed when *Trichoderma* grows on *S. rolfsii* lectin-coated nylon fibers, even if no chitin is present in this system (Inbar and Chet 1995). To test the expression of different chitinases during interaction with different hosts, dual cultures of *T. harzianum* and *R. solani* or *S. rolfsii*, respectively, were tested for chitinase activity (Haran *et. al.* 1996). The mycoparasitic attack of *R. solani* involved the expression of three endochitinases (CHIT52, CHIT42 and CHIT33) and the N-acetylglucosaminidase CHIT102. In contrast to *Rhizoctonia*, *S. rolfsii* is only hardly controlled by *T. harzianum* and during this non-efficient mycoparasitic interaction the activity of only two exochitinases, CHIT73 and CHIT102, could be detected (Haran *et. al.* 1996). These results demonstrate that the regulation of chitinase gene expression *in situ* is rather complex and does not only require the presence of chitin. Additionally, the expression patterns in various antagonist – host combinations differ significantly (Inglis *et. al.* 2001).

In conclusion, the induction of the chitinolytic system is not regulated by one single mechanism but by both specific stimuli, as lectin-recognition and chitin or chitooligomers, as well as unspecific stimuli, like stress.

3.1.4. Chitinase-encoding genes isolated from *Trichoderma*

Although numerous chitinolytic enzymes are known in *Trichoderma*, only a moderate number of corresponding genes have been isolated by now, the first being the gene encoding for CHIT42 which has been cloned from several strains. In *T. harzianum* this gene was designated as *chit42* and *chi1*, in *T. atroviride* as *ech42* and *ThEn-42* (Carsolio *et. al.* 1994, Garcia *et. al.* 1994, Hayes *et. al.* 1994, Draborg *et. al.* 1996), *cht42* in *T. virens* (Bark *et. al.* 1999) and *th-ch* in *T. hamatum* (Fekete *et. al.* 1996, Giczey *et. al.* 1998); the 42 kDa chitinase encoding gene from *T. viride* was isolated and sequenced by Zhang *et al.* (1999, accession number:AF208842). The genes coding for the 42 kDa endochitinase of *Trichoderma* have high similarity to other genes for fungal chitinases. As these genes are the most often isolated chitinase-encoding genes of *Trichoderma*, they provide a useful tool for phylogenetic analysis of the genus (Lieckfeld *et. al.* 2000). Recently three 42 kDa endochitinase-encodig genes were isolated by Kim *et al.* (2002) from *T. virens*. These three genes, *ech1*, *ech2* and *ech3*, share 50% identity on DNA level to each other and to both *Tv-cht42* and *Thchit42*.

The only other endochitinase-encoding gene which has been characterized in detail is *chit33*, coding for a 33 kDa endochitinase from *T. harzianum* (Limon *et. al.* 1995). The enzyme has high similarity to plant chitinases but no other fungal homologue has been found yet.

chit36, a gene encoding a 36 kDa endochitinase from *T. harzianum* TM was isolated by Viterbo *et al.* (2001). The same group isolated and sequenced further three *chit36* homologues from *T. asperellum, T. inhamatum and T. harzianum* CECT2413, showing high similarity of the proteins. Surprisingly, the enzymes encoded by *chit36* in *T. harzianum* TM and *T. atroviride* are 100% identical (Viterbo *et. al.* 2002). Two N-acetylglucosaminidase-encoding genes, *Tv-nag1* and *Tv-nag2*, were isolated by Kim *et al.* (2002) from *T. virens*. The genes share 51% identity to each other and are highly similar to *T. atroviride nag1*.

Draborg *et al.* (1995) isolated two N-acetylhexosaminidase-encoding genes, *exc1* and *exc2*, from *T. harzianum* with the corresponding enzymes showing 72% aa- identity to each other, whereas only one gene coding for a N-acetylhexosaminidase, namely *nag1* was found in *T. harzianum* (Peterbauer 1996).

Gene	Strain	Encoded protein	Reference
Th-En42	*T. atroviride* P1	42 kDa endochitinase	Hayes, 1994
ech42	*T. atroviride* IMI206040	42 kDa endochitinase	Carsolio, 1994
chit42	*T. harzianum* CECT2413	42 kDa endochitinase	Garcia, 1994
cht42(ech1)	*T. virens* Gv29-8	42 kDa endochitinase	Baek, 1999, Kim 2002
th-ch	*T. hamatum* Tam-61	42 kDa endochitinase	Fekete, 1996
ech2	*T. virens* Gv29-8	42 kDa endochitinase	Kim 2002
ech3	*T. virens* Gv29-8	42 kDa endochitinase	Kim 2002
chi1	*T. harzianum* T25-1	42 kDa endochitinase	Draborg, 1996
chit33	*T. harzianum* CECT2413	33 kDa endochitinase	Limon, 1995
chit36	*T. atroviride* P1	36 kDa endochitinase	Viterbo, 2002
	T. harzianum TM	36 kDa endochitinase	Viterbo, 2001
	T. asperellum	36 kDa endochitinase	Viterbo, 2002
	T. harzianum CECT2413	36 kDa endochitinase	Viterbo, 2002
	T. inhamatum CECT2424	36 kDa endochitinase	Viterbo, 2002
nag1	*T. atroviride* P1	73 kDa N-acelylglucosaminidase	Peterbauer, 1996
Tv-nag1	*T. virens* Gv29-8	73 kDa N-acelylglucosaminidase	Kim, 2002
Tv-nag2	*T. virens* Gv29-8	73 kDa N-acelylglucosaminidase	Kim, 2002
exc1	*T. harzianum* T25-1	73 kDa N-acelylglucosaminidase	Draborg, 1995
exc2	*T. harzianum* T25-1	73 kDa N-acelylglucosaminidase	Draborg, 1995

Table 2. Overview of chitinase-encoding genes from different *Trichoderma* strains.

3.1.5. References

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

Bruce A., Sinivasan U., Staines H.J., Highley T.L. 1995. Chitinase and laminarase production in liquid culture by *Trichoderma* spp. And their role in biocontrol of wood decay fungi. Int. Biodeterior. Biodegrad. 35:337-353

Carsolio C, Gutierrez A, Jiménez B, Van Montagu M, Herrera-Estrella A. 1994. Characterization of *ech42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA 91, 10903-10907

Chet, I. 1987. Application, mode of action, and potential as a biocontrol agent of soil borne plant pathogenic fungi. In Chet (ed.), Innovative Approches to Plant Disease Control, Wiley, NY, 221-255

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.

Deane E.E., Peperdy J.F., Whippes. J.M., Lynch J.M. 1995. Isolation of a partial chitinase clone from *Trichoderma harzianum*. Fifth International *Trichoderma*and Gliocladium workshop, April 1995

De la Cruz J., Rey M., Lora J.M., Hidalgo-Gallego A., Dominguez F., Pintor-Toro J.A., Llobell A., Benitez T. 1993. Carbon source control on glucanase, chitobiase and chitinose from *Trichoderma*harzianum. Arch. Microbiol. 159:316-322

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

Donzelli, B.G. and Harman, G.E. (2001) Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. Appl Environ Microbiol. 67, 5643 - 5647.

Draborg H, Kaupinnen S, Dalboge H, Christgau S. 1995. Molecular cloning and expression in *Saccharomyces cerevisiae* of two exochitinases from *Trichoderma harzianum*. Biochem Mol Biol Inter 36, 781 – 791 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28, 350-356

Fekete C, Weszely T, Hornok L. 1996. Assignment of a PCR-amplified chitinase sequence cloned from *Trichoderma*hamatum to resolved chromosomes of potential biocontrol species of Trichoderma. FEMS Microbiol Lett. Dec 15;145(3):385-91

Garcia I, Lora JM, de la Cruz J, Benitez T, Llobell A, Pintor-Toro A. 1994. Cloning and characterization of a chitinase (*chit42*) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet 27, 83 - 89

Giczey G, Kerenyi Z, Dallmann G, Hornok L. 1998. Homologous transformation of *Trichoderma*hamatum with an endochitinase encoding gene, resulting in increased levels of chitinase activity.FEMS Microbiol Lett. Aug 15;165(2):247-52.

Gooday, G.W. 1990. Physiology of microbial degradation of chitin and chitisan. Biodegradation 1: 177 – 190

Haran S, Schickler H, Oppenheim A, Chet I. 1995. New components of the chitinolytic system of *Trichoderma harzianum*. Mycol Res 99, 441 – 446

Haran S., Schickler H., Chet I. 1996. Differential expression of *Trichoderma*harzianum chitinases during mycoparasitism. Phytopathology 86:980-985

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Tronsmo A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology 83, 313-318.

Inbar J, Chet I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. J Bacteriol. Feb;174(3):1055-9.

Inbar J, Chet I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. Microbiology 141, 2823 - 2829 Kelly JM, Hynes MJ. 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J 4: 475-479

Inglis GD, Kawchuk LM. Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi.Can J Microbiol. 2002 Jan;48(1):60-70.

Kullnig CM, Krupica T, Woo SL, Mach RL, Rey M, Benitez T, Lorito M, Kubicek CP. 2001.Confusion abounds over identity of *Trichoderma* biocontrol isolates. Mycol. Res. 105, 769 – 772

Lieckfeld E., Cavignac Y. Fekete C., Börner T. 2000. Endochitinase based phylogenetic analysis of *Trichoderma*. Microbiological Research 155 :1-6.

Limon MC, Lora JM, Garcia I, de la Cruz J, Llobell A, Benitez T, Pintor-Toro JA. 1995 Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet. Oct;28(5):478-83.

Lo C.T. Nelson, E.B., Harman, G.E. 1996. Biological control of turfgrass with a rhizosphere competent strain of *Trichoderma harzianum*. Plant Dis. 80: 736 – 741.

Lorito M, Hayes CK, Di Pietro A, Harman GE. Biolistic transformation of *Trichoderma*harzianum and Gliocladium virens using plasmid and genomic DNA. Curr Genet. 1993 Oct;24(4):349-56.

Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE. 1994. Purification, characterization and synergistic activity of a glucan 1,3-â-glucosidase and N-acetyl-ß-glucosaminidase from *Trichoderma harzianum*. Phytopathology 84, 398-405.

Lorito M. 1998. Chitinolytic enzymes ands their genes. In: *Trichoderma* and *Gliocladium*, Vol. 2 (Harman GE, Kubicek CP, eds) pp 73 - 99. Taylor and Francis, London UK

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

Peterbauer C, Lorito M, Hayes CK, Harman GE, Kubicek CP. 1996. Molecular cloning and expression of *nag1* (N-acetyl-ß-D-glucosaminidase-encoding) gene from *Trichoderma harzianum* P1. Curr Genet 29, 812-820

Ridout C.J., Coley-Smith J.R., Lynch J.M. 1988,
Samuels G.J. 1996. *Trichoderma*: a review of biology and systematics of the genus . Mycol. Res. 100:923-935

Sahai AS, Manocha MS. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol Rev 11, 317 – 338

Ulhoa CJ, Peberdy JF. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol. 1991 Sep; 137:2163-9.

Viterbo A, Haran S, Friesem D, Ramot O, Chet I. 2001. Antifungal activity of a novel endochitinase gene (*chit36*) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200, 169-174

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, Chet I. Expression regulation of the endochitinase chit36 from *Trichoderma*asperellum (T. harzianum T-203). Curr Genet. 2002 Nov;42(2):114-22. Epub 2002 Nov 21

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

3.2. *ech42* gene expression under carbon starvation conditions in *Trichoderma atroviride*

3.2.1. Specific Introduction

The 42 kDa endochitinase is the most prominent chitinase of *Trichoderma* being essential for biocontrol (Woo *et. al.* 1998, Baek *et. al.* 1999, Carsolio *et. al.* 1999). *Ech42* is expressed before contact with the host *R. solani* (Zeilinger *et. al.* 1999) *in vitro and* is induced by fungal cell walls (Carsolio *et. al.* 1994, Mach *et. al.* 1999), regulated by carbon and nitrogen levels (Lorito *et. al.* 1996, Donzelli *et. al.* 2001, Mach *et. al.* 1999) and responds to physiological stress (Mach *et. al.* 1999). However, until now only few analysis on the molecular mechanism of its regulation have been performed. In this report *ech42::goxA* reporter strains, bearing truncated or partially deleted *ech42* promoters, and electophoretic mobility shift assays were used to identify binding sites for transcription factors affecting *ech42* gene expression during carbon starvation. These studies were performed with the 815 bp fragment of the upstream regulatory sequence of the *T. atroviride ech42* gene, which has previously been shown to be sufficient for regulation upon carbon source starvation (Mach *et. al.* 1999). Three putative binding sites for the carbon catabolite repressor Cre1 are present on this fragment at positions -522, -408 and -333. Lorito *et al.* (1996) demonstrated that binding of Cre1 is involved in the regulation of *ech42* expression under mycoparasitic conditions *in vitro*. Furthermore, four consensus sequences for the yeast stress response regulator Msn2/4 can be found at -790, -542, -400 and -318. The *MSN2* and *MSN4* genes both encode zinc finger proteins. A disruption of both genes resulted in a higher sensitivity to different stresses, including carbon source starvation, heat shock and severe osmotic and oxidative stresses (Marchler *et. al.* 1993). Additionally, three binding sites for the *Aspergillus nidulans* conidiophore development regulator BrlA are present on this 815 bp fragment of the upstream regulatory sequence of *ech42* at positions -524, -326 and -203. BrlA mediates the switch from vegetative growth to asexual sporulation in *Aspergillus* and, like *ech42*, *brlA* responds to carbon and nitrogen starvation (Adams *et. al.* 1988, Skromne *et. al.* 1995).

3.2.2. Results

***In vivo* identification of areas involved in *ech42* gene expression under carbon starvation conditions**. Reporter strains of *T. atroviride* containing the *A. niger goxA* gene under control of the *ech42* upstream regulatory sequence were constructed; from these strains KB1 bears the whole 815 bp fragment of the 5' noncoding sequences, whereas KB11 bears a truncated regulatory region of only 349 bp. This truncated promoter lacks two putative Cre1 binding sites (-522 and –408), three Msn2/4 (-790, -542 and –400) and one BrlA (-524) site. Three additional strains were constructed containing promoters with deletions of specific areas. In strain KB12 an area of 48 bp containing one putative Cre1, one Msn2/4, and one BrlA binding site, was deleted from the *ech42* promoter. Strain KB13 lacks a 82 bp fragment with only one BrlA-like site and in strain KB14 both, the 48 bp and the 82 bp fragments are deleted from the promoter. Another strain, KB15 bears the *goxA* reporter gene under control of a truncated *ech42* upstream regulatory sequence of only 193 bp, now lacking all the regulatory elements described above. Strains with only one copy of the reporter construct were chosen for further experiments. A detailed overview of the deleted promoter areas is given in Figure 1.

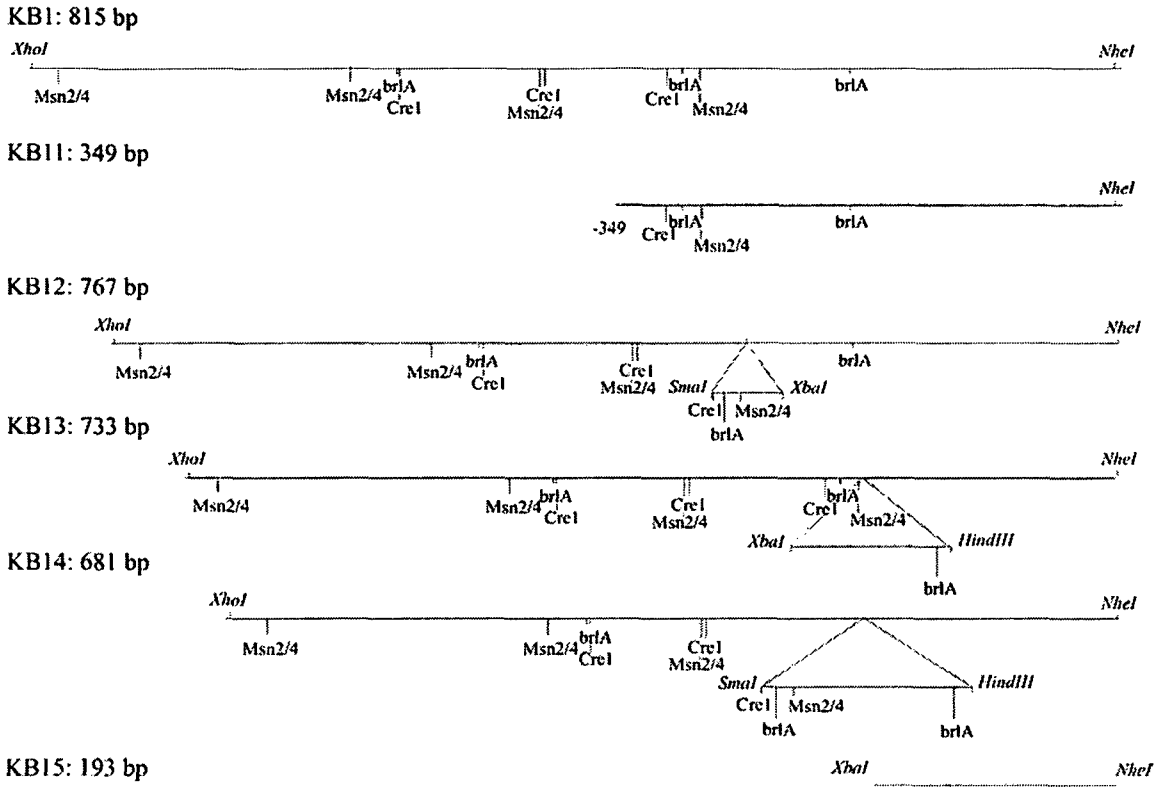

Figure 1. Structure of the 815 bp fragment of the *ech42* promoter and truncations or deletions thereof used throughout this study.

The reporter strains described above were precultivated on 1% glycerol, then transferred to carbon-free or glycerol-containing medium as a control. After 48 hours the *goxA* reporter gene activity in the culture supernatants was determined by measuring glucose oxidase enzyme activity. Truncation of half the length of the *ech42* promoter (KB11) and the deletion of the two small fragments of 49 bp (KB12) and 82 bp (KB13) only had a moderate effect on reporter gene expression during carbon starvation. In contrast, deletion of both, the 49 bp and the 82 bp fragment, led to an approximately three-fold increase of reporter gene expression during carbon source starvation.

Strain	Deleted promoter area	Relative activity of glucose oxidase (%) Glycerol control	Carbon starvation
KB1	---	<5(±0.3)	100 (±5)
KB11	-815 to –349	<5(±0.3)	130 (±16)
KB12	-332 to –285	<5(±0.3)	107 (±11)
KB13	-286 to –193	<5(±0.3)	93 (±10)
KB14	-332 to –193	<5(±0.3)	283 (±33)
KB15	-815 to –193	<5(±0.3)	469 (±18)

Table 1. Effect of deletion of different *ech42* promoter areas on reporter gene expression. The 100% of strain KB1 on carbon-free medium correspond to 8.1 U/g mycelial dry weight.

A *T. atroviride* protein contacts the BrlA-like binding site in the *ech42* promoter. After pregrowth on glycerol, *T. atroviride* mycelium was transferred to carbon-free medium or medium containing glycerol as control. After 48 hours cell-free extracts were prepared as described in materials and methods. To identify the sequences responsible for the enhanced *ech42* expression in more detail, electrophoretic mobility shift assays (EMSA) were performed. For the preliminary test, two fragments were used, one spanning the region from –815 to –350 and another from –350 to –1 to demonstrate that proteins in the cell-free extract bind to the upstream regulatory regions of *ech42 in vitro*. EMSAs with both fragments resulted in a single protein-DNA complex which was significantly smaller under carbon-starved conditions. Subsequently, three synthetic oligonucleotides of 28 to 38 bp in length were used in further EMSA experiments (see fig 2.). The fragments ech1 and ech2 still bound proteins, whereas ech3 failed to form a protein-DNA complex. These initial experiments were performed by Manuel Monteras in 1999.

Figure. 2. Positions of the synthetic oligonucleotides ech1, ech2 and ech3 in the *ech42* 5' noncoding region. Synthetic fragments are grey shadowed and putative BrlA-like binding sites are shown in bold letters.

As oligonucleotide ech1 contains only the putative BrlA-like binding site and no consensus sequence for binding of Cre1 or Msn2/4, and as the BrlA site is not present in ech3, it can be considered that rather the BrlA-binding site than one of the others is responsible for protein binding. Oligonucleotides used for EMSAs in previous studies of the *T. reesei xyn1* promoter, containing a Cre1 binding site (Mach *et al.*, 1996), or ckp2 from the *T. atroviride nag1* promoter, bearing a Msn2/4 like binding site (peterbauer mgg 2002), were tested for competition of complex formation. Both fragments did not contain any other similarities to the ech1 or ech2 fragments but the above mentioned motifs. Neither the *xyn1*-fragment nor ckp2 succeeded in competing for binding proteins. Additionally, vice versa competition experiments with ech1 and ech2 were carried out, showing that ech1 could not be competed by ech2 but the use of ech1 as competitor for ech2 abolished complex formation. This

experiment demonstrated that the same protein binds to the ech1 and ech2 fragments, but only ech1 bears a correct BrlA-like binding site, whereas ech2 differs from the consensus motif in the third base. Hence only ech2 could perfectly be competed with ech1.
In order to test whether the BrlA-like motif is indeed involved in protein binding to the *ech42* promoter, mutated versions of the oligonucleotides ech1 and ech2 were created.

Oligonucleotide	Sequence
ckp2	ACGTGTCGCGTGTC**AGGGG**GCCGTGCT
ech1	AGAACCCCGAAAGGGAAGCTTCATAAGTT
ech11	AGAACCCCGAAA**CCC**AAGCTTCATAAGTT
ech2	CCGCCGAGCCCCGGGCACGGGACAGGGGCCACAAGCT
ech21	CCGCCGAGCCCC**CCC**CACGGGACAGGGGCCACAAGCT

Table 2. Oligonucleotides used throughout these studies. Consensus sequences for Cre1 or Msn2/4 binding sites are given in bold, mutated nucleotides from the *ech42* promoter are bold and underlined.

Performing EMSAs with the mutated fragments as probes showed that their ability to bind proteins differs significantly from the unmutated oligonucleotides. Oligonucleotides with the mutated BrlA-binding sites bound proteins only weakly or resulted in complexes of different size compared to the unmutated fragments.

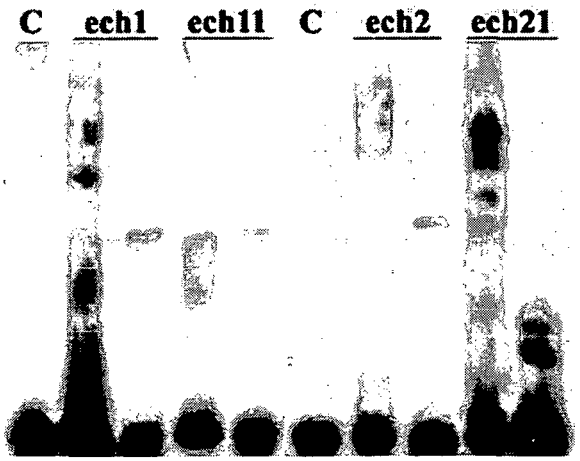

Fig. 3. EMSAs using the unmutated and the mutated fragments as probes. C is the control without cell-free extracts.

To test whether the labelled unmutated oligonucleotides can be competed by the mutated versions, a 50-fold molar excess of the competitors was used. The mutated fragments nearly completely lost their ability to compete, again demonstrating that the binding of proteins to their corresponding motifs is much weaker than with the unmutated *ech42* fragment.

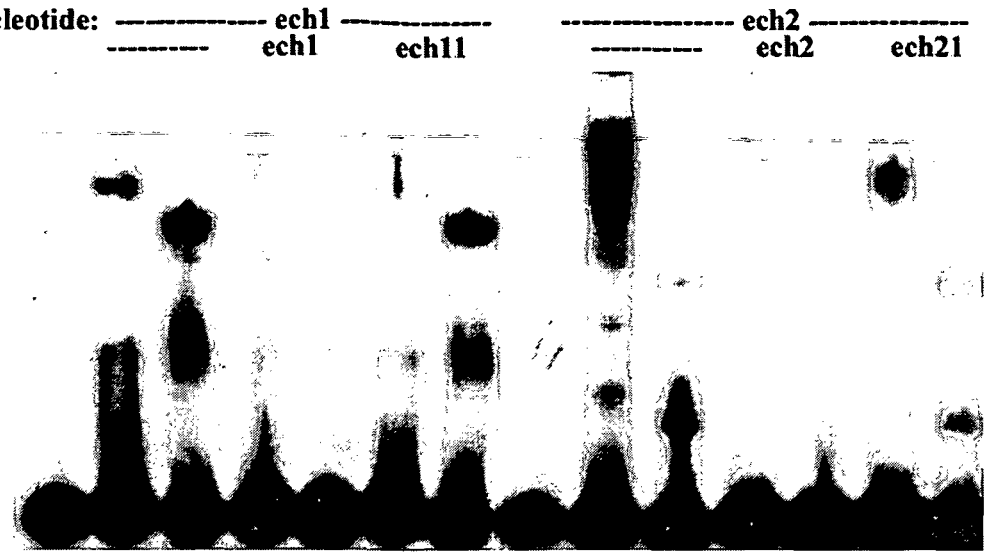

Fig. 4. Competition experiments with wild type and mutated fragments. Lane 1 and 8 are the labelled probes without cell-free extracts.

3.2.3. Discussion

In this study, the first molecular investigations on *ech42* gene expression during carbon starvation are presented. Experiments with reporter strains containing the *A. niger goxA* gene under control of different truncated or deleted versions of the *ech42* upstream regulatory sequences gave first insights on the regulation of this gene. A 349 bp promoter fragment was sufficient for derepression under carbon starvation. Further experiments with the reporter strains KB12, KB13 and KB14 pointed to the importance of the area between bp –331 to –194, as deletion of this region resulted in an about three-fold increased expression of the reporter gene. The deleted region contains one Msn2/4 and two BrlA-like binding sites. EMSAs with oligonucleotides bearing these motifs revealed that the Msn2/4 binding site does not seem to be responsible for protein binding, but that the BrlA motif is the only one to be bound by proteins in the area –331 to –194. Additionally, the interaction of the two BrlA-like sites could be demonstrated in reporter strains by separate deletion of each of them. As long as only one of the BrlA-binding motifs between –331 and –194 was deleted, no significant changes in reporter gene expression could be detected, whereas simultaneous deletion of both motifs significantly increases repoter gene expression. When the third BrlA-binding site at position –524 was also deleted, derepression was raised again for an unproportionally high factor. These findings argue for a synergistic interaction of all three motifs and are confirmed by the fact that the distance between these motifs is about 150bp which is close to a DNA loop around a nucleosome (Hayes *et. al.* 2001).
Until now, only few investigations dealing with the molecular mechanism of *ech42* induction in *T. atroviride* have been published. Lorito *et al.* (1996) examined the proceedings on the *ech42* promoter before and during the mycoparasitic interaction with a host fungus and provided indications that the carbon catabolite repressor Cre1 is involved in *ech42* gene transcription. In *Aspergillus* BrlA is known for its role as the developmental switch from vegetative growth to asexual reproduction as it is necessary and sufficient to induce sporulation through its role in regulating expression of conidiation-specific genes (Adams *et. al.* 1988). Endochitinolytic activity has been detected on spores of *T. atroviride*

and *ech42::goxA* reporter strains exhibit glucose oxidase activity on their conidia (Brunner K., unpublished findings). Another link between *Trichoderma ech42* and *Aspergillus brlA* is provided by the fact, that both genes respond to carbon and nitrogen starvation (Skromme *et. al.* 1995).
Although this investigations seem to provide evidence for the presence of a protein in *T. atroviride* that binds to the BrlA sequence, *in silico* analysis could not confirm such a protein homologue existing in the *Neurospora crassa* sequence database (C. P. Kubicek and C. K. Peterbauer, unpublished findings). Additional attempts to demonstrate binding of the BrlA protein to its binding motif failed *in vitro* (Adams 1999). Therefore, it can be considered that not a BrlA homologue of *T. atroviride* has been found; it rather seems to be a new transcriptional regulator of *ech42* gene transcription which binds to a sequence very similar to the BrlA-binding motif.

3.2.4. Materials and Methods

Strains. *T. atroviride* strain P1 (*T. "harzianum"* ATCC 74058) was used throughout this study and maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany). *Escherichia coli* JM109 (Yanisch-Perron *et. al.* 1985) was the host for plasmid amplification.

Cultivation conditions. *T. atroviride* and recombinant strains prepared from it were grown in liquid synthetic medium (SM) as described previously (Mach *et. al.* 1999) containing either glucose or glycerol (10 g/l) as a carbon source. Carbon starvation experiments in replacement cultures were performed by transferring *T. atroviride*, pre-cultivated on glycerol as carbon source for 36 hrs, to fresh medium lacking the carbon source and further cultivation for up to 48 h (Mach *et. al.* 1999).

Plasmids and plasmid constructions. Plasmid pSJ3, containing the *Aspergillus niger goxA* gene under the control of the *T. atroviride nag1* 5′ regulatory region (Mach *et. al.* 1999), was used for the construction of the reporter vectors with truncated promoter fragments. To this end, 815-bp of the *ech42* promoter were amplified by PCR, cloned into pGEM-T, appropriate fragments cut out and exchanged against the *nag1* promoter in pSJ3. All fragments were verified by sequencing.

Fungal transformation. Plasmids were introduced into *T. atroviride* by cotransformation with plasmid pHATα, confering hygromycin B resistance (Herrera-Estrella *et. al.* 1990) as described previously (Peterbauer *et. al.* 2002). 10 µg of the *goxA*-bearing plasmids and 2 µg of pHATα were used per 5 x 107 protoplasts. Protoplasts were regenerated on PDA supplemented with 1M sorbitol and 100µg/ml hygromycin B (Calbiochem, La Jolla, CA). Mitotically stable transformants were obtained by at least three sequential transfers of conidia from non-selective to selective media.

DNA manipulation. Plasmid DNA was isolated by using a midiprep kit (Qiagen Inc., Chatsworth, CA) as recommended by the manufacturer. Other molecular techniques were performed according to standard protocols (Sambrook *et. al.* 1989, Ausubel *et. al.* 1990).

Glucose oxidase assay. The conditions for using glucose oxidase as a reporter of *ech42* gene expression and the spectrophotometric assay of its activity in the culture supernatant were described previously (Mach *et. al.* 1999). One unit (1 U) of activity is

defined as the amount of enzyme required to oxidize 1μmol of glucose per min at pH 5.8 and 25oC.

Electrophoretic Mobility Shift Assay (EMSA). The oligonucleotide fragments used were end-labelled with [α-32P]dCTP using Sequenase Version 2.0 (USB, Cleveland, OH) and purified by non-denaturing polyacrylamide gel electrophoresis. Annealing and end-labelling of synthetic oligonucleotides used for EMSA's was done as described previously (Peterbauer *et. al.* 2002). Binding assays and non-denaturing polyacrylamide gel electrophoresis were performed as described previously (Peterbauer *et. al.* 2002). For competition experiments, synthetic oligonucleotides were annealed and filled in using Sequenase Version 2.0 as described above, and used in a 10, 50, or 150-fold molar excess.

3.2.5. References

Adams T.H, Boylan M.T, Timberlake W.E. brlA is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell. 1988 Jul 29;54(3):353-62.

Adams, T. H. and Wieser, J.K. (1999) Asexual sporulation: conidiation. In: Molecular Fungal Biology (Oliver, R.P. and Schweizer, M., Eds.) pp. 185 – 207. Cambridge University Press, NY.

Adams, T.H., Deising, H. and Timberlake, W.E. (1990) BrlA requires both zinc fingers to induce development. Mol. Cell. Biol. 10, 1815 – 1817

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1990) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley Interscience, New York

Baek, J.M., Howell, C.R. and Kenerley, C.M. (1999) The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani.* Curr. Genet. 35, 41-50

Carsolio, C., Gutierrez, A., Jiménez, B., Van Montagu, M., and Herrera-Estrella, A. (1994) Characterization of *ech42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc. Natl. Acad. Sci. USA 91, 10903-10907

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I, Herrera-Estrella A. Role of the *Trichoderma harzianum* endochitinase gene, *ech42*, in mycoparasitism. Appl Environ Microbiol. 1999 Mar;65(3):929-35.

Cortés, C., Gutiérrez, A., Olmedo, V., Inbar, J., Chet, I., and 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

Donzelli, B.G. and Harman, G.E. (2001) Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. Appl Environ Microbiol. 67, 5643 - 5647.

Harman, G.E., and T. Björkman. (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: *Trichoderma and*

Gliocladium (Harman, G.E. and Kubicek, C.P., Eds.) Vol. 2, pp. 229 – 265. Taylor and Francis Ltd., London, UK.

Hayes, J.J. and Hansen, J.C. (2001) Nucleosomes and the chromatin fiber. Curr. Opin. Genet. Dev. 11, 124 – 129

Herrera-Estrella, Goldman G.H., and Van Montagu, M. (1990) High efficiency transformation system for the biocontrol agents *Trichoderma* spp. Mol. Microbiol. 4, 839 - 843

Kubicek, C.P., Mach, R.L., Peterbauer, C.K. and Lorito, M. (2001) *Trichoderma*: from genes to biocontrol. J. Plant Pathol. 83, 11 – 23

Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J., and Felenbok, B. (1993) Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol. Microbiol. 7, 847 - 857.

Lorito, M., Mach, R.L., Sposato, P., Strauss, J., Peterbauer, C.K., and Kubicek, C.P. (1996) Mycoparasitic interaction relieves binding of Cre1 carbon catabolite repressor protein to promoter sequence of *ech-42* (endochitinase-encoding) gene of *Trichoderma harzianum*. Proc. Natl. Acad. Sci. USA 93, 14868-14872

Mach, R.L., Strauss, J., Zeilinger, S., Schindler, M., and Kubicek, C.P. (1996) Carbon catabolite repression of xylanase I (*xyn1*) gene expression in *Trichoderma reesei*. Mol. Microbiol. 21, 1273 – 1281

Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S, Woo, S.L., Zeilinger, S., Kullnig, C.M., Lorito, M., and Kubicek, C.P. (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

Marchler, G., Schüller, C., Adam, G. and Ruis, H. (1993) A *Saccharomyces cerevisiae* AUS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 12, 1997 - 2003

Margolles-Clark, E., Harman, G.E., and Penttilä, M. (1996) Enhanced expression of endochitinase in *Trichoderma harzianum* with the *cbh1* promoter of *Trichoderma reesei*. Appl. Environ. Microbiol. 62, 2152-2155

Peterbauer, C.K., Brunner, K., Mach, R.L. and Kubicek, C.P. (2002) Identification of the N-acetyl-ß-D-glucosamine-inducible element in the promoter of the *Trichoderma*atroviride *nag1* gene encoding N-acetyl-glucosmainidase. Mol. Gen. Genom. 267, 162 - 170

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed

Skromne I, Sanchez O, Aguirre J. Starvation stress modulates the expression of the Aspergillus nidulans brlA regulatory gene. Microbiology. 1995 Jan; 141:21-8.

Woo, S.L., Donzelli, B., Scala, F., Mach, R.L., Harman, G.E., Kubicek, C.P., Del Sorbo, G., and Lorito, M. (1998) Disruption of *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* strain P1. Mol. Plant-Microbe Interact. 12, 419-429

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103 – 119

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

3.3. Involvement of *nag1* from *T. atroviride* in chitinase gene induction and biocontrol

3.3.1 Specific introduction

Trichoderma spp. produce a set of chitinolytic enzymes during mycoparasitic interaction with a host. Nevertheless, only few chitinase-encoding genes have been isolated and characterized until now (see introduction, chapter 3.1. for further details).
Although two N-acetyl-β-D-glucosaminidases are present in *T. virens* and *T. harzianum* (Draborg *et. al.* 1995, Kim *et. al.* 2002), only a single Nag-encoding gene (*nag1*) has thus far been found from *T. atroviride*. *nag1* was isolated by Peterbauer *et al.* (1996) and it was shown to encode for a protein of 580 amino acids, interrupted by two short introns. The N-acetyl-β-D-glucosaminidase activity is specifically induced upon cultivation on chitin, fungal cell walls, or N-acetylglucosamine and during mycoparasitic interaction after contact with the host (Ulhoa *et. al.* 1993, Chet *et. al.* 1995, Mach *et. al.* 1999, Zeilinger *et. al.* 1999)
.
In the present study, a Δ*nag1*-disruption strain (P1ND1) constructed by C. K. Peterbauer was used to determine the function of *nag1* in induction of the chitinolytic enzyme system and its relevance in biocontrol applications.

3.3.2 Results

***nag1* deletion effects autolysis of *T. atroviride*.** As chitinases are involved in cell-wall turnover (Reyes *et. al.* 1989a and b, Sahai *et. al.* 1993, White *et. al.* 2002), effects of the deletion of the *nag1* gene on general morphology was tested. However, the *nag1* disruptant strain behaved exactly as the wild type concerning morphology, growth rates, and sporulation behaviour on different media.
In contrast, a major difference was observed concerning the autolysing capability of the disruptant strain. When both strains were pre-cultivated on glycerol and then transferred to media with chitin as sole carbon source, autolysis of the wild type strain P1 started after 16 h as indicated by the continuous release of soluble carbohydrates into the culture medium. Significantly less carbohydrates could be detected in culture fluids of the Δ*nag1* disruptant strain. A *nag1* retransformant strain P1ND1r3 behaved essentially like the wild type strain, indicating that the lack of chitinolytic activity in P1ND1 is due to the lost function of *nag1*.

	Soluble carbohydrates [mg/ml]		
	P1	P1ND1	P1ND1r3
1 h	0.10±0.02	0.03±0.01	0.05±0.04
16 h	4.70±0.61	0.10±0.03	3.85±0.60
26 h	20.66±3.47	0.14±0.03	16.10±2.8

Table 1. Soluble carbohydrates released by *T. atroviride* wild type and mutant strain during cultivation on colloidal chitin.

The major extracellular N-acetylglucosaminidase activity of *T. atroviride* is encoded by *nag1*. The Δ*nag1* disruptant strain was used to test whether the N-acetylglucosaminidase activity of *T. atroviride* corresponds solely to this gene. The fungus was cultivated on N-acetylglucosamine and N-acetylglucosaminidase activity was determined in the culture supernatant and a mycelial suspension. Whereas NAG activity in the supernatant of strain P1ND1 was reduced to less than 1 % of that found in the wild type P1, the mycelial suspension of the Δ*nag1* disruptant strain still exhibited 10 % of the activity found in P1. This fact provides evidence for a second N-acetylglucosaminidase in *T. atroviride* which is almost exclusively located in the cell wall.

	Enzyme activity [mU/ml]	
	P1	P1ND1
Mycelial suspension	500±52	22±3.9
Culture filtrate	293±18	2±0.5
Cell walls	207±15	20±4.0

Table 2. Extracellular N-acetylglucosaminidase activity of *T. atroviride*. Activity in the cell wall was determined from the difference in the measurement of mycelial suspensions and mycelium-free culture supernatants.

Effect of *nag1* deletion on the expression of other chitinases. The *nag1*-deficient strain and the wild type *T. atroviride* P1 were pregrown on glycerol and then transferred to media containing colloidal chitin or glucose. Dialyzed and concentrated culture filtrates were used for determination of chitinase activity using the p-nitrophenyl derivatives of N-acetylglucosamine, diacetylchitobiose and triacetylchitotriose as substrates. No chitinase activity could be detected in the Δ*nag1* disruptant strain, whereas activity was clearly present in the wild type strain P1.

Substrate	Enzyme activity (units/ml)	
	T. atroviride P1	*T. atroviride P1ND1*
p-Nitrophenyl-N-acetylglucosamine	1003±61	7.2±1.1
p-Nitrophenyl-N,N'-diacetylchitobiose	65±4	1.0±0.3
p-Nitrophenyl-N,N',N''-diacetylchitotriose	40±2	0.5±0.2

Table 4. Expression of chitinases in the Δ*nag1* disruptant strain and the wild type P1. Data are means ± SD from three separate experiments.

To test whether this lack of chitinase activity in the Δ*nag1* disruptant strain is due to a lack of gene transcription, northern analyses with the *ech42* gene as probe were performed. In contrast to the wild type P1, the *nag1*-negative mutant showed no transcripts of *ech42* (see Figure 1.).

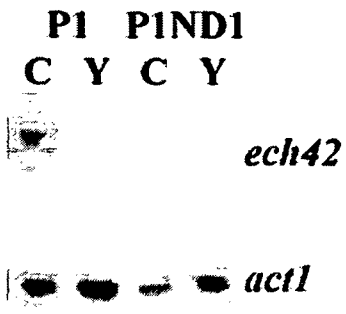

Figure 1. *ech42* northern blot analysis of the Δ*nag1*-disruptant P1ND1 and wild type strain P1. In lanes indicated with C, RNA from mycelium grown on colloidal chitin was used and in lanes indicated with Y, the RNA is from fungi cultivated on glycerol.

These results indicate that *ech42* transcription cannot be induced by colloidal chitin as sole carbon source in a Δ*nag1* disruptant strain. To find out whether this effect on chitinase expression is indeed caused by the disruption of the *nag1* gene and not due to any other effect, strain P1ND1 was retransformed with an intact copy of the *nag1* gene. The plasmid pCN2 (constructed by C. K. Peterbauer) contains the *nag1* structural gene including a functional promoter and terminator and was chosen for biolistic transformation of P1ND1. A strain containing only one copy of *nag1* (see Fig 2.) was chosen for further experiments. Culture supernatants of the re-transformed strain P1ND1r3 were used for enzymatic assays with p-nitrophenyl-N-acetylglucosamine as substrate. The N-acetylglucosaminidase activity of this strain was 0.65 U/ml compared to 1.20 U/ml of the wild type P1. Both results refer to the same biomass determined as dry weight. The N-acetylglucosaminidase activity could not be restored completely by retransformation with pCN2, probably due to a locus effect as the plasmid integrated randomly into the genome. The regain of chitinase gene transcription in the re-transformed strains was demonstrated by northern analysis using the *ech42* gene as probe (see Figure 2).

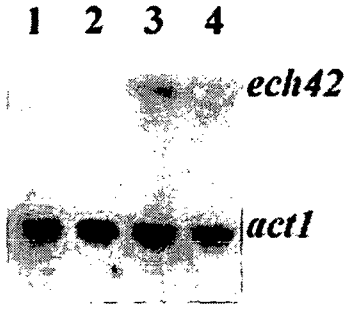

Figure 2. Northern blot analysis of the re-transformed strain P1ND1r3. In lanes 1 and 2 RNA from mycelia grown on Glycerol is used and lanes 3 and 4 is RNA from cultures on colloidal chitin.

Addition of exogenous Nag1 cannot restore the effect of *nag1* disruption on *ech42* gene expression. Culture filtrates of *T. atroviride* grown on N-acetylglucosamine as sole carbon source were dialyzed, concentrated and subsequently used to add exogenous Nag activity to media. P1ND1 was pregrown on glycerol and then transferred to media containing colloidal chitin as the sole carbon source, augmented with the above described culture concentrate to a final volumetric N-acetylglucosaminidase activity of 0.04 U/ml. However, this addition of Nag1 enzyme failed to rescue the induction of *ech42* transcription or the formation of chitinase enzyme activity. This lack of effect was not due to inhibition of *ech42* transcription by an unknown component in the added Nag-concentrate as transcription in the wild type strain incubated with colloidal chitin and Nag-concentrate resulted in the expected *ech42* transcripts. Therefore, the effect of *nag1* deletion on chitinase gene induction is not due to the loss of the extrcellular Nag1 activity.

***Nag1* deletion has a major effect on biocontrol ability of *T. atroviride*.** Although the Δ*nag1* disruptant strain lost almost its complete chitinolytic activity *in vitro*, plate confrontation assays with *R. solani* and *S. sclerotiorum* exhibited no difference in overgrowing or lysis of the plant pathogens between P1 and P1ND1. In contrast to confrontation experiments, *in planta* biocontrol assays revealed a significant loss of antagonistic abilities of the disruptant strain, as the growth of plants was reduced to 61 % (*R. solani*) and 65 % (*S. sclerotium*), respectively.

	Plant height [cm]			
Days	*Rhizoctonia solani*		*Sclerotinia sclerotiorum*	
	P1	P1ND1	P1	P1ND1
19	6.9 [± 1.3]	4.3 [± 0.6]	14.2 [± 7.8]	6.2 [± 4.3]
31	15.7 [± 2.3]	10.8 [± 0.5]	20.0 [± 1.3]	12.8 [± 7.3]
41	23.7 [± 1.5]	17.8 [± 3.8]	23.4 [± 4.2]	15.5 [± 8.2]

Table 4. Antagonistic activity of *T. atroviride* P1 and the *nag1*-negative mutant P1ND1 against *R. solani* and *S. sclerotiorum*. 42 beans were used for each assay.

3.3.3. Discussion

The results presented in this study show that the N-acetylglucosaminidase Nag1 has a major influence on the induction of chitinase encoding genes by colloidal chitin in *T. atroviride*. The deletion of *nag1* blocks the formation of virtually all other chitinases, including *ech42*. This finding is of great interest as previous studies describe chitinase gene expression in *Trichoderma* as a response to external signals including stress, nitrogen and carbon source starvation or cultivation on fungal cell walls or chitin (Carsolio *et. al.* 1994, Garcia *et. al.* 1994, Zeilinger *et. al.* 1999, Mach *et. al.* 1999, de la Mercedes Dana *et. al.* 2001). While both endochitinase-encoding genes *ech42* and *chit33* are induced by chitooligomers in *T. harzianum* (de la Mercedes Dana *et. al.* 2001), no such induction was observed in *T. atroviride* (Mach *et. al.* 1999). These findings were previously interpreted simply as a lack of inducibility of *T. atroviride ech42* by chitin degradation products. However, present data suggest that the effect of Nag1 on chitinase gene induction is due to its enzymatic activity, thereby being involved in the formation of the physiological inducer of other chitinase genes. The fact that *ech42* is not induced by chitin degradation products released by the action of Nag1 on chitin does not contradict this possibility, as the role of Nag1 in chitinase expression may be to modify them by its transglycosylating acticity (Koga *et. al.* 1991). The fact that the *Δnag1* disruptant strain has a reduced ability to autolyse suggests that this gene plays a key role in autolytic cell wall degradation. These findings are in agreement with Reyes *et al.* (1989), who demonstrated that a N-acetylglucosaminidase is involved in cell wall autolysis in *Aspergillus*. Thus, for the induction of *ech42* of *T. atroviride*, an inducer derived rather from its own cell wall than from chitin is necessary. The structure of chitooligosides released from a fungal cell wall can differ from those released from chitin, as the cell wall chitin is covalently bound to β-glucan via peptide bridges (Wrthall *et. al.* 1973). Lorito *et al.* (Manuscript in preparation) recently demonstrated that *ech42* is induced by chitooligosaccharides released from fungal cell walls. Additionally, these findings are in context with chitinase gene induction by cold, osmotic and pH stress, as under these conditions cell wall synthesis stops immediately and therefore autolysis can be caused. The

fact, that the loss of chitinase expression cannot be restored by extracellular addition of Nag1 does not contradict this assumption as autolysis occurs in the inner part of the hyphae. Thus far only a single gene coding for a N-acetylglucosaminidase has been isolated from *T. atroviride* (Peterbauer *et. al.* 1996), nevertheless the results of this study provide evidence for the presence of a second one. The Δ*nag1* disruptant strain exhibited N-acetylglucosaminidase activity, mainly cell wall located, accounting for 4% of the extracellular activity.
Although this strain lost almost its complete chitinolytic activity, there was no complete reduction in biocontrol ability. In plate confrontation assays the *nag1*-negative strain antagonized as well as the wild type P1 and *in planta* assays revealed a reduction of biocontrol ability of approximately 33%. As all the experiments were carried out in synthetic media containing chitin as carbon source, no evidence is given for the induction of chitinase genes during overgrowth of a host on plates or in the rhizosphere of a plant. Dal Soglio *et al.* (1998) reported that soybean seedlings incubated with *T. harzianum* and *R. solani* showed high expression of a plant N-acetylglucosaminidase together with the fungal 42 kDa endochitinase. Thus, the effect on chitinase gene induction caused by the lack of Nag1 may not be relevant, as the plant itself can be able to compensate this deficiency.
In addition the lack of an enzyme is sometimes compensated by the overexpression of another. Similar findings were made with a cellulase-negative mutant of *T. reesei* (Seiboth *et. al.* 1997, Suominen *et. al.* 1993). In fact a high glucanase activity was detected in the Δ*nag1* disruptant strain (Brunner, K. unpublished data).
In situ investigations with strain P1ND1 have to be performed to further clarify the effect of the lack of *nag1* on chitinase expression in mycoparasitism.

3.3.4. Materials and Methods

Strains.*T. atroviride* strain P1 (*T. "harzianum"* ATCC 74058; Kullnig et al. 2001) was used throughout this study and maintained on potato dextrose agar (PDA; Merck, Darmstadt, (Germany). *Botrytis cinerea* strain 26 and *Sclerotinia sclerotiorum* strain 1450 were used as model pathogens and obtained from the collection of the Institute of Plant Pathology, Universita degli Studi di Napoli "Federico II (Naples, Italy). *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) was the host for plasmid amplification.

Cultivation conditions. *T. atroviride* and recombinant strains prepared from it were grown in liquid synthetic medium (SM) containing (in g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; urea, 0.6; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 2H_2O$, 0.0028; $CoCl_2 \cdot 6H_2O$, 0.0032 (pH 5.4). Glucose or glycerol (10 g/l, except when stated otherwise) were used as carbon sources. For induction experiments, *T. atroviride* was precultivated for 36 hrs in SM containing glycerol as carbon source, harvested by filtration through sterile Miracloth (Calbiochem, La Jolla, CA), washed with sterile tap water and transferred to 100 ml Erlenmeyer flasks containing 25 ml of SM medium and 1.5 % (w/v) of colloidal chitin. Mycelia were harvested after different times of incubation as indicated at the respective results. *B. cinerea* and *S. sclerotiorum* were cultivated on malt extract-peptone- and potato dextrose broth or agar, respectively.

DNA and RNA manipulation. Details of all these methods have been reported previously (Peterbauer et al. 2002a). Briefly, DNA was then isolated by the CTAB method (Ausubel et al. 1990), and plasmid DNA by means of a midiprep kit (Qiagen Inc., Chatsworth, CA). Northern blots of total RNA were done using Biodyne B nylon membranes (Pall Corp., Ann Arbor, MI). All restriction enzymes and DNA modifying

enzymes were obtained from Promega Corp. (Madison, WI). DNA probes for hybridizations were radioactively labelled by random priming (Ausubel et al. 1990). Other molecular techniques were performed according to standard protocols (Sambrook et al. 1989; Ausubel et al. 1990).

Fungal transformation. Transformation of the *nag1*-delta strain *T. atroviride* P1ND1 with pCN2 was performed by cotransformation with pHATα as described previously (Peterbauer et al. 2002a).

Preparation of N-acetyl-ß-D-glucosaminidase from *T. atroviride.* N-acetyl-ß-D-glucosaminidase from *T. atroviride* was prepared by inducing mycelia, pregrown on glycerol, with N-acetyl-ß-D-glucosamine as described previously (Mach et al. 1999). SDS-PAGE showed that the 73-kDa N-acetyl-ß-D-glucosaminidase accounted for more than 95 % of total protein in culture filtrates harvested after 4 h of incubation. They were thus dialyzed, twentyfold concentrated, lyophilized, and kept on ice until use.

Enzyme assays. Extracellular culture filtrates were filtered through a 0.22 µm membrane (Millipore, Bradford, MA), dialyzed against distilled water for 24 hrs at 4°C, concentrated about 20-fold with polyethylene glycol (8000 MW, Fluka Biochemika, Buchs, Switzerland), and stored at –20°C with 20% glycerol until use. Enzymatic assays were performed as previously described (Harman et al. 1993; Lorito et al., 1994), using *p*-nitrophenyl N-acetyl-α-D-glucosamine, *p*-nitrophenyl α-D-N,N′-diacetylchitobiose and *p*-nitrophenyl α-D-N′-N′′-triacetylchitotriose (all from Sigma, St. Louis, MO) as substrates.

Biocontrol assays. Tests of inhibition of *Botrytis cinerea* spore germination were performed in ELISA plates containing 20 µl culture filtrate of *T. atroviride* P1 and the recombinant strains thereof and 2000 *Botrytis* conidia in 100 µl 5 mM potassium phosphate buffer pH 6.7 per well. The plates were incubated at 25°C for 5 hrs and the number of germinated spores was counted under an inverted microscope (Lorito et al. 1993). Plate confrontation assays were performed as described previously (Lorito et al. 1996). For *in vivo* biocontrol tests, bean seeds were coated with a 10% (w/v) suspension of Pelgel (Liphatech, Milwaukee, WI) in 20 mM potassium phosphate buffer augmented with 20 mM glucose. 1 ml of a *Trichoderma* conidial suspension (108 conidia/ml) were used per 10 g of seeds. Pathogen infested soil was prepared by adding 1.5 - 3 g fungal biomass (*R. solani* and *S. sclerotiorum* wet weight) to 1 liter of sterile soil. After 2 days the infested soil was diluted 1:4 with sterile soil and used for biocontrol assays as described above. *Trichoderma* coated seeds were planted 4 cm deep into the pathogen infested soil. The number and height of surviving plants was evaluated for a period of up to 4 weeks. Control experiments without *Trichoderma* were also included.

3.3.5. References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1990. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

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

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 22

Carsolio C, Gutierrez A, Jiménez B, Van Montagu M, Herrera-Estrella A. 1994. Characterization of *ech42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA 91, 10903-10907

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 application. Taylor and Francis Ltd., London, UK, 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. Dal Soglio FK, Bertagnolli BL, Sinclair JB, Yu G-Y, Eastburn DM. 1998. Production of chitinolytic enzymes and endoglucanase in the soybean rhizosphere in the presence of *Trichoderma harzianum* and *Rhizoctonia solani*. Biol Contr 12, 111 - 117

de la Mercedes Dana M, Limon CM, Meijas R, Mach RL, Pintor-Toro JA, Kubicek CP. 2001. Regulation of chitinase 33 (*chit33*) gene expression in *Trichoderma harzianum*. Curr Genet 38, 335 - 342

Draborg H, Kaupinnen S, Dalboge H, Christgau S. 1995. Molecular cloning and expression in *Saccharomyces cerevisiae* of two exochitinases from *Trichoderma harzianum*. Biochem Mol Biol Inter 36, 781 – 791 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28, 350-356

Garcia I, Lora JM, de la Cruz J, Benitez T, Llobell A, Pintor-Toro A. 1994. Cloning and characterization of a chitinase (*chit42*) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet 27, 83 - 89

Griffiths D. 1994. Fungal Physiology. Wiley and Sons.

Haran S, Schickler H, Oppenheim A, Chet I. 1995. New components of the chitinolytic system of *Trichoderma harzianum*. Mycol Res 99, 441 - 446

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Tronsmo A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology 83, 313-318.

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

Hjeljord L, Tronsmo A. 1998. *Trichoderma* and *Gliocladium* in biological control: an overview. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, Vol.2: Enzymes, biological control and commercial application. Taylor and Francis Ltd, London, UK, pp 129-151

Inbar J, Chet I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum.* Microbiology 141, 2823 - 2829 Kelly JM, Hynes MJ. 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J 4: 475-479

Kim DJ, Baek JM, Uribe P, Kenerley CM, Cook DR. 2002. Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*. Curr Genet 40, 374 - 384. Koga K, Iwamoto Y, Sakamoto H, Hatano K, Sano M, Kato I. 1991. Purification and characterization of ß-N-acetylhexosaminidase from *Trichoderma harzianum*. Agric Biol Chem 55, 2817 - 2833

Koga D, Mitsutomi M, Kono M, Matsumiya M. Biochemistry of chitinases. EXS. 1999; 87:111-23.

Kubicek CP, Mach RL, Peterbauer CK, Lorito M. 2001. *Trichoderma* chitinases: from genes to biocontrol. J Plant Pathol 83, 11 - 23

Kullnig CM, Krupica T, Woo SL, Mach RL, Rey M, Benitez T, Lorito M, Kubicek CP. 2001.Confusion abounds over identity of *Trichoderma* biocontrol isolates. Mycol. Res. 105, 769 – 772

Lorito M. 1998. Chitinolytic enzymes ands their genes. In: *Trichoderma* and *Gliocladium*, Vol. 2 (Harman GE, Kubicek CP, eds) pp 73 - 99. Taylor and Francis, London UK

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A. 1993. Chitinolytic Enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83, 302-307.

Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE. 1994. Purification, characterization and synergistic activity of a glucan 1,3-â-glucosidase and N-acetyl-ß-glucosaminidase from *Trichoderma harzianum*. Phytopathology 84, 398-405.

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

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

Margolles-Clark E, Harman GE, Penttilä M (1996) Enhanced expression of endochitinase in *Trichoderma harzianum* with the *cbh1* promoter of *Trichoderma reesei*. Appl Environ Microbiol 62, 2152-2155

Penttila M, Nevalainen H, Ratto M, Salminen E, Knowles J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61, 155-164

Peterbauer C, Lorito M, Hayes CK, Harman GE, Kubicek CP. 1996. Molecular cloning and expression of *nag1* (N-acetyl-ß-D-glucosaminidase-encoding) gene from *Trichoderma harzianum* P1. Curr Genet 29, 812-820

Peterbauer CK, Brunner K, Mach RL, Kubicek CP. 2002a. Identification of the N-acetyl-D-glucosamine-inducible element in the promoter of the *Trichoderma atroviride nag1* gene encoding N-acetyl-glucosaminidase. Mol Genet Genomics 267, 162 - 170.

Peterbauer CK, Litscher D, Kubicek CP. 2002b. The *Trichoderma atroviride seb1*-(stress response element binding) gene encodes an AGGGG-binding protein which is involved in osmotic stress response. Mol. Genet. Genomics 268, 223 – 231.

Reyes F, Calatayud J, Vazquez C, Martinez MJ. 1989a. Beta-N-acetylglucosaminidase from *Aspergillus nidulans* which degrades chitin oligomers during autolysis. FEMS Microbiol Letts 53, 83 - 87

Reyes F, Calatayud J, Martinez MJ. 1989b. Endochitinases from Aspergillus nidulans Beta-N- acetylglucosaminidase from *Aspergillus ndulans* implicated in the autolysis of the cell-wall. FEMS Microbiol Letts 51, 119 - 124

Sahai AS, Manocha MS. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol Rev 11, 317 – 338

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.

Seiboth B, Hakola S, Mach RL, Suominen PL, Kubicek CP. 1987. Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei*. J Bacteriol 179, 5318-5320

Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen H. 1993. High frequency one- step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. Mol Gen Genet 241, 523-530

Ulhoa CJ, Peberdy JF. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol. 1991 Sep; 137:2163-9.

Viterbo A, Haran S, Friesem D, Ramot O, Chet I. 2001. Antifungal activity of a novel endochitinase gene (*chit36*) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200, 169-174

White S, McIntyre M, Berry DR, McNeil B. 2002. The autolysis of industrial filamentous fungi. CRC Crit Rev Biotechnol 22, 1 – 14.

Woo SL, Donzelli B, Scala F, Mach RL, Harman GE, Kubicek CP, Del Sorbo G, Lorito M. 1998. Disruption of *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* strain P1. Mol Plant-Microbe Interact 12, 419-429

Wrathall CR, Tatum EL. 1973. The peptides of the hyphal wall of *Neurospora crassa*. J Gen Microbiol. 78, 139 - 153.

Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC 19 vectors. Gene 33, 103-119

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

Chapter 4

Improvement of the biocontrol activity of *Trichoderma atroviride* P1 by expression of multiple copies of the *Aspergillus niger* glucose oxidase encoding gene *goxA* under a chitinase (*nag1*) promoter

4.1. Summary

I investigated whether a transgenic strain of *T. atroviride,* which expresses the *A. niger* glucose-oxidase encoding gene *goxA* under a homologous chitinase (*nag1*) promoter would show an improved ability for biocontrol of plant pathogenic fungi. The respective transgenic strains exhibited no differences compared to the wild-type regarding sporulation or growth rates. The *nag1* promoter triggered *goxA* expression immediately after contact with the respective plant pathogen, resulting in secretion of the formed glucose oxidase into the media. Simultaneously, the transgenic strain produced significantly reduced N-acetyl-glucosaminidase and endochitinase activities. Glucose oxidase containing culture filtrates exhibited a 3-fold increase in the inhibition of germination of *Botrytis cinerea* spores. Consistent with these findings, the transgenic strain showed an increased rate in overgrowing and lysing the plant pathogenic fungi *Rhizoctonia solani* and *Pythium ultimum.* In contrast, the transgenic strain had no effect *in planta* against low inocula concentrations of these pathogens. However, beans planted into heavily infested soil and treated with conidia of the transgenic *Trichoderma* strain still retained the ability to germinate, whereas the wild type spores could not provide any protection against fungal root rot. We conclude that the transformation of *T. atroviride* with *nag1*-driven *Aspergillus* glucose oxidase improves its biocontrol properties.

4.2 Introduction

T. atroviride is a filamentous soil fungus which is known as an effective biocontrol agent against a wide range of economically important aerial and soil borne plant pathogens (Chet *et. al.* 1987, Papavizas *et. al.* 1985). Its mycoparasitic activity is due to the combination of successful nutrient competition (Chet et. el. 1987), production of cell wall degrading enzymes (Schirmböck *et. al.* 1994) and antibiosis (Ghisalberti at. al. 1993). The use of several strains of the genus *Trichoderma* as an alternative to chemical fungicides has received remarkable attention in recent years. However, the full-scale application of *Trichoderma* for biological control of plant pathogens has remained the exception because of their lower efficacy in biocontrol when compared with the respective chemical agents. For this reason, several attempts to improve the biocontrol ability of *Trichoderma* were carried out. At a molecular genetic level, attempts were focusing towards an increased chitinase or proteinase formation by either increasing the copy number of the respective genes, fusing them to strong promoters (e.g.: p*cbh1::ech42*) or constitutively overexpressing them. However, these strategies did not result in enhanced *in situ* biocontrol activity as the levels of chitinases secreted by *Trichoderma* might be enough for efficient biocontrol (Limon *et. al.* 1999, Margolles-Clark 1996, Carsolio 1999).
Talaromyces flavus is known as a potential biocontrol agent against the plant pathogens *Verticillium dahliae* (Marois *et. al.* 1982), *Sclerotinia sclerotiorum* (McLaren *et. al.* 1986) and *Rhizoctonia solani* (Boosalis *et. al.* 1956). *In vitro* experiments performed with culture filtrates of *T. flavus* grown on glucose indicated that glucose oxidase is responsible for the main part of the growth inhibition of *V. dahliae* microsclerotia and hyphae (Murray *et. al.* 1997, Stosz *et. al.* 1996), which was further supported by the findings that a glucose oxidase deficient strain of *T. flavus* failed to antagonize *Verticillium* wilt of eggplant in greenhouse experiments (Fravel *et. al.* 1991). Glucose oxidase catalyzes the oxygen-dependent oxidation of D-glucose to D-glucono-1,5-lactone and hydrogen peroxide. Glucose oxidase, glucose and gluconate (which is spontaneously formed from D-glucono-1,5-lactone in aqueous solutions) are not inhibitory to *V. dahlia* when used individually (Kim *et. al.* 1988),

but low concentrations of hydrogen peroxide significantly inhibited the growth of *Pythium ultimum*, *P. aphanidermatum*, *R. solani* and *V. dahliae*. Therefore the antifungal effect of the glucose oxidase system is due to hydrogen peroxide (Kim *et. al.* 1990).
Several species of *Trichoderma* are considerably more resistant to the products of glucose oxidase activity than the plant pathogens named above (Kim *et. al.* 1993). On the other hand, *Trichoderma* does not have a glucose oxidase orthologue (Mach *et. al.* 1999). As increased secretion of chitinolytic enzymes did not result in appropriate enhancement of plant protection, I therefore reasoned that the transformation of *T. atroviride* with a heterologous glucose oxidase-encoding gene may increase the biocontrol abilities of the respective strain. In order to test this hypothesis, a transgenic strain of the fungus which contains 12-14 copies of the *Aspergillus niger goxA* (glucose oxidase-encoding) gene under the *nag1* (N-acetyl-ß-D-glucosaminidase) promoter was used, and its biocontrol properties *in vitro, in vivo* and *in planta* was tested.

4.3. Results

Physiological properties of *T. atroviride* strain SJ3 4 expressing the *goxA* gene under control of the *nag1* promoter. Recombinant strain SJ3 4 bearing 12-14 copies of the p*nag1*::*goxA* fusion exhibited a similar growth rate on PDA or SM augmented with 1% glucose as the wild-type P1. (Tab.1) Similar results were obtained for biomass production in replaced liquid cultures: 8.0 vs. 7.2 g mycelial dry weight/l for P1 and SJ3 4, respectively (Tab. 1).

strain	colony diameter (mm)[a]		biomass (g dryweight/100ml PDB)[b]
	PDA	SM+Glucose	
P1	50[±6][c]	32[±4]	0.8
SJ3 4	46[±4]	32[±4]	0.7

Table 1. Comparison of growth rates of *T. atroviride* P1 and SJ3 4
[a] growth on solid media
[b] in liquid culture
[c] standard deviation from five separate experiments

Formation of chitinolytic enzyme activities by the two strains was also tested with culture filtrates from mycelia pregrown on PDB and replaced to SM media containing glucose or colloidal chitin (both 1.5 %, w/v), respectively. No chitinolytic enzyme activities were detected in filtrates from both *Trichoderma* strains grown on glucose; however, they were clearly detectable in filtrates from both strains after replacement to medium containing colloidal chitin as a sole carbon source. Under these conditions, SJ3 4 exhibited only 55% and 70% of the N-acetyl-ß-glucosaminidase and endochitinase activities produced by the wild type (Tab. 2).

strain	enzyme activity (mU/ml) [a] Nag[b]	Chb[c]	Ech[d]	GoxA[e]
P1	293[e][±13][f]	65[±2]	40[±3]	n.d.
SJ3 4	163[±13]	63[±3]	29[±2]	300[±19]

Table 2. Enzyme production of *T. atroviride* strain P1 and SJ3 4 on colloidal chitin.
[a] normalized to the biomass production of strain P1
[b] N-acetyl-β-D-glucosaminidase, [c] 1,4-β-chitobiosidase, [d] endochitinase, [e] glucose oxidase
[f] standard deviation from five separate experiments

In contrast, the activity of chitobiosidase was virtually the same in both strains. From these data it can be concluded that the transgenic strain SJ3 4 suffers from no general reduction in viability and fitness, but exhibits a reduced efficacy in its expression of two biocontrol-related chitinase genes.

Glucose oxidase expression is induced by chitin and during mycoparasitic interaction in the transgenic strain. We have previously reported that *T. atroviride* P1 does not produce any glucose oxidase activity (Lorito *et. al.* 1994). However, the strain SJ3 4 produced 4 [± 1] and 300 [± 19] mU/ml of glucose oxidase activity on media containing glucose or colloidal chitin, respectively. These findings are in perfect agreement with previous observations that expression of the *nag1* gene is induced by chitin (Mach *et. al.* 1999, Peterbauer *et. al.* 1996). The low level of glucose oxidase activity on glucose is most probably due to the high copy number of the integrated p*nag1*::*goxA* fusion (12-14 copies; (15) in this particular strain as no such expression could be measured in other *nag1*::*goxA* transgenic strains bearing less copies (R.L. Mach, K. Payer and S. Jaksits unpublished data). To prove that glucose oxidase expression is not only induced by chitin but also by direct contact with a potential host, plate confrontation assays with *B. cinerea* were carried out. To detect glucose oxidase formation, we used methyl red as pH indicator for the gluconic acid formed. A red halo was observed around *Trichoderma* strain SJ3 4 right after contact (1 to 2 hours) with its host. The development of these halos at this confrontation stage was specific for the presence of glucose oxidase as it was only observed with glucose oxidase-producing transformants of *T. atroviride*, whereas neither the wild type nor strains transformed with the hygromycin B resistance conferring vector pHATα (Herrera-Estrella *et. al.* 1990) exhibited such a fast pH shift (see Figure 1)

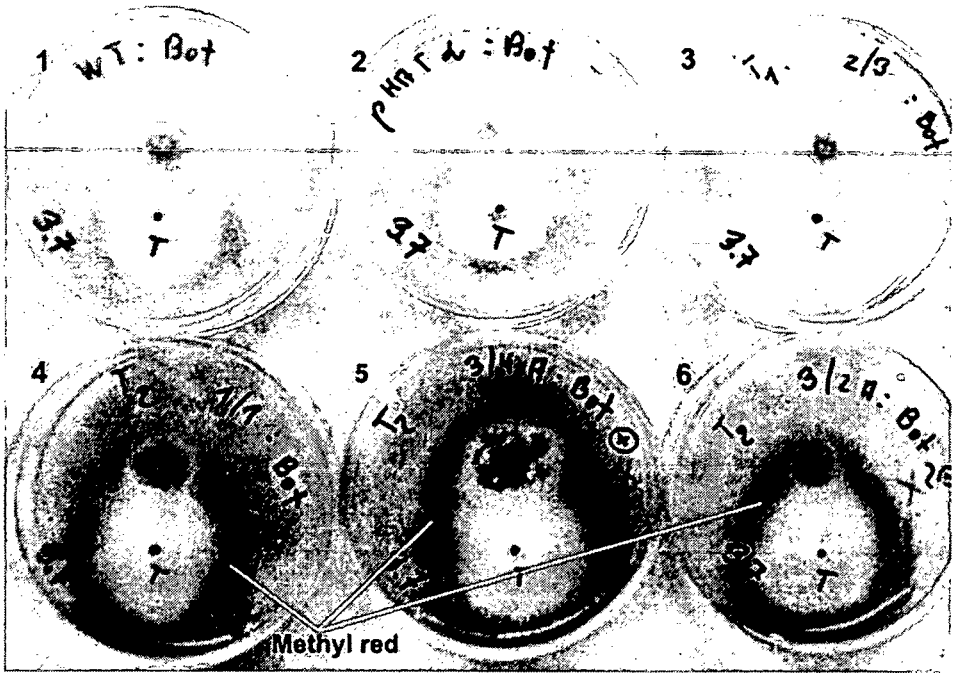

Figure 1.: Production of glucose oxidase by *T. atroviride* strain SJ3 4 during confrontation with *Botrytis cinera*. The medium contained methyl red as a pH indicator to monitor the acidification of the medium by the gluconate formed after the oxidation of glucose to glucono-1,5- δ-lactone by glucose oxidase. *Trichoderma* wild type (1), transformation with pHATα, a plasmid bearing the *hph* gene encoding for hygromycin B phospho transferase used for co-transformation (2), unsuccessfully transformed strain not containing any copy of *goxA* (3), isolates with different copy numbers of the *goxA* gene (4-12). Strain SJ3 4 on plate number 5 was chosen for further experiments.

A general acidification leading to the occurrence of red halos could be observed with all strains during later stages of biocontrol (18 to 24 hours after contact). These data strongly indicate coherence between host contact-induced *nag1* gene expression and glucose oxidase production in the transgenic *T. atroviride* strain SJ3 4.

***GoxA* expression leads to enhanced inhibition of *Botrytis* spore germination.** The inhibition of *B. cinerea* spore germination by culture supernatants of *T. atroviride* strains P1 and SJ3 4 was tested *in vitro*. The addition of hydrogen peroxide at a final concentration of 10 mM and 100 mM respectively was used as a control. At the lower H_2O_2 concentration, 39% of the spores germinated, whereas only 5% in the experiment with the higher amount of peroxide. All percent values given refer to a germination percentage of 100 in a control assay using sterile water instead of any culture filtrate. Culture filtrates denaturated for 5 minutes at 95°C had no inhibitory effect (data not shown). The culture filtrates obtained from the glucose oxidase producing strain grown on colloidal chitin exhibited a significant improvement in antifungal activities as the inhibitory effect of SJ3 4 was three times enhanced compared with the wild type (see Fig. 2).

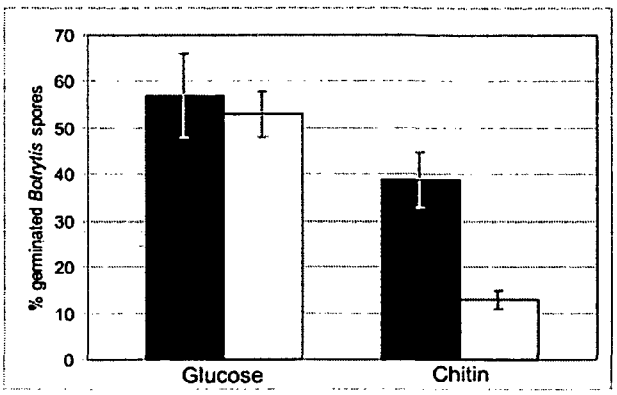

Figure 2.: Effect of culture filtrates from *T. atroviride P1* and SJ3 4 on inhibition of *Botrytis* condial germination. Percentage (referring to a control with water as 100%) of germinated *Botrytis* spores in media augmented with culture filtrates from *Trichoderma* wild type (indicated by black bars) and the glucose oxidase producing strain SJ3 4 (grey bars). Filtrates were obtained from media containing glucose or colloidal chitin as sole carbon source.

***T. atroviride* strain SJ3 4 shows improved mycoparasitism against selected plant pathogenic fungi.** To investigate whether the expression of glucose oxidase enhances the mycoparasitic ability of *T. atroviride*, plate confrontation assays against two soil born plant pathogens which have widely been used in the literature for this purpose (i.e. *R. solani* and *P. ultimum*) were performed. Fig. 3 shows that strain SJ3 4 exhibited an enhanced antagonism (sporulation and host lysis) against *R. solani*, whereas no significant differences in the rate of growth on the host were found. An even stronger difference was observed during confrontation with the oomycete *P. ultimum*: while the wild type strain P1 exhibited only a minor mycoparasitic activity, SJ3 4 completely lysed the pathogen within 7 days and at that time already started to sporulate on the host (Fig. 3).

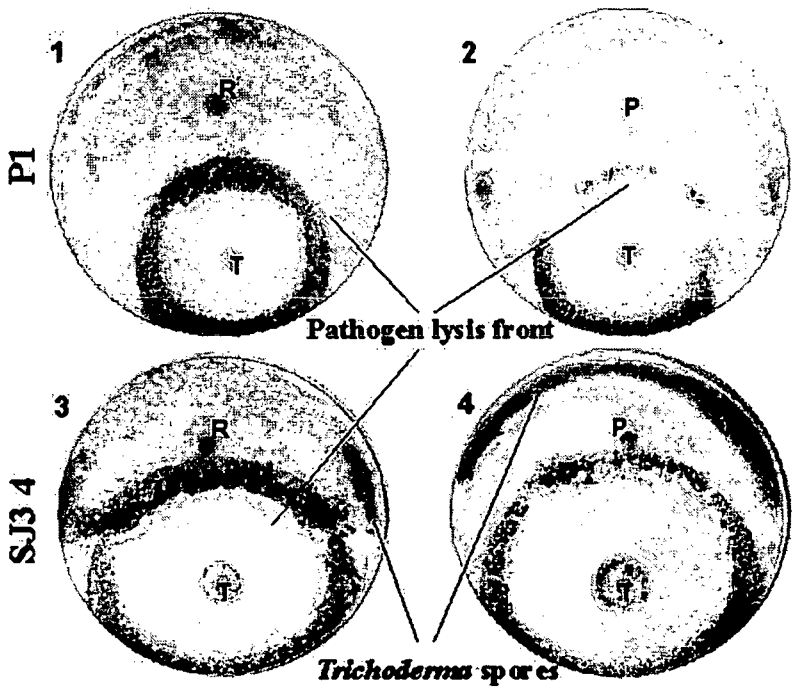

Fig. 3.: Performance of *T. atroviride* P1 and SJ3 4 in plate confrontation assays on PDA with *Rhizoctonia* (R) or *Pythium* (P) as hosts.

Strain SJ3 4 can overcome a high disease pressure of soil borne pathogens such as *R. solani* and *P. ultimum*. To investigate whether this increased activity in plate confrontation tests would be reflected in a correspondingly increased biocontrol activity, *in planta* experiments were carried out. To this end bean seeds coated with conidia of either strain P1 or SJ3 4 were planted into pathogen infested soil. The germination ability of the beans was followed for 2 weeks. In soil tests with low amounts of pathogen (*R. solani* 1g biomass, *P. ultimum* 4 homogenized plates per liter soil) the glucose oxidase producing strain exhibited approximately the same level of biocontrol against *R. solani* and *P. ultimum* as the wild type strain. Both the number of germinated seeds and the height of the grown plants were comparable to the results previously described by Woo *et al.* (1999). Increasing the disease pressure by applying either 2g *Rhizoctonia* biomass per liter soil or 8 PDA plates of *Phytium* for the primary infection of 1 liter of sterile soil caused a nearly complete rot of seeds coated with wild type conidia and of uncoated seed. Almost all beans treated with the glucose oxidase producing strain retained the ability to germinate in soil infested with high amounts of the two pathogens (Table 3, Fig. 4).

	germinated seeds P1	germinated seeds SJ3 4
Control without pathogen	40	39
Rhizoctonia	4	36
Pythium	3	36
dpc (*Pythium*)	0	
dpc (*Rhizoctonia*)	0	

Table 3. *In planta* biocontrol assays. 42 beans were used for each assay. Beans not protected by *Trichoderma* are indicated as dpc (disease pressure control).

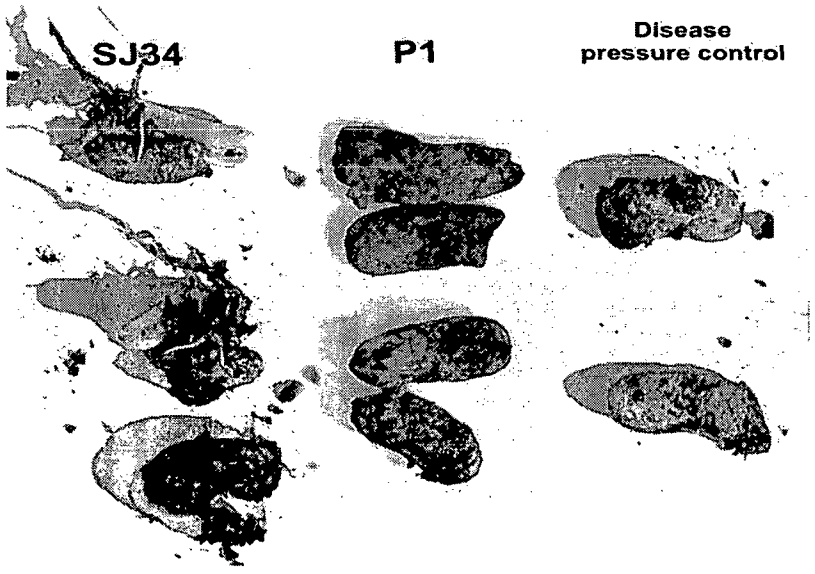

Fig 4.: Biocontrol activites of *T. atroviride* P1 and SJ3 4 in *in planta* assays under high disease pressure. Beans were coated with *Trichoderma* P1 or SJ3 4 spores and were planted into *Rhizoctonia*-infested soil. Beans not protected by *Trichoderma* are indicated as dpc (disease pressure control).

4.4. Discussion

Fravel *et al* (1991) demonstrated that glucose oxidase plays a key role in the biocontrol of *Verticillium* Wilt by *T. flavus*. Since *T. atroviride* does not form glucose oxidase, it had to be proven whether overexpression of a glucose oxidase in *T. atroviride* would enhance its biocontrol ability. To test for this hypothesis, a transgenic strain of *T. atroviride* (SJ3 4) which contains multiple copies of a p*nag1:goxA* construct was used. The *nag1* promoter was used for this purpose to encure that glucose oxidase is produced only upon contact with the plant pathogen. Mach *et. al.* (1999) reported that the *nag1* gene is active after contact of *Trichoderma* hyphae with its host but is not expressed at a constitutive level. However, the multicopy strain also expressed a low level of glucose oxidase constitutively.
T. atroviride SJ3 4 displayed growth rates comparable to the wild type strain on solid media as well as in liquid culture, proving that its general physiological functions were not influenced by genomic location and expression of the p*nag1:goxA* multicopies. However, N-acetyl-ß-D-glucosaminidase activity was reduced to about 45 % of that of the wild-type during growth on colloidal chitin. This is most likely due to competition for transacting factors by the multiple *nag1* promoter copies. In addition the expression of endochitinase activity is also significantly reduced. This finding may be interpreted by assuming that both the *nag1* gene as well as the endochitinase genes share the same transcription factors. However, at least *nag1* and *ech42* are not induced by the same chitooligomers (Lorito *et. al.* 1994), which argues against this interpretation. Another possibility is that the product(s) of the enzymatic activity of N-acetyl-ß-D-glucosaminidase are required for full endochitinase gene expression. Support for this hypothesis comes from our previous findings that a *nag1* negative *T. atroviride* strain produces only about 1 % of the wild type endochitinase activity (Brunner *et. al.* 2002). Whatever the mechanism of influence is, it is intriguing that the activity of chitobiosidase was not affected by *nag1:goxA* overexpression, thus indicating that its gene is apparently subject to a different regulatory circuit. Unfortunately, the respective gene has not been characterized yet.
Despite the reduction in the activities of the cell wall lytic enzymes, the p*nag1::goxA* multicopy strain easily compensated this handicap and even exceeded the wild type's biocontrol abilities in several standard assays, i.e. SJ3 4 culture filtrates showed a three times enhanced inhibitory effect on *Botrytis* spore germination. In plate confrontation assays, the transgenic strain performed better in overgrowing and lysis of *Rhizoctonia*.
The most obvious benefit of SJ3 4 was observed in confrontation assays with the oomycete *Pythium*. Whereas the *T. atroviride* wild type only moderately controlled this host, the mutant strain SJ3 4 immediately overgrew the entire plate and completely lysed *Pythium*. Such a distinct difference between the two strains could be due to the fact that the cell wall of *P. ultimum* does not contain any chitin thus excluding the action of the previously described pathogenicity factor chitinases (Brunner *et. al.* 2002, Woo *et. al.* 1999) and/or an exceedingly high sensitivity against hydrogen peroxide of this particular host (Klessing *et. al.* 1994). Apparently, the *nag1* promoter is induced during contact with a host bearing no chitin in its cell wall. Nevertheless, neither sophorose nor cellobiose could induce this chitinase promoter (Brunner, unpublised data). However, Inglis *et al.* (2002) recently demonstrated that *T. harzianum* expresses a set of chitinases upon cultivation on *Pythium* cell walls and furthermore, in a biomimetic system *R. solani* lectins could activate transcriptiojn of a 102 kDa N-acetylglucosaminidase in *T. harzianum* (Inbar *et. al.* 1995). These findings complete previous oservations that *nag1* expression is elicited by N-acetyl-glucosamine, chitooligomers and enzymatic released chitooligosacharides from *R. solani* cell walls (Mach *et. al.* 1999, Peterbauer *et. al.* 1996, Zeilinger *et. al.* 1999). Further investigations are required to gain deeper insights into the induction of *nag1* in mycoparasitism.

In the crucial experiment, the *in planta* assays, bean seeds were coated with conidia of the two different *Trichoderma* strains and planted into *Rhizoctonia* or *Pythium* infested soil, respectively. No detectable difference between the glucose oxidase producing strain and the wild type (with respect to both the number of germinated seeds and the height of the plants) could be observed under conditions of a low disease pressure. When the pathogen concentration was elevated, however, only seeds coated with SJ3 4 conidia retained the ability to germinate. Beans treated with conidia of the wild type strain P1 rotted completely within two weeks. This can be explained by the use of glucose (20 mM) in the coating procedure, so that every single bean is provided with 0.30 to 0.45 mg glucose (depending on the size of the seeds) as carbon source for adequate spore germination conditions and as a starting substrate for the glucose oxidase produced by the transgenic *Trichoderma* strain in pathogen infested soil. After successful germination of the seeds and formation of roots, a continued supply of glucose is probably guaranteed by root exudates. Fravel *et al.* (1991) demonstrated that eggplant roots have the potential to supply the *Talaromyces* glucose oxidase with enough glucose to harm *V. dahliae* microsclerotia. In contrast cotton roots are not able to provide enough glucose to the glucose oxidase from *Talaromyces* to control *Verticillium* Wilt (Murray *et. al.* 1997). Consequently, the impact of glucose oxidase on fungal biocontrol efficiency seems to be dependent both on the pathogen and also on the host plant used. The results from this study show that the main benefit of the glucose oxidase producing *Trichoderma* strain is its application in heavily infested soil, i.e. seeds planted into fields with high disease pressure and/or close to pathogen infested weeds or organic matter. This is of special importance as the mycelia or sclerotia of some plant pathogens such as *Rhizoctonia* are known to overwinter in high concentrations on infected roots of weeds and on colonized plant material (Griesbach 1980).

Hydrogen peroxide is known to be involved in the systemic acquired resistance (SAR) of plants, by inducing the expression of pathogenesis related proteins during the oxidative burst after a pathogen attack (Klessing *et. al.* 1994). Potatoes transformed with the *Aspergillus niger* glucose oxidase encoding gene and therefore consequently forming an elevated H_2O_2 level exhibited enhanced resistance both to fungal and bacterial pathogens (Wu *et. al.* 1995). Some strains of *Trichoderma* can penetrate the host root epidermis, progress toward the cortical area by intercellular growth (Yedida *et. al.* 1999), and may thus secrete glucose oxidase inside of the plant. Wu et al (1995) demonstrated that intercellularly secreted glucose oxidase elevates the hydrogen peroxide concentration in potatoes. The use of *Trichoderma* for this purpose would have the advantage that the *nag1* promoter is tightly regulated by the presence of the pathogen, thereby avoiding the unnecessary accumulation of hydrogen peroxide concentrations in the roots in the absence of a pathogen attack.

Summarizing, a new transgenic *Trichoderma* was characterized, showing enhanced antifungal activities and a potential for induction of the systemic acquired resistance in plants. Its special applicability against various fungal plant pathogens in heavily infested soil was demonstrated.

4.5. Materials and methods

Strains. *T. atroviride* strain P1 (*T. "harzianum"* ATCC 74058) was used throughout this study and was maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany). The glucose oxidase producing strain *T. atroviride* SJ3 4 has previously been described by (Mach *et. al.* 1999). *B. cinerea* strain 26 was cultivated on malt extract peptone agar, *R. solani* strain 19 and *P. ultimum* strain 8 on potato dextrose agar. *Botrytis, Rhizoctonia* and *Pythium* were obtained from the collection of the Institute of Plant Pathology, Università degli Studi di Napoli "Federico II" (Naples, Italy).

Cultivation conditions. *T. atroviride* strains were grown in liquid synthetic medium (SM) containing (in g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7\ H_2O$, 0.3; urea, 0.6; (mg/l): $FeSO_4 \cdot 7H_2O$, 10; $ZnSO_4 \cdot 2H_2O$, 2.8; $CoCl_2 \cdot 6H_2O$, 3.2 (pH 5.4), and augmented with either glucose or glycerol as carbon source (15 g/l, except when otherwise stated).

Determination of enzyme activities involved in cell wall degradation. *T. atroviride* was precultivated in PDB (potato dextrose broth; Merk, Darmstadt, Germany) for 48 hrs, harvested by filtration through miracloth (Calbiochem, La Jolla, CA) washed with sterile tap water and transferred to SM media containing either 1.5 % (w/v) glucose or colloidal chitin. After 3 days culture filtrates were obtained via filtration through a 0.22 µm filter followed by a dialyzing step against distilled water for 24 hrs at 4°C. Enzyme enrichment was carried out by covering the dialysis bags with polyethylene glycol 8000, (Fluka Biochemika, Buchs, Switzerland) and leaving them for 10 hours at 4°C leading to a 20-fold concentrated solution. The filtrates were stored at –20°C with 20% (v/v)glycerol until use. Enzymatic assays were performed as described previously (14) using the respective substrates *p*-nitrophenyl N-acetyl-β-D-glucosaminidine for N-acetyl-β-D-glucosaminidase, *p*-nitrophenyl β-D-N,N′-diacetylchitobiose for 1,4-β-chitobiosidase and *p*-nitrophenyl β-D-N′-N′′-triacetylchitotriose for endochitinase (all substrates from Sigma-Aldrich, Traufkirchen, Germany).

Determination of glucose oxidase activities. Culture filtrates were prepared following essentially the same growth conditions as describes above. Crude culture supernatants were tested for glucose oxidase activity as described previously (5, 15). For the determination of glucose oxidase activity produced during plate confrontation assays with *B. cinerea,* plates contained (in g/l): glucose 10, $(NH_4)_2SO_4$ 6, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, agar 15 and trace elements (see above) supplemented with the pH indicator methyl red (0.01 g/l) were used. Disks (3 mm in diameter) of *Botrytis* and *Trichoderma* were placed in a distance of 1.5 cm on the plate and the confrontation assay was performed in the dark. The formation of a red colour due to the pH shift caused by the oxidation of glucose to gluconate catalyzed by the produced glucose oxidase was monitored.

Biocontrol assays. *In vitro B. cinerea* spore germination inhibition was tested in ELISA plates essentially following (Lorito *et. al.* 1994). Briefly, a suspension of $3 \cdot 10^3$ *Botrytis* spores, 50 µl PDA with 5mM potassium phosphate buffer, pH 6.7, was placed into one well of an ELISA plate and 10 µl of the culture supernatants of strain P1 and SJ3 4 grown on colloidal chitin were added. The number of germinated spores was counted after 8 hours of incubation

For plate confrontation assays 5mm disks of *T. atroviride, R. solani* and *P. ultimum* were placed on PDA in a distance of 4 cm. The plates were incubated for several days in absence of light.

To test the germination ability of beans in pathogen infested soil, the seeds were coated with a 10% (w/v) suspension of Pelgel (Liphatech, Milwaukee, WI) in 20 mM potassium phosphate buffer, additionally augmented with 20 mM glucose. 1 ml of a 10^8 conidia/ml suspension of *Trichoderma* was used for coating 10 g of seeds. As a control, the same suspension was used without *Trichoderma.* Pathogen infested soil was prepared as follows: 500 ml of PDB media was inoculated with *R. solani* mycelium from one 4 d old 8-cm PDA plate. 2 g of the resulting biomass was used for 1 liter of sterile soil. For *P. ultimum* 1 liter of sterile soil was infested with four 3 d old 8 cm plates of the pathogen homogenized in a

blender for 30 s. After 2 days the infested soil was diluted 1:4 with sterile soil and used for biocontrol assays as described above. The coated seeds were planted 4 cm deep into infested soil and their germination was monitored for 2 weeks.

4.6. References

Boosalis, M. G. 1956. Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 46:473-478.

Brunner, K., C. K. Peterbauer, R. L. Mach, M. Lorito, S. Zeilinger, and C. P. Kubicek. 2003. The Nag1 N-acetylglucosaminidase of Trichoderma atroviride is essential for chitinase induction by chitin and of major relevance to biocontrol. Curr Genet.

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 22

Chet, I. 1987. *Trichoderma*-application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. Wiley & Sons, New York, NY.

Fravel, D. R., Roberts D. P. 1991. *in situ* Evidence for the Role of Glucose Oxidase in the Biocontrol of Verticillium Wilt by *Talaromyces flavus*. Biocontrol Science and Technology 1:91-99.

Geisen, R. 1995. Expression of the *Aspergillus niger* glucose oxidase gene in *Penicillium nalgiovense*. World Journal of Microbiology and Biotechnology 11:322-325.

Ghisalberti, E. L., and C. Y. Rowland. 1993. Antifungal metabolites from Trichoderma harzianum. J Nat Prod 56:1799-804.

Griesbach, E. 1980. [The importance of weeds for the transmission of Rhizoctonia solani Kuhn. IV. The role of weeds in the overwintering of the pathogen (author's transl)]. Zentralbl Bakteriol Naturwiss 135:195-200.

Herrera-Estrella, A., G. H. Goldman, and M. Van Montagu. 1990. High-efficiency transformation system for the biocontrol agents, Trichoderma spp. Mol Microbiol 4:839-43.

Inbar J, Chet I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum.* Microbiology 141, 2823 - 2829 Kelly JM, Hynes MJ. 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J 4: 475-479

Inglis GD, Kawchuk LM. 2002. Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi.Can J Microbiol. Jan;48(1):60-70

Kim, K. K., F. D.R., and P. G.C. 1988. Identification of a Metabolite Produced by *Talaromyces flavus* as Glucose Oxidase and its Role in the Biocontrol of *Verticillium dahliae*. Phytopathology 78:488-492.

Kim, K. K., Fravel D. R., Papavizas G. C. 1993. Glucose oxidase as the antifungal prinziple of talaron from *Talaromyces flavus*. Can. J. Microbiol. 36:760-764.

Kim, K. K., D. R. Fravel, and G. C. Papavizas. 1990. Glucose oxidase as the antifungal principle of talaron from Talaromyces flavus. Can J Microbiol 36:760-4.

Klessing, D. F., Malamy J. 1994. The salicylic acid signals in plants. Plant Mol. Biol. 26:1439-1458.

Limon, M. C., Pintor-Toro J. A., Benitez T. 1999. Increased Antifungal Activity of Trichoderma harzianum Transformants That Overexpress a 33-kDa Chitinase. Phytopathology 89:254-261.

Lorito, M., C. Peterbauer, C. K. Hayes, and G. E. Harman. 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology 140:623-9.

Mach, R. L., C. K. Peterbauer, K. Payer, S. Jaksits, S. L. Woo, S. Zeilinger, C. M. Kullnig, M. Lorito, and C. P. Kubicek. 1999. Expression of two major chitinase genes of Trichoderma atroviride (T. harzianum P1) is triggered by different regulatory signals. Appl Environ Microbiol 65:1858-63.

Margolles-Clark, E., C. K. Hayes, G. E. Harman, and M. Penttila. 1996. Improved production of Trichoderma harzianum endochitinase by expression in Trichoderma reesei. Appl Environ Microbiol 62:2145-51.

Marois J. J., J. S. A., Dunn M. T., Papavizas G. C. 1982. Biological Control of Verticillium wilt of eggplant in the field. Plant Dis. 66:1166-1168.

McLaren, D. L., Huang H.C. 1986. Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. J. Plant Pathol. 8:43-48.

Murray, F. R., D. J. Llewellyn, W. J. Peacock, and E. S. Dennis. 1997. Isolation of the glucose oxidase gene from Talaromyces flavus and characterisation of its role in the biocontrol of Verticillium dahliae. Curr Genet 32:367-75.

Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annu. Rev. Phytopathology 23:23-54.

Peterbauer, C. K., M. Lorito, C. K. Hayes, G. E. Harman, and C. P. Kubicek. 1996. Molecular cloning and expression of the nag1 gene (N-acetyl-beta-D- glucosaminidase-encoding gene) from Trichoderma harzianum P1. Curr Genet 30:325-31.

Schirmbock, M., M. Lorito, Y. L. Wang, C. K. Hayes, I. Arisan-Atac, F. Scala, G. E. Harman, and C. P. Kubicek. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of Trichoderma harzianum against phytopathogenic fungi. Appl Environ Microbiol 60:4364-70.

Stosz, S. K., D. R. Fravel, and D. P. Roberts. 1996. In vitro analysis of the role of glucose oxidase from Talaromyces flavus in biocontrol of the plant pathogen Verticillium dahliae. Appl Environ Microbiol 62:3183-6.

Woo, S. L., Donzelli, B., Scala, F., Mach R., Harman G. E., Kubicek C. P., Del Sorbo, G., Lorito, M. 1999. Disruption of the *ech42* (Endochitinase-Encoding) Gene Affects Biocontrol Activity in *Trichoderma harzianum* P1. MPMI 12:419-429.

Wu, G., B. J. Shortt, E. B. Lawrence, E. B. Levine, K. C. Fitzsimmons, and D. M. Shah. 1995. Disease resistance conferred by expression of a gene encoding H2O2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-68.

Yedidia, I. I., N. Benhamou, and I. I. Chet. 1999. Induction of defense responses in cucumber plants (Cucumis sativus L.) By the biocontrol agent trichoderma harzianum. Appl Environ Microbiol 65:1061-70.

Zeilinger, S., C. Galhaup, K. Payer, S. L. Woo, R. L. Mach, C. Fekete, M. Lorito, and C. P. Kubicek. 1999. Chitinase gene expression during mycoparasitic interaction of Trichoderma harzianum with its host. Fungal Genet Biol 26:131-40.

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