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BARD

FINAL REPORT

PROJECT NO. US-2325-93

**Discovery and Use of Genes and Gene Combinations
Coding for Proteins Useful in Biological Control**

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(Principal listed first)

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ABSTRACT

Discovery and use of genes and gene combinations coding for proteins useful in biological control

The objectives of the research in this proposal were to (A) identify synergy among proteins that provide enhanced activity over single proteins for control of plant pathogenic fungi, (B) clone and characterize genetic sequences coding for proteins with ability to control pathogenic fungi, (C) produce transgenic organisms with enhanced biocontrol ability using genes and gene combinations and determine their efficiency in protecting plants against plant pathogenic fungi. A related objective was to produce disease-resistant plants. Fungal cell wall degrading enzymes from any source are strongly synergistic with any membrane active compound and, further, different classes of cell wall degrading enzymes are also strongly synergistic. We have cloned and sequenced a number of genes from bacterial and fungal sources including five that are structurally unrelated. We have prepared transgenic fungi that are deficient in production of enzymes and useful in mechanistic studies. Others are hyperproducers of specific enzymes that permit us, for the first time, to produce enzymes from *T. harzianum* in sufficient quantity to conduct tests of their potential use in commercial agriculture. Finally, genes from these studies have been inserted into several species of crop plants where they produce a high level of resistance to several plant pathogenic fungi.

OBJECTIVES OF THE ORIGINAL PROPOSAL

- (A) identify synergy among proteins that provided enhanced activity over single proteins for control of plant pathogenic fungi.
- (B) clone and characterize genetic sequences coding for proteins with ability to control pathogenic fungi.
- (C) produce transgenic organisms with enhanced biocontrol ability using genes and gene combinations and determine their efficiency in protecting plants against plant pathogenic fungi.
 1. Produce transgenic bacteria with heterologous genes and secretory signals.
 2. Produce transgenic *Trichoderma* strains with heterologous or homologous genes for biocontrol enzymes under the control of constitutive (35S) or mycoparasitism suppressed (pgk) promoters. Such transgenic strains will still contain the normal complement of inducible biocontrol enzymes and the additional gene/promoter combinations will augment these naturally-occurring biocontrol systems.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

Note: In the USA, research is not yet complete. Two postdoctoral associates employed on this project left for other employment and so we have requested a nonfunded extension from the USDA, which funded the US side of the project. This was granted and more progress was made. However, the project is now complete.

Identify synergy among proteins that provide enhanced activity over single proteins for control of plant pathogenic fungi - In the USA, very strong synergistic relationships have been found between cell wall degrading enzymes produced from plants, bacteria or biocontrol fungi in the genus *Trichoderma*, and membrane active compounds. Examples of cell wall degrading enzymes are chitinolytic and β -1,3-glucosidases. Some of the membrane active compounds include synthetic fungicides, especially those inhibiting sterol demethylation, phospholipases, osmotin or osmotin-like proteins produced in plants as induced resistance factors, and various antibiotic compounds (Lorito et al., 1996). Further, the various enzymes, so long as they represent different classes, such as exo- or endo-acting enzymes, or β -1,3-glucosidases and chitinases, all are synergistic with one another. The level of synergy may reach a very high level; combinations of certain cell wall degrading enzymes and membrane active compounds are more than 100-fold more effective as antifungal agents than either used alone (Lorito et al., 1996).

In Israel, the chitinolytic system of *T. harzianum*, strain TM was found to have six distinct chitinolytic enzymes, two of which were identified as B-1,3 N-acetylglucosaminidases (E.C. 3.2.1.30) which hydrolyze chitin to N-acetylglucosamine (GlcNAc) monomers in an exo-type

fashion (CHIT 102 and CHIT 73) and four were characterized as endochitinases (EC 3.2.1.14) which cleave randomly at internal sites over the entire length of the chitin microfibril (CHIT 52, CHIT 42, CHIT 33 and CHIT 31) (Haran et al., 1995). This is at variance with results from other strains, for example, strain P1, which has been primarily used in the USA, lacks CHIT 33 but does have a 38 kDa chitobiosidase that is absent from strain TM. Differences in the enzymes that different strains produce may have important implications for their use and the molecular biology of these fungi.

Characterization of the *T. harzianum* chitinolytic system was performed in liquid cultures containing different carbon sources. These growth conditions facilitated the identification of the chitinolytic components induced in *T. harzianum* for the hydrolysis of chitin, but they do not represent the conditions during the antagonistic interactions between this parasitic fungus and its hosts. It had been reported that *Trichoderma* isolates produced chitinase and glucanase activities when grown on live mycelium of *S. rolfii* and *R. solani*, and a correlation between the expression levels of these enzymes and the biocontrol efficiency of the *Trichoderma* strains was found. However, the specific enzymes(s) actually involved in this activity was not identified.

To find which of the chitinolytic enzymes is involved in the interactions, we have used dual culture experiments, in which *T. harzianum* was separately confronted with two important plant pathogens, both containing chitin as a major cell wall component. One strain of *T. harzianum*, that was capable of overgrew *R. solani* hyphae in agar dual cultures, but was hardly overgrowing *S. rolfii* under the same conditions, was used in this study. To investigate which chitinases are expressed during these complex biological processes we have developed a procedure for the simultaneous detection of the various chitinases after their separation on SDS-polyacrylamide gels. A mixture of two enzyme substrates facilitated the simultaneous detection of chitinases with different modes of action.

The expression of the various N-acetylglucosaminidases and endochitinases during mycoparasitism was found to be regulated in a very specific and finely tuned manner, which was affected the host. When *T. harzianum* was antagonizing *Sclerotium rolfii*, an N-acetylglucosaminidase of 102 kDa (CHIT 102) was the first to be induced. As early as 12 h after contact its activity diminished, and another N-acetylglucosaminidase of 73 kDa (CHIT 73) was expressed at high levels. However, when *T. harzianum* was antagonizing *Rhizoctonia solani*, the chitinase expression patterns differed considerably. Twelve h after contact, CHIT 102 activity was elevated and the activities of three additional endochitinases at 52 kDa (CHIT 52), 42 kDa (CHIT 42) and 33 kDa (CHIT 33) were detected. The differential expression of *T. harzianum* chitinases may influence the overall antagonistic ability of the fungus against a specific host.

In Israel, we have purified and characterized the 33 kDa endochitinase (CHIT 33) from the culture filtrate of *T. harzianum*. This enzyme was not expressed when the fungus was grown on

glucose, but was induced and excreted into the growth medium when the fungus was grown on chitin as the sole carbon source. CHIT 33 is involved in the mycoparasitic interaction of *T. harzianum* with the plant pathogen *R. solani* and may therefore be an important component of the antagonistic activity of *Trichoderma* against this pathogen.

Clone and characterize genetic sequences coding for proteins with ability to control plant pathogenic fungi - The following genes have been cloned and sequenced.

An gene encoding a 40 kDa endochitinase from *T. harzianum* (Hayes et al, 1994).

Two endochitinases from *T. virens* (one nearly identical to that from *T. harzianum* strain P1).

An N-acetylglucosaminidase from *T. harzianum* (Peterbauer et al., 1996).

An α -D-glucosidase from *T. harzianum*.

An gene encoding an endochitinase from the biocontrol bacterium *Enterobacter agglomerans* (Chernin et al., 1997).

A gene encoding a strongly antifungal chitinase from the biocontrol bacterium *Chromobacterium violaceum* (unpublished).

All of the proteins produced by these genes are strongly antifungal. Except for the different endochitinases from *T. harzianum*, the genes are unrelated or possess only limited homology with one another. Therefore, the different genes give rise to unrelated proteins useful in different applications.

Produce transgenic microorganisms with enhanced biocontrol ability using genes and gene combinations and determine their efficacy in protecting plants against plant pathogenic fungi - There are two basic ways we can obtain results to accomplish this objective. One method is to create strains that produce very high levels of the various enzymes, and to use these enzymes directly in plant therapy. For example, these enzymes, if effective, would be of great interest in control of post-harvest fruit diseases caused by *Penicillium*, *Botrytis*, and other pathogens. The other method is to obtain fungal or bacterial strains that produce very high levels of the enzymes and to use them directly as biocontrol fungi.

Either approach requires production of transgenic strains with altered or enhanced levels of enzyme production. We provided our endochitinase gene to researchers in Finland, who have long specialized in producing transgenic strains of *Trichoderma* that produce high levels of specific proteins. This gene was inserted into *T. reesei* under the control of the *cbh1* promoter, and strains that produce high levels of endochitinase were identified. From the native strain, we were able to produce only about 1-2 mg of purified endochitinase, but they were able to make a strain that produced very high levels of the enzyme, with our assistance (Margolles-Clark et al., 1996a). However, the enzyme from this strain was without antifungal activity even though it was strongly

active against purified chitin. They also prepared transgenic strains of *T. harzianum* that overexpress endochitinase under the control of the *cbhl* promoter (Margolles-Clark et al., 1996b). In the USA, we have been characterizing the enzyme mixtures produced by these transgenic strains and find that, in the proper medium, this strain also produces high levels of N-acetylglucosaminidase and chitobiosidase, enzymes that are strongly synergistic with endochitinase. In other media, the strain gives rise to almost pure endochitinase that is also strongly antifungal. Even the culture filtrates of this strain are strongly antifungal due to the activity of these enzymes *in vitro* even though the enzymes from the native strain produced in our original medium unless they were highly purified.

We have developed production methods using the transgenic strain of *T. harzianum* overexpressing fungal endochitinase that give rise to high levels of almost pure endochitinase. Similarly, using the wild strain we have developed production techniques that produce high levels of a mixture of enzymes. If properly prepared, both are very strongly antifungal even without further concentration. This is a very important step in our view. In the past we have been unable to evaluate the potential of enzymes for use directly in control of disease because it required months even to produce a few mg of strongly antifungal preparations.

Probably more importantly, we have made strides in determining the structure and activity of control elements for the genes encoding biocontrol enzymes from *T. harzianum*. In general, these genes are controlled separately and interactively by nitrogen and glucose nutrition and by the presence or absence of inducers such as colloidal chitin. Understanding this regulation will permit us to readily manipulate the production of both transgenic and native enzymes in this fungus.

Further, we have prepared a strain of *T. harzianum* that has reduced endochitinase activity by gene disruption techniques. It produces an aberrant enzyme that is not antifungal. Interestingly, the N-acetylglucosaminidase gene also is disrupted. This strain, along with other disrupted strains, is expected to be highly useful in mechanistic studies.

In related research, our colleagues are placing chitinase genes from *T. harzianum* into plants. This research is providing some exciting results. Fungal genes are expressed well in a variety of plant species. Transgenic lines of tobacco, potato, and apple all express high resistance to species of *Alternaria*, *Botrytis*, and *Venturia*. These fungal genes may have a prominent role in disease-resistance plants in the future.

In Israel, the soilborne *Enterobacter agglomerans* strain IC1270 has a broad spectrum of antifungal activity. This strain produces and secretes a number of chitinolytic enzymes, including two N-acetyl- β -D-glucosaminidases and an endochitinase. The importance of the chitinolytic enzymes in the strain IC1270 biocontrol activity was demonstrated by using *Rhizoctonia solani* in cotton as a model, and comparing the parent IC1270 strain with Tn5 mutants deficient in chitinolytic activity. The bacteria decreased the incidence of disease caused by *R. solani* in cotton,

but the biocontrol effect disappeared when the plants were treated with the chitinase null mutants. *Escherichia coli* strain JM109 carrying the *E. agglomerans chiA* gene produced and secreted the endochitinase. The transformed strain was shown to inhibit *Rhizoctonia solani* growth on a plate and in cotton seedlings, and the antifungal activity of the secreted chitinase was demonstrated by inhibition of *Fusarium oxysporum* spore germination.

EVALUATION OF THE RESEARCH ACHIEVEMENT WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL AND ITS OBJECTIVES

This research follows the objectives set forth in the original proposal and is on target with the expected accomplishments. In some respects, such as the contribution of genes cloned in this project to the production of transgenic plants with resistance to plant diseases, the results have gone beyond expectations. However, we have focused less on the production of transgenic bacteria than we originally anticipated. Further, we have used other promoter regions for transgenic fungal studies than those originally envisioned.

DESCRIPTION OF THE COOPERATION

The PIs were in frequent contact both at meetings and via email and other mechanisms. Research progress on the two parallel and complimentary approaches were discussed and evaluated. The data obtained provides a more complete understanding of the natural genes available for plant disease control and a better conceptual framework of how to use them than if either group had worked alone.

CONCLUSIONS

This work was quite successful. Advances were made in several important areas, including a much more complete understanding of the genetic components of biocontrol in several microbes and a better appreciation of the great diversity that exists among antifungal chitinases from different sources. A number of genes have been cloned that are useful in various applications. In some cases, such as the production enzymes for evaluation in agricultural disease control of agricultural plant diseases, and in production of transgenic plants, these discoveries offer substantial promise for commercial utility. These strongly antifungal enzymes, especially in

synergistic compounds with other enzymes or other materials, would seem to be very useful in agriculture in a variety of applications.

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- Lorito, M., D'Ambrosia, M., Woo, S. L., Harman, G. E., Hayes, C. K., and Scala, F. 1996. Synergistic interaction between cell wall-degrading enzymes and membrane-affecting compounds. *Molec. Plant Microbe Interact.* 3:206-213.
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- Haran, S., Schickler, H. and Chet, I. 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiol.* 142: 2321-2331.
- Chernin, L., Fuente, L., Sobolev, V., Haran, S., Vorgais, C. E., Oppenheim, A., and Chet, I. Molecular cloning, structural analysis, and expression in *Eschericia coli* of a chitinase from *Enterobacter agglomerans*. *J. Appl. Environ. Microbiol.* 63:834-839.

*These cooperative research papers do not list BARD support in the acknowledgments.

PATENTS SUBMITTED AND ISSUED

PATENT ISSUING

Gene Encoding for Endochitinase

Inventors: Gary Harman, Arne Tronsno, Christopher Hayes, Sonja Klemsdahl & Matteo Lorito

US Patent Appl. 08/371,608 (D-1424A) (Notice of Allowance Received)

PATENTS FILED

Gene encoding β -1,3 glucosidase (provisional filed May, 1999)

Inventors: Gary Harman, Bruno Donzelli and Matteo Lorito

Fungal signal peptide sequences for chitinolytic enzymes and uses thereof (provisional filed July 6, 1998, full application filed before July 6, 1999)

Inventors: Gary Harman, Matteo Lorito, Elizabeth Earle, Aigars Brants, Clemens Peterbauer, C. Kubicek, Arne Tronsmo, Sonja Klemsdahl

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Investigators' Names Investigators' Institutions
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A gene encoding a strongly antifungal chitinase from the biocontrol bacterium *Chromobacterium violaceum* (unpublished).

All of the proteins produced by these genes are strongly antifungal. Except for the different endochitinases from *T. harzianum*, the genes are unrelated or possess only limited homology with one another. Therefore, the different genes give rise to unrelated proteins useful in different applications.

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Probably more importantly, we have made strides in determining the structure and activity of control elements for the genes encoding biocontrol enzymes from *T. harzianum*. In general, these genes are controlled separately and interactively by nitrogen and glucose nutrition and by the presence or absence of inducers such as colloidal chitin. Understanding this regulation will permit us to readily manipulate the production of both transgenic and native enzymes in this fungus.

Further, we have prepared a strain of *T. harzianum* that has reduced endochitinase activity by gene disruption techniques. It produces an aberrant enzyme that is not antifungal. Interestingly, the N-acetylglucosaminidase gene also is disrupted. This strain, along with other disrupted strains, is expected to be highly useful in mechanistic studies.

In related research, our colleagues are placing chitinase genes from *T. harzianum* into plants. This research is providing some exciting results. Fungal genes are expressed well in a variety of plant species. Transgenic lines of tobacco, potato, and apple all express high resistance to species of *Alternaria*, *Botrytis*, and *Venturia*. These fungal genes may have a prominent role in disease-resistance plants in the future.

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but the biocontrol effect disappeared when the plants were treated with the chitinase null mutants. *Escherichia coli* strain JM109 carrying the *E. agglomerans* *chiA* gene produced and secreted the endochitinase. The transformed strain was shown to inhibit *Rhizoctonia solani* growth on a plate and in cotton seedlings, and the antifungal activity of the secreted chitinase was demonstrated by inhibition of *Fusarium oxysporum* spore germination.

EVALUATION OF THE RESEARCH ACHIEVEMENT WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL AND ITS OBJECTIVES

This research follows the objectives set forth in the original proposal and is on target with the expected accomplishments. In some respects, such as the contribution of genes cloned in this project to the production of transgenic plants with resistance to plant diseases, the results have gone beyond expectations. However, we have focused less on the production of transgenic bacteria than we originally anticipated. Further, we have used other promoter regions for transgenic fungal studies than those originally envisioned.

DESCRIPTION OF THE COOPERATION

The PIs were in frequent contact both at meetings and via email and other mechanisms. Research progress on the two parallel and complimentary approaches were discussed and evaluated. The data obtained provides a more complete understanding of the natural genes available for plant disease control and a better conceptual framework of how to use them than if either group had worked alone.

CONCLUSIONS

This work was quite successful. Advances were made in several important areas, including a much more complete understanding of the genetic components of biocontrol in several microbes and a better appreciation of the great diversity that exists among antifungal chitinases from different sources. A number of genes have been cloned that are useful in various applications. In some cases, such as the production enzymes for evaluation in agricultural disease control of agricultural plant diseases, and in production of transgenic plants, these discoveries offer substantial promise for commercial utility. These strongly antifungal enzymes, especially in

synergistic compounds with other enzymes or other materials, would seem to be very useful in agriculture in a variety of applications.

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PATENTS SUBMITTED AND ISSUED

PATENT ISSUING

Gene Encoding for Endochitinase

Inventors: Gary Harman, Arne Tronsno, Christopher Hayes, Sonja Klemsdahl & Matteo Lorito

US Patent Appl. 08/371,608 (D-1424A) (Notice of Allowance Received)

PATENTS FILED

Gene encoding β -1,3 glucosidase (provisional filed May, 1999)

Inventors: Gary Harman, Bruno Donzelli and Matteo Lorito

Fungal signal peptide sequences for chitinolytic enzymes and uses thereof (provisional filed July 6, 1998, full application filed before July 6, 1999)

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