



# DISCOVERY AND ISOLATION OF A BACTERIAL CHITOSANASE GENE WITH POTENTIAL FOR GENETICALLY ENGINEERED FUNGAL RESISTANCE

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## RESEARCH PROBLEM

Fungal pathogens like *Rhizoctonia*, *Thielaviopsis*, and *Fusarium* thrive in the soils of Arkansas and are a major threat to both emerging cotton seedlings and established crops. For the Arkansas cotton farmer, these and other fungal pathogens make fungicide application a necessary part of production. The toxicity, environmental harm, and expense that come with fungicides, though, leave many farmers looking for other options. In the future, genetically-engineered cotton plants that produce enzymes to degrade key structural polymers found in fungal cell walls may provide farmers with a more attractive option. One such enzyme, chitinase, has the potential to slow or prevent fungal infection by degrading the structural chitin found in cell walls of many fungi. This is a preliminary report of a study to evaluate the efficacy of chitinase to increase fungal resistance.

## BACKGROUND INFORMATION

Chitinase is well known as a plant defensive enzyme that degrades chitin, the major polymer of crustacean shells and insect exoskeletons, but it is also a component in the cell walls of some fungi. Chitin is a hard polymer of glucosamine in which the amine has an acetyl group attached. When the acetyl group is absent, the result is a more flexible polymer known as chitosan that is not susceptible to degradation by chitinases. This form is probably more abundant in fungal cell walls. It is now recognized that chitinases are also produced in many plants, bacteria, and fungi. While chitinases also are considered as pathogenesis-related (PR) proteins, they exist in many forms in plants. This redundancy has prompted the suggestion that the varied isoforms may have functions other than pathogen defense. In addition to pathogen

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attack, chitosanases are induced in response to arbuscular mycorrhizal symbiosis (Pozo et al., 1998) and cold stress (de los Reyes, personal communication).

El Quakfaoui et al. (1995) were the first to report a successful plant transformation with a bacterial chitosanase (*Streptomyces* sp. strain N174). Their chitosanase gene construct was driven by the 35S cauliflower mosaic-virus promoter and retained activity without adversely affecting the growth of the explants.

### RESEARCH DESCRIPTION

Many ecological niches were sampled to find organisms that produced chitosanase. Samples from each niche were collected and streaked onto agar plates with chitosan as the sole carbon source. The prospective organisms were isolated and streaked on opaque chitosan plates augmented with LB medium to verify chitosanase activity as determined by plate clarification. A bacterium with high activity was selected, and the 16s rRNA gene was sequenced to identify the organism. Its genome was cloned in a plasmid expression vector in *E. coli*. A colony with chitosanase activity was chosen. The Erase-a-base system (Promega) was used to generate overlapping DNA fragments for sequencing of the chitosanase gene. The sequence was compared to the Genbank database to confirm function and sequence location of the gene. Primers flanking the mature protein region were synthesized (Sigma-Genosys), and a PCR-amplified sequence was fused into pGEM-T vector to verify that a functional protein had been isolated. The fragment was subsequently modified to contain a 3' signal sequence and cloning sites on each end. The modified fragment was cloned in a plant transformation vector already possessing a plant promoter and an essential selectable antibiotic resistance gene.

Following complete sequence confirmation of the gene, *Nicotiana tabacum* will be transformed via *Agrobacterium tumefaciens* to test for chitosanase efficacy against fungi. Assuming positive results, cotton will then be transformed with the gene construct.

### RESULTS

Strong extra-cellular expression of chitosanase by the bacteria was determined by clear halos around the test colonies growing on medium made opaque by insoluble chitosan. A bacterium capable of utilizing chitosan as an energy source was selected and most closely matched a *Paenibacillus* species by the 16S rRNA gene sequence. One colony in the *E. coli* genomic DNA library of this bacterium showed strong chitosanase activity and was used for the construction of the Erase-a-Base library. Most of the gene sequence could be obtained from this second library, but construction of specific PCR primers based on flanking sequences was necessary to span one gap. The resulting sequence showed high homology in GenBank with chitosanases produced by *Bacillus ehimensis* and *B. circulans*.

New primers were designed to PCR amplify the mature protein region of the chitosanase. When cloned in an expression vector, this fragment retained chitosanase activity as confirmed by chitosan clarification by a total protein extraction obtained by boiling. The sequence corresponding to the mature protein gene has been modified to contain a signal peptide sequence and cloned into a plant transformation vector with a strong promoter and 3' termination sequence.

Following confirmation of the correct DNA sequence, the vector will be placed in *A. tumefaciens* and transformed into tobacco (*Nicotiana tabacum*). Tobacco is used because it can be regenerated from callus quickly in order to test the efficacy of the gene construct against fungi. If the transgenic tobacco plants show improved fungal resistance, then the gene construct will be used to transform cotton, which requires a much longer regeneration period.

### PRACTICAL APPLICATION

In addition to potentially reducing fungicide use and increasing the efficiency with which Arkansas cotton farmers can combat fungi, this study may answer questions regarding chitosanase function in plant systems. It has been shown that chitinase, another "anti-fungal" PR protein, is involved in leaf abscission, response to cold and drought stress, response to ozone, and freezing tolerance (anti-freeze protein action) (de los Reyes et al., 2001). We suspect that chitosanase may also be involved in similar activities and plan to investigate such possibilities.

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