delivery system would have to distribute the material over the volume, or one would have to allow adequate time for slow internal mixing. The possibility that the vortex region exchanges and processes air from lower latitudes (*32*) could lead to some losses of the added alkanes. If instead the gases would be introduced into the stratosphere before the vortex sets up (when there is more mixing), much larger quantities would be needed, and their chemical fate would be more uncertain. Experiments can be imagined with vertically thin atmospheric layers wherein the injected hydrocarbons would be consumed (reaction 1) and the present concept could be tested. Before any actual injection experiment is undertaken there are many scientific, technical, legal, and ethical questions to be faced, not the least of which is the issue of unintended side effects (*33*).

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Transgenic Plants with Enhanced Resistance to the Fungal Pathogen *Rhizoctonia solani*

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The production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi is an important component of the defense response of plants. The timing of this natural host defense mechanism was modified to produce fungal-resistant plants. Transgenic tobacco seedlings constitutively expressing a bean chitinase gene under control of the cauliflower mosaic virus 35*S* promoter showed an increased ability to survive in soil infested with the fungal pathogen *Rhizoctonia solani* and delayed development of disease symptoms.

PLANTS RESPOND TO ATTACK BY pathogenic fungi by mobilizing a complex network of active defense mechanisms (*1*). These include modifications designed to strengthen the plant cell wall and thereby restrict or inhibit access of the pathogen to the plant cell. More aggressive tactics involve the synthesis of toxic antimicrobial compounds (phytoalexins) and the induction and accumulation of proteinase inhibitors and lytic enzymes such as chitinase and β-1,3-glucanase. The success of the plant in warding off phytopathogen invasion appears to depend on the coordination between the different defense strategies and the rapidity of the overall response (*2*).

Chitinase catalyzes the hydrolysis of chitin, a β-1,4–linked polymer of *N*-acetyl-D-glucosamine and a major component of the cell wall of most filamentous fungi except the Oomycetes (*3*). Although chitinase is generally found at low or basal levels in healthy plants, its expression is increased

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during pathogen attack (*4*), and it accumulates intracellularly in the central vacuole (*5*) or extracellularly in the intercellular space (*6*). Evidence for the role of chitinase in the defense response of the plant has been largely dependent on data obtained in vitro. The purified enzymes from tomato and bean hydrolyze isolated fungal cell walls (*7, 8*). The enzyme from pea alone or in combination with β-1,3-glucanase inhibits the growth of certain pathogenic fungi in culture (*9*). In addition, the activation profile of a bean chitinase promoter during fungal infection indicates that chitinase expression is intimately associated with the response of the plant to pathogen invasion (*10*).

Because the timing of the defense response contributes to the outcome of the interaction between host and pathogen, we have eliminated the temporal factor in chitinase gene expression. We find that constitutive expression of a bean endochitinase gene in transgenic tobacco and canola plants affords increased protection against disease caused by the fungal pathogen *R. solani*.

A hybrid chitinase gene was constructed by the replacement of the 5′ regulatory region of a bean endochitinase CH5B gene (*11*) with the promoter region of the cauliflower mosaic virus (CaMV) 35*S* transcript, a highly active promoter that is able to function in a wide variety of plant cell types (*12*). The chimeric 35*S*-chitinase genes, contained in the plasmids pK35CHN641 and pK35CHN695 (Fig. 1A) (*13*), were mobilized from *Escherichia coli* HB101 into *Agrobacterium tumefaciens* strain GV3850 (*14*) and used to infect leaf disks of *Nicotiana tabacum* cv. Xanthi (*15*).

Of the 47 kanamycin-resistant primary transformants that were regenerated, 22 were screened for expression of the bean chitinase polypeptide by immunoblot analysis (Fig. 1B) (*16*). All were found to contain a band that was immunoreactive with polyclonal antibodies to bean chitinase and identical in size to the protein from bean. No cross reaction was detected with soluble protein isolated either from wild-type tobacco or from tobacco transformed with an otherwise identical construct pKNK (*17*) that lacked the 35*S*-chitinase gene (control plants). The size of the protein, indistinguishable from the size of the native bean polypeptide, suggested that the precursor protein was correctly processed in the heterologous tobacco system. Somewhat higher amounts of bean chitinase protein were found in the roots of the transgenic plants than in the leaves. Out of four randomly selected transformants, 238 displayed the lowest amount of bean chitinase; 373 showed the strongest expression of the bean chitinase polypeptide. Assays of protein extracts indicated increased chitinase enzyme activity in the 35*S*-chitinase transformed plants (Table 1) (*18*). Homozygous progeny showed a two- to fourfold increase in the roots and a 23- to 44-fold increase over control plants in chitinase enzyme activity in the leaves. These results demonstrate that the chimeric 35*S*-chitinase gene was functional in tobacco and gave rise to constitutive expression of the bean polypeptide in healthy plants. Transgenic tobacco plants constitutively expressing bean chitinase showed no obvious difference in growth and development when compared to control or wild-type tobacco plants.

Table 1. Chitinase enzyme activity of leaf, stem, and root samples of control and 35*S*-chitinase tobacco plants (*18*). The specific activity [nanoKatal (nkat) per milligram of protein] (*8*) is defined as that amount of enzyme which releases 1 nmol *N*-acetyl-D-glucosamine per second per milligram of protein; 548, control plants; and 238 and 373, 35*S*-chitinase tobacco plants.

Plant no.	Chitinase activity (nkat) per milligram of protein		
	Leaf	Stem	Root
548	1.3	1.5	13.4
238	29.9	16.8	24.4
373	57.2	35.4	58.4

The 35*S*-chitinase tobacco plants were assayed for resistance to the phytopathogen *R. solani* (*19*), an endemic, chitinous, soilborne fungus that infects numerous plant species, including corn and soybeans (*20*). Seeds planted in soils heavily infested with *R. solani* typically have problems with stand establishment and early season growth. Damping-off, seedling blight, and brown-girdling root rot are diseases that are attributable to *R. solani* infection.

Homozygous progeny of 35*S*-chitinase transgenic tobacco plants were grown in the presence of *R. solani* to determine their susceptibility to fungal attack. Eighteen-day-old tobacco seedlings were transplanted

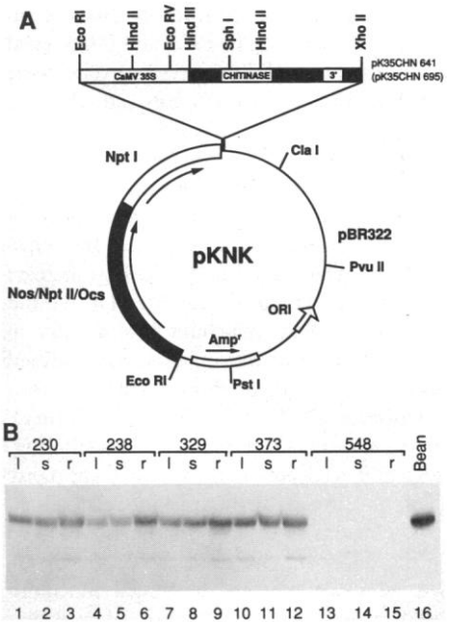

Fig. 1. (**A**) Structure of the intermediate plasmid pKNK (*17*), pK35CHN641, and pK35CHN695. The plasmids pK35CHN641 and pK35CHN695 contain an Eco RI–Bgl II DNA fragment encoding the chimeric 35*S*-chitinase gene (*13*). (**B**) Immunoblot of protein from control and 35*S*-chitinase tobacco plants. Soluble protein from leaf (l), stem (s), and root (r) tissue was fractionated by SDS–polyacrylamide gel electrophoresis (*29*), and the bean chitinase polypeptide was visualized by alkaline phosphatase-linked detection of antibodies to chitinase (*16*). Plants 230 and 238 were transformed with pK35CHN695, and plants 329 and 373 were transformed with pK35CHN641. Plant 548 was transformed with pKNK. Lanes 1 to 15, 10 μg of tobacco protein; lane 16, 20 μg of ethylene-treated bean leaf protein.

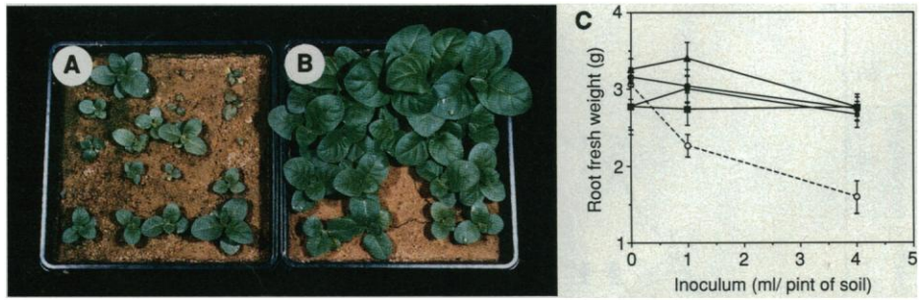

Fig. 2. Resistance of 35*S*-chitinase tobacco plants to *R. solani*. (**A** and **B**) Comparison of control plants (A) and 35*S*-chitinase transformants 373 (B) 18 days after growth in *R. solani*–infested sand (1.0 ml per pint of soil) (*19*). (**C**) Root fresh weight of 23-day-old control and 35*S*-chitinase tobacco seedlings 11 days after growth in soil containing increasing amounts (0, 1, and 4 ml per pint of soil) of *R. solani* inoculum. 548 (control), ○; 373, ●; 230, ▲; 238, ■; 329, ▪. Two independent experiments were performed by use of ten replicates for each experimental condition. Bars indicate standard error of the determinations.

into *R. solani*–inoculated soil and grown for 13 to 16 days (*19*). Under these conditions, an average of 53% of the plants without the 35*S*-chitinase gene died. A lower percentage of seedling mortality, ranging from 22.7 to 37.1% (*21*), was observed for plants containing the 35*S*-chitinase gene. Transgenic plants with higher amounts of the bean polypeptide showed greater resistance to the development of disease symptoms. When the 35*S*-chitinase plants were grown in the presence of a pathogen, *Pythium aphanidermatum*, that lacks a chitin-containing cell wall, no difference in survival was detected compared to control plants. Twenty days after transplanting into infested soil, seedling mortality ranged from 48.4 ± 6.6 to 63.7 ± 1.9% for the 35*S*-chitinase plants and 56.5 ± 2.6% for control transgenic plants (*22*).

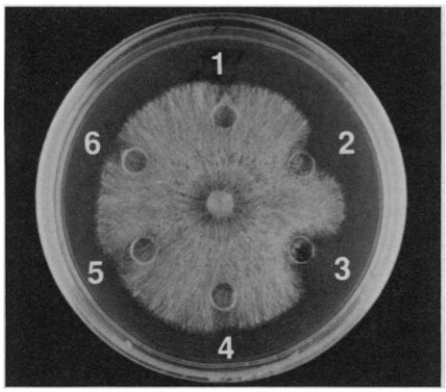

Fig. 3. Inhibition of fungal growth by bean chitinase. Samples added (40 μl per well) to 1-day-old *R. solani* cultured on a potato dextrose agar, and incubated for 12 hours at room temperature are as follows: 1, buffer (50 mM sodium phosphate, pH 7.0, 0.1 M NaCl) containing 100 μg of BSA per milliliter; 2, buffer containing 100 μg of bean chitinase per milliliter; 3, buffer containing 400 μg of bean chitinase per milliliter; 4, buffer containing 400 μg of BSA per milliliter; 5, buffer containing 100 μg of boiled bean chitinase per milliliter; 6, buffer containing 400 μg of boiled bean chitinase per milliliter.

In certain plants, susceptibility to infection by *R. solani* decreases with increasing age of the plant. For tobacco, lettuce, cauliflower, and bean (*23*), young seedlings are most severely affected. On more mature plants, stem and roots may be damaged, but the plant is still able to survive. Control tobacco plants grown in infested soil were noticeably stunted (Fig. 2A), while the 35*S*-chitinase tobacco plants were larger and hardier (Fig. 2B) and showed minimal root damage. To quantitate the effect of *R. solani* infection on root development, conditions were chosen such that the plants did not die; instead, the extent of disease was monitored by the loss of root fresh weight (Fig. 2C). For the 35*S*-chitinase plants the loss in root fresh weight ranged from an average of 5 to 15%, compared to 46% for the control plants.

The enhanced resistance of the 35*S*-chitinase plants is consistent with the inhibitory effect of bean chitinase on the growth of *R. solani* in vitro (Fig. 3). Inhibition was proportional to the concentration of added chitinase. No inhibition was detected in the presence of either boiled enzyme or bovine serum albumin (BSA). The observed growth inhibitory effect may arise from enzyme-catalyzed hydrolysis of newly formed chitin (*24*) and resultant disruption of the growing fungal-hyphal tips (*25*). It is not known whether modification of chitinase expression alone is sufficient to provide protection against a wide range of chitinous fungal pathogens. For example, tobacco plants containing transgenically increased amounts of a tobacco chitinase enzyme were only slightly more resistant than control tobacco plants to attack by the fungal pathogen *Cercospora nicotinae*, indicating that factors other than chitinase may be limiting in the defense against this pathogen (*26*).

We have also introduced the chimeric 35*S*-chitinase gene into canola, *Brassica napus* cv. Westar (*27*). In 1983 and 1984, yield losses of 36 and 23%, respectively, were attributed to root rot diseases caused by *R. solani* infection (*28*). Regenerated transgenic canola plants were assayed for bean chitinase expression by immunoblot analysis and homozygous progeny generated by self-fertilization of primary transformants. One transformant, which exhibited a 33-fold increase in chitinase activity in leaves and a twofold increase in roots over chitinase activity in untransformed tobacco plants, was analyzed and was found to be more resistant to root rot disease of *R. solani*. The 35*S*-chitinase plants showed more vigorous growth (Fig. 4A) and a decrease in seedling mortality (Fig. 4B) during growth in inoculated soil.

The extent of disease resistance observed in the 35*S*-chitinase tobacco or canola varied with the amount of fungal inoculum used, a property characteristic of quantitative resistance. However, the delay in the appearance of symptoms as well as the lower severity of disease may enable young seedlings to survive the critical period during stand establishment in the field when they are most susceptible to attack by soil-borne pathogens.

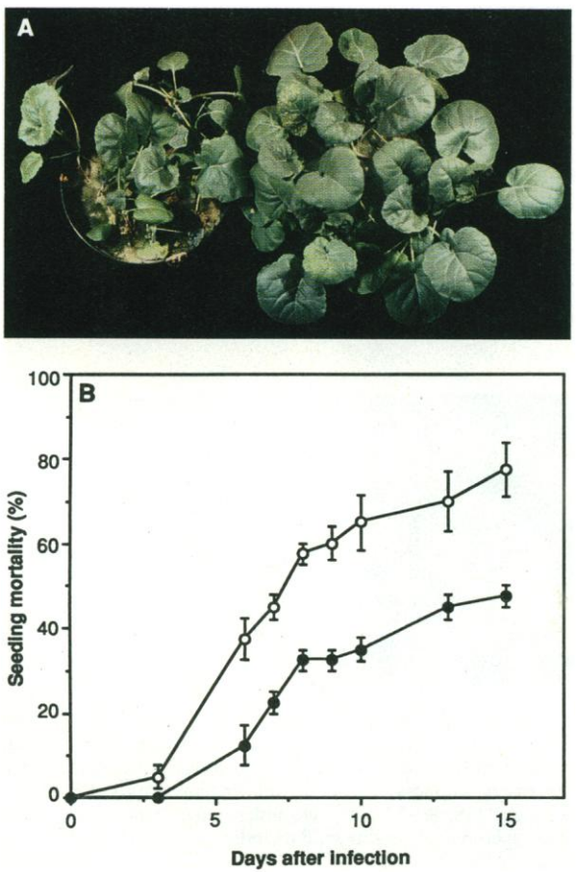

Fig. 4. (**A**) Wild-type canola (left) and 35*S*-chitinase canola (right) 14 days after growth in sand inoculated with *R. solani* (0.25 ml per pint) (*19*). (**B**) Rate of seedling mortality of 35*S*-chitinase canola plants (closed circles) and wild-type canola plants (open circles). Data represent four independent experiments with 12 plants used in each. Error bars are standard errors of the determinations.

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Binding of ARF and β-COP to Golgi Membranes: Possible Regulation by a Trimeric G Protein

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The binding of cytosolic coat proteins to organelles may regulate membrane structure and traffic. Evidence is presented that a small guanosine triphosphate (GTP)–binding protein, the adenosine diphosphate ribosylation factor (ARF), reversibly associates with the Golgi apparatus in an energy, GTP, and fungal metabolite brefeldin A (BFA)–sensitive manner similar to, but distinguishable from, the 110-kilodalton cytosolic coat protein β-COP. Addition of βγ subunits of G proteins inhibited the association of both ARF and β-COP with Golgi membranes that occurred upon incubation with guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S). Thus, heterotrimeric G proteins may function to regulate the assembly of coat proteins onto the Golgi membrane.

CYTOSOLIC PROTEINS THAT BIND REversibly to membranes of the Golgi complex have been identified (*1, 2*) and shown to exist as a high molecular weight complex in the cytosol referred to as the coatomer (*3*). Cycling between the cytosol and Golgi membrane of at least one of these proteins, the 110-kD coat protein β-COP (*4*), is affected by nonhydrolyzable analogs of GTP such as GTP-γ-S and by BFA (*5–7*). BFA inhibits the association of cytosolic β-COP with Golgi membranes. Aluminum fluoride and GTP-γ-S both promote association of β-COP with Golgi membranes, suggesting that one or more GTP-binding proteins participate in initiating the association of β-COP with the membrane (*6–8*).

Two general classes of regulatory GTP-binding proteins have been defined: the signal transducing trimeric G proteins, believed to exert their effects at the plasma membrane, and the family of small GTP-binding proteins (the RAS superfamily). Several of the small GTP-binding proteins have been implicated in the control of intracellular membrane traffic (*9, 10*). One of these GTP-binding proteins, the adenosine diphosphate ribosylation factor (ARF), is associated with Golgi membrane and is also present in the cytosol (*9*).

We used immunofluorescence microscopy to study the effects of various agents that influence energy status, disrupt the Golgi complex, or alter the activity of G proteins or small GTP-binding proteins on the cellular localization of ARF and β-COP in normal rat kidney (NRK) cells. Immunolabeling of ARF and β-COP in untreated cells revealed a predominant Golgi-like staining pattern, which was juxtanuclear and half-moon shaped (Fig. 1). A low amount of ARF staining was also observed throughout the cytosol. Double labeling with antibodies to mannosidase II confirmed the Golgi distribution of both ARF and β-COP (*11*). Treatment of cells with 50 mM 2-deoxyglucose and 0.05% Na azide (DOGAz) for 10 min at 37°C to

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