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Full Length Research Paper

Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with a rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease

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Stylosanthes guianensis **is a diverse tropical and subtropical forage legume of great potential. Foliar blight disease, caused by** ***Rhizoctonia solani*** **AG-1, can be a significant constraint to the legume's production in some areas. The pathogen has a broad range of host plant species, and can survive as sclerotia for long periods in soil or plant debris. No sources of resistance in** ***Stylosanthes*** **are known. Cost-effective disease management strategies are, therefore, needed for this host-pathogen system. Chitinases, which catalyze the hydrolysis of the β-1,4 linkages of the N-acetyl-D-glucosamine polymer chitin of fungal cell walls, are expressed in plants in response to infections by pathogens and some abiotic stresses. In this study, a basic chitinase-encoding gene, isolated from rice, was introduced into the widely distributed** ***S. guianensis*** **accession CIAT 184, using** ***Agrobacterium tumefaciens*****. A 1.1-kb rice genomic DNA fragment containing the chitinase gene was cloned into a transformation vector, pCAMBIA2301. The vector carried the CaMV 35S promoter, the neomycin phosphotransferase (nptII) gene and the gusA reporter gene. The presence of the chitinase gene in transgenic** ***Stylosanthes*** **plants was determined by dot blot analysis. Transgenic plants expressed higher levels of resistance to** ***R. solani*** **than did control plants. Progenies of a selfed transgenic plant showed segregation for resistance at a ratio of 3:1.**

Key words: Chitinase gene, disease control, plant transformation, *Rhizoctonia solani*, *Stylosanthes guianensis*, transgenic plant.

INTRODUCTION

The genus *Stylosanthes* consists of more than 44 species and subspecies (Edye and Cameron, 1984) that are naturally distributed in Central and South America, southern USA, Africa and Southeast Asia (Williams et al., 1984). Species of *Stylosanthes* are used for soil improvement through nitrogen fixation, reclaiming degraded wastelands, and water and soil conservation. In southern China, dried leaves are also used for making meal for livestock feed, whereas, in Central and South America, the legume is a significantly important tropical forage.

Foliar blight, caused by *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* Donk) and reported in many parts of the world (O'Brien and Pont, 1977; Lenné, 1990), affects *Stylosanthes* species in tropical America wherever the annual rainfall is more than 1500 mm (Lenné and Calderón, 1984). The disease initially appears as water-soaked spots on infected leaves. Under favorable conditions such as prolonged humidity, the spots progress into rotting and extensive

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foliar death. The pathogen has an extensive host range and can produce substantial foliar damage on susceptible genotypes (Baker, 1970), including various tropical and subtropical forage legumes and crops (Hepperly et al., 1982; Galindo et al., 1983; Lenné, 1990; Yang et al., 1990).

Sclerotia of the fungus can survive in soil or on plant debris for long periods. They first appear as white masses on infected plant tissues and, as they mature, turn brown and loosely attached. They shed easily, becoming primary sources of inoculum.

Chitinases have been reported from several plants and various microbes (Punja and Zhang, 1993; Ohme-Takagi et al., 1998). In plants, both biotic and abiotic stresses can induce chitinase expression at relatively high levels, although, constitutionally, many plant chitinases are often expressed at low levels. Chitinase catalyzes the hydrolysis of the β- 1,4 linkages of the N-acetyl-D-glucosamine polymer chitin, a structural component in many organisms, but absent in plants (Boller et al., 1983). In contrast, chitin constitutes between 3% and 60% of cell walls in fungi (Bartnicki-García, 1968). Purified chitinases obtained from:

1. bean were effective against the cell walls of *Fusarium solani* (Boller et al., 1983) and *Trichoderma viride* (Schlumbaum et al., 1986);
2. tomato (Young and Pegg, 1982), tobacco (Mauch et al., 1988) and pea (Sela-Buurlage et al., 1993) could inhibit fungal growth by lysis of fungal tips; and
3. leaves of *S. guianensis* could kill *Colletotrichum gloeosporioides* hyphae (Brown and Davis, 1992).

Chitinases have also been reported as pathogenesis-related proteins in cucumber (Métraux et al., 1988). These and other findings support the hypothesis that plant chitinases have antibiotic functions and thus probably constitute a defense mechanism in plants against pathogens. Recombinant DNA techniques permit the isolation of specific genes and their introduction into plants, which otherwise would not have been possible with conventional breeding:

1. Complementary DNA clones and genomic clones have been isolated, and the amino acid sequences deduced for several chitinases obtained from several plants (Broglie et al., 1986; Métraux et al., 1989; Swegle et al., 1989; Samac et al., 1990; Huang et al., 1991; Zhu and Lamb, 1991; Huynh et al., 1992; Van Damme et al., 1993).
2. Some transgenic plants with chitinase-encoding genes have also been generated to control fungal pathogens such as canola (Benhamou et al., 1993), cucumber (Raharjo et al., 1996; Tabei et al., 1998), rice (Lin et al., 1995), rose (Marchant et al., 1998) and tobacco (Broglie et al., 1991).
3. A rice-chitinase gene, controlled by the CaMV 35S promoter and introduced into indica rice, was shown to enhance resistance to *Rhizoctonia solani* (Lin et al., 1995).
4. A rice-chitinase gene in a transgenic rose plant reduced the development of blackspot disease caused by the fungus *Diplocarpon rosae* Wolf (Marchant et al., 1998).
5. Tabei et al. (1998) had shown that transgenic cucumber plants ccarrying a rice-chitinase gene had enhanced resistance to gray mold disease caused by *Botrytis cinerea*.
6. Increased chitinase activity has also been reported to enhance anthracnose resistance in *S. guianensis* (Brown and Davis, 1992).

In this study, we introduced a rice-chitinase gene (Huang et al., 1991) into the widely grown *S. guianensis* accession CIAT 184 to enhance foliar blight resistance. This accession has broad adaptation to the humid tropics and has been released as a cultivar in various countries, including Peru and southern China. It has also performed well in parts of Africa. This work provides a potential application in breeding for resistance to a fungal disease to which no sources of resistance are available within the plant species.

MATERIALS AND METHODS

DNA manipulation

A 1.5-kb DNA fragment, carrying a 1.1-kb rice-chitinase gene and the CaMV 35S promoter, was recovered from the *Hind*III -digested plasmid pBSKS-G11 (R) with the chitinase gene (provided by Dr. S. Muthukrishnan, Department of Biochemistry, Kansas State University), using a DNA recovery kit (BIO-RAD) according to the manufacturer's instructions. This DNA fragment was ligated to the *Hind*III site of pCAMBIA2301. The ligated DNA product was used to transform *Escherichia coli* DH5α. A construct, designated pCIATCH2, was selected. All recombinant DNA techniques were carried out according to standard procedures (Sambrook et al., 1989).

Bacterial transformation

Competent cells of *E. coli* DH5α were prepared, and the subsequent transformations with plasmid DNA carried out according to the protocol described by Inoue (1990). Transformed cells were cultured on Luria agar medium (10 g bacto-tryptone; 5 g bacto-yeast-extract; 10 g NaCl; 15 g agar per liter of distilled water) with appropriate antibiotics (100 μg/mL ampicillin for pBSKS-G11 (R) (Huang et al., 1991) and 50 μg/mL kanamycin for pCAMBIA2301 (Roberts, 1997) and its derivatives), and incubated overnight at 37°C. For α-complementation screening, 40 μL of X-gal and 4 μL of IPTG per plate were used.

pCIATCH2 DNA was directly transformed into the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983). Transformed cells were selected by plating them on Luria agar medium containing 25 μg/mL streptomycin, and 50 μg/mL kanamycin. Recombinant DNA was isolated from transformed *A. tumefaciens*

cells and digested with *Hind*III for verification. All transformants were maintained at –80°C in 20% glycerol.

Plant transformation and regeneration

Seeds of *S. guianensis* CIAT 184 were surface-sterilized with 3% NaOCl solution for 15 min and rinsed with sterilized distilled water, then treated for 5 min with 70% ethanol and rinsed three times with sterilized distilled water. Seeds were then germinated on basal MS medium (Murashige and Skoog, 1962). Cultures were maintained under fluorescent light at 55 μE/m^2 per second at 24°C, with a 12-h photoperiod. Segments were excised from leaves for transformation.

Agrobacterium tumefaciens LBA4404, containing plasmid pCIATCH2, was incubated overnight at 28°C, with agitation at 200 rpm, in 10 mL of Luria broth (LB) containing 100 μM acetosyringone, 25 μg/mL streptomycin and 50 μg/mL kanamycin. For co-culturing, cells were collected from the overnight cultures by centrifuging and re-suspended in fresh LB liquid medium.

Leaf-segment explants were inoculated by swirling for 2-5 min in the bacterial suspension, blotted dry on sterilized filter paper, plated onto regeneration medium (basal medium with 1.0 μg/mL α-NAA and 4.0 μg/mL BAP) (Sarria et al., 1994) and incubated at 28°C in the dark for 2 days. The explants were then washed in sterilized distilled water, blotted dry on sterile filter paper and cultured on regeneration medium, containing 15 μg/mL kanamycin and 250 μg/mL carbenicillin.

After 2 weeks, all growing calli were transferred to a fresh regeneration medium for further selection. The selected green calli were then transferred to basal medium, containing 0.01 μ g/mL α-NAA and 4.0 μg/mL BAP for shoot induction. After shoots appeared, the regenerated plantlets were transferred to basal medium, containing 0.1 μg/mL α-NAA and 0.4 μg/mL BAP for elongation. Shoots were excised and cultured on basal medium with quarter-strength salt and 0.1 μg/mL α-NAA for rooting. Kanamycin and carbenicillin were used in all regeneration steps. Regenerated plantlets were transferred to pots containing autoclaved soil and placed in a greenhouse.

Genomic and plasmid DNA isolations

Genomic DNA was isolated from fresh leaves of *S. guianensis*, using DNeasy Plant Mini Kit (QIAGEN). DNA concentration was estimated, using a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia Biotech, USA).

Medium-scale plasmid DNA isolations were done, using the protocol described by Marko (1982). Mini-preparations of plasmids were made according to the protocol described by Birnboim and Doly (1979). Highly purified plasmids were extracted with the QIAGEN plasmid kit (QIAGEN, USA).

Molecular analyses

For dot blot analysis, 5 μg DNA was used. A 1.5-kb *Hind*III DNA fragment containing the chitinase gene from pCIATCH2 was used as a probe. The probe was labeled, using the DIG High Prime DNA Labeling and Detection Kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. All hybridizations and detections were carried out according to manufacturers' instructions.

PCR amplifications were carried out in 25-μL reaction mixtures containing 0.25 mM dNTPs, 3.0 mM $MgCl_2$, 0.6 μ M primers (*npt*II gene primers, using 0.12 μM), 1 unit of Taq DNA polymerase, 1X PCR buffer and 250 ng of template DNA. The reaction was performed with pre-denaturation at 94°C for 1 min, and then 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min. Sequences of the *npt*II gene primers used in this study were 5'-ATCGGGAGCG-GCGATACCCTA-3' and 5'-GAGGCTATTCGGCTATGACTG-3'. The primers were synthesized by Operon Technologies, USA.

Inoculum production

Sclerotia of *R. solani* AG-1 were produced in a peptone sucrose yeast broth (PSY: 20 g peptone, 20 g sucrose, 5 g yeast and 1 L deionized water) (McCoy and Kraft, 1984). The methods were as described in Kelemu et al. (1995ab). Mycelial discs (4 mm in diameter) were taken from a 4 to 5-day-old culture of *R. solani* AG-1 grown on potato dextrose agar (Difco) . One disc was added to each of several 250-mL Erlenmeyer flasks, each containing 30 mL PSY. The flasks were wrapped with aluminum foil and incubated as still cultures at room temperature (about 25°C) for 10 days. Sclerotia were harvested with sterilized forceps by separating them from the mycelial mats. They were then air-dried overnight in a laminar flow hood. Dry sclerotia were placed in sterilized glass tubes and stored at 4°C until use.

Plant inoculations and disease evaluations

Seedlings of both transformed and non-transformed *S. guianensis* plants were handled as described by Kelemu et al. (1999). Ten-week-old plants were artificially inoculated with sclerotia by placing one sclerotium on the soil surface in contact with each plant's stem. Humidity was maintained at 100% by placing the inoculated plants in a plastic box with one side made of cheesecloth, and immersing the entire box in a tray of water until disease symptoms developed. Plants were then evaluated for their reactions to *R. solani*, starting 6 days after inoculation. The number of sclerotia produced per plant, number of diseased and defoliated leaves per plant, and upward progress of disease per plant were measured.

Inheritance of resistance attributed to chitinase

Seeds were collected from selfed, transformed and non-transformed plants, germinated in petri dishes containing moist filter papers, and subsequently transplanted into potted soil in the greenhouse, as described by Kelemu et al. (1999). The transgenic plant, designated as "plant No. 22", was chosen for subsequent study on inheritance in the progenies. Progeny seedlings were transplanted into individual pots: 156 selfed transgenic progeny and 20 selfed control. Inoculations and disease evaluations were conducted as described above. Probability values for goodness of fit to expected ratios were calculated, using Chi-square analysis.

RESULTS

Plant transformation and regeneration

Calli were induced from leaf segments infected by *A. tumefaciens* on selective medium, containing 15 mg L^{-1} kanamycin. Shoots were induced from kanamycin-resistant calli cultured on regeneration medium with half-strength salt. These shoots grown from kanamycin-resistant calli, were elongated on regeneration medium,

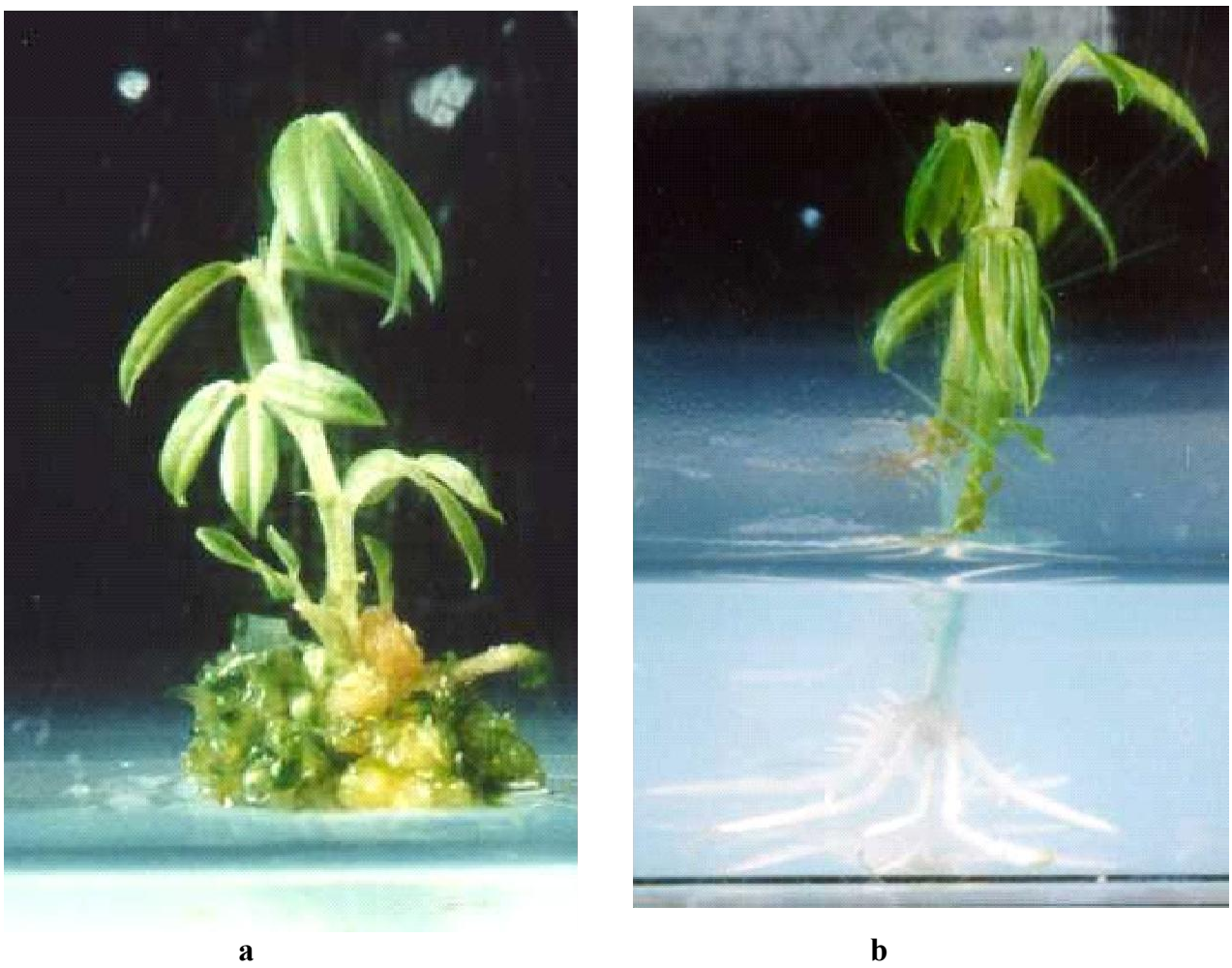

Figure 1. Shoots of *Stylosanthes* plantlets growing from putatively tranformed calli on a regeneration medium (a), and rooting in a selective medium (b).

containing 20 mg L^{-1} kanamycin (Figure 1a). Rooting occurred from shoots excised from calli (Figure 1b) . Fifty putatively transformed kanamycin-resistant plants were generated.

Molecular analyses

DNA, isolated from the putative plant transformants, was tested, using dot blot analysis (Figure 2). No positive signal was detected in DNA from untransformed control plants.

DNA from randomly picked progenies of transgenic plants was tested, using *npt*II gene primers in PCR reactions. These progenies segregated into either generating an amplified DNA product or not (Figure 3). DNA from progenies of the control plant showed no amplified products.

Reactions of transgenic and control plants to *R. solani*

Transgenic *Stylosanthes* plants constitutionally expressing the rice-chitinase gene showed no visible differences in growth and vigor when compared with control plants. Results showed that transgenic plants had a higher level of resistance to *R.solani* than did control plants (Figure 4). More sclerotia were produced on the infected adult control plant (137 sclerotia) than on the transgenic plant No. 22 (11 sclerotia). This trend was also observed in the segregating progenies of the selfed

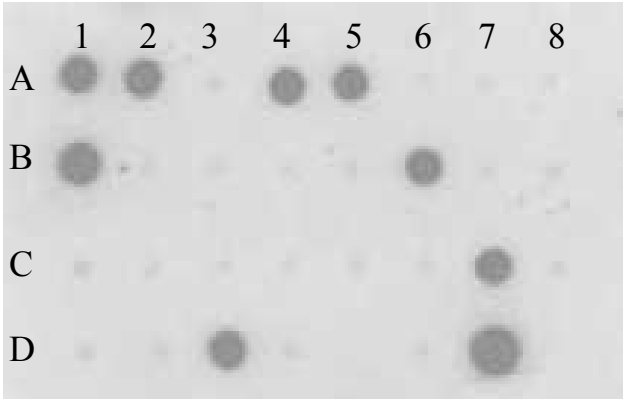

Figure 2. Dot blot analysis of plant DNA, using DIG-labeled chitinase probe. Entries are A1 = putative tranformant (PT) No. 18, A2 = PT No. 22, A3 = PT No. 31, A4 = PT No. 34, A5 = PT No. 44, A6 = PT No. 62, A7 = PT No. 63, A8 = PT No. 64, B_1 = PT No. 65, B_2 = PT No. 66, B_3 = PT No. 67, B_4 = PT No. 69, B_5 = PT No. 71, B_6 = PT No. 72, B_7 = PT No. 74, B_8 = PT No. 75, C_1 = PT No. 76, C_2 = PT No. 79, C_3 = PT No. 81, C_4 = PT No. 82, C_5 = PT No. 83, C_6 = PT No. 84, C_7 = PT No. 85, C_8 = PT No. 86, D_1 = PT No. 87, D_2 = PT No. 88, D_3 = PT No. 89, D_4 = PT No. 91, D_6 = untransformed control plant, D_7 = ~1.5-kb fragment carrying the chitinase gene (probe).

Figure 3. PCR amplifications of genomic DNA isolated from randomly picked selfed progenies and untransformed control plant with *npt* II gene primers. Lane 1 = marker 100-bp ladder; lane 2 = positive control; lane 3 = negative control; lane 4 = untransformed control plant; lanes 5-20 = selfed progenies of transformed plants. *Note:* All progenies lacking the amplified band were susceptible to *Rhizoctonia solani*.

transgenic plant. Control plants also had more infected and defoliated leaves than did transgenic ones (data not shown).

These results have practical significance. When mature, sclerotia produced on infected plants fall to the soil, where they can survive for long periods, forming sources of inoculum for the next disease cycle. Transgenic plants therefore not only confer resistance to *Rhizoctonia* foliar blight disease, but also drastically help reduce sclerotia production and thus sources of inoculum, thereby maintaining healthier soil.

Inheritance of resistance to *R. solani* in selfed progeny

To determine the inheritance of resistance to *R. solani*, seeds were collected from a selfed transgenic mother (T_0) plant, plant No. 22. The plant showed high levels of foliar blight resistance, strong dot- blot signal and positive PCR results with the *npt*II gene primers. Foliar blight, seedling blight, root rot and brown-girdling are among the disease symptoms caused by *R. solani*, a chitinous soil-borne pathogen with a wide host range. The number of diseased and defoliated leaves, number of sclerotia produced on diseased tissues of each plant and upward progress of disease were determined in each plant, as follows:

1. 118 plants were rated as resistant (i.e. showing either no or limited symptoms) and 38 plants as susceptible as control plants (Figure 6). This segregation in the progenies agreed with the expected Mendelian ratio of 3 resistant to 1 susceptible, when analyzed by the Chi-square test (Table 1).
1. 2. The number of infected and defoliated leaves per resistant progeny was lower than for susceptible ones (Figure 6).
2. Susceptible plants sustained more sclerotia per plant than did resistant ones (Figure 6).
3. Upward progress of disease was higher in susceptible segregants than in resistant ones (Figure 6).
4. Average root length and root dry weight were higher in resistant segregants than in susceptible ones. The average root length in each resistant progeny was 25 cm versus 21 cm in susceptible ones.
5. Average root dry weight of resistant segregants was 0.228 g versus 0.156 g for susceptible segregants.
6. Disease development and progress over time in each susceptible progeny was faster and more severe than in resistant ones (Figure 7).

These results confirm that the introduced rice- chitinase gene in the transgenic *S. guianensis* plant was inherited by the progenies along with resistance to *Rhizoctonia* foliar blight disease.

DISCUSSION

Chitinases have no known function and have no known endogenous substrate in higher plants, but because their substrate, chitin, is a major component of cell walls of

Figure 4. Reactions of transgenic (left) and control *Stylosanthes guianensis* accession CIAT 184 plants to *Rhizoctonia solani* AG-1, 8 days after inoculations. *Note* the blighted leaves on the lower part of the control plants.

Figure 5. Reactions of two of the segregating selfed progenies (susceptible [left] and resistant) to *Rhizoctonia solani* AG-1, 6 days after inoculations.

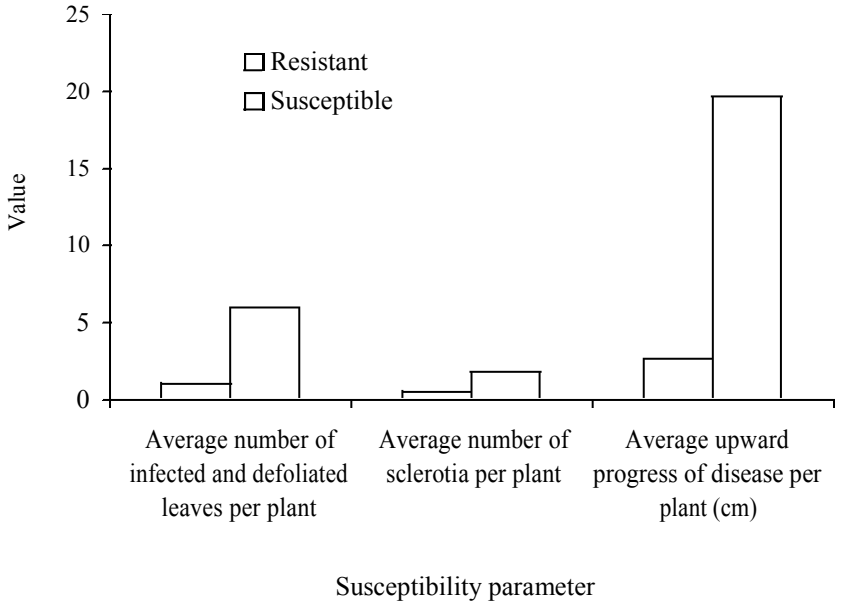

Figure 6. *Rhizoctonia* foliar blight disease development in selfed progenies of transgenic plant No. 22 according to the number of defoliated and infected leaves, number of sclerotia formed in infected plant tissues and disease progress.

Table 1. Inheritance of resistance to *Rhizoctonia solani* AG-1 conferred by a rice-chitinase gene in progenies from a selfed transgenic *Stylosanthes guianensis* plant.

Plant description	Observed		Expected		Chi-square value	P (%)
	Resistant	Susceptible	Resistant	Susceptible		
Selfed transgenic	118*	38**	117	39	0.03418	85
Selfed control (accession CIAT 184)	-	20	-	20	-	-

*64 plants with no macroscopic symptoms of *Rhizoctonia* foliar blight, and 54 plants with some visible symptoms with upward progress of disease of less than 6 cm.
**38 progenies were as susceptible as the progenies of the selfed control plant.

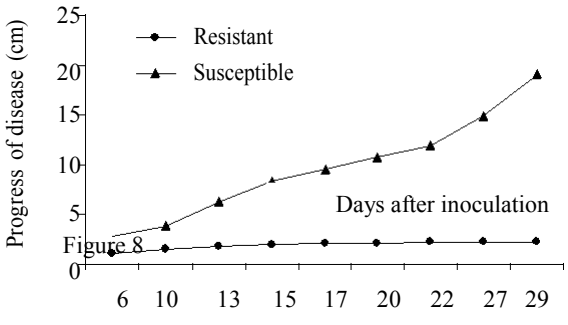

Figure 8

Figure 7. Progress of *Rhizoctonia* foliar blight disease in selfed progenies of transgenic plant No. 22 over time.

many filamentous fungi, except Oomycetes, they may have a plant protection function (Pegg, 1977). Plants naturally respond to microbial invasion by activating defenses such as antibiotic synthesis, stimulation of enzymes and reinforcement of cell walls (Dixon and Lamb, 1990). Chitinases belong to a group of pathogenesis-related proteins that can be induced in response to attack by plant pathogens and some abiotic stresses (Boller, 1988).

In this paper, we have shown that the presence of a rice-chitinase gene under the control of the CaMV 35S promoter in transgenic *S. guianensis* accession CIAT 184 confers resistance to foliar blight disease caused by *R. solani*. This resistance is manifested by a significant reduction in the disease's upward progress, reduced number of infected and defoliated leaves, greater root biomass and length, and fewer fungal sclerotia. *Stylosanthes* is a perennial forage legume and the production of significantly fewer fungal sclerotia on transgenic plants than on control plants has important implications on curtailing the disease cycle through reductions of inoculum sources. Constitutional production

of chitinase in transformed tobacco plants reduces plant mortality in soil infested with *R. solani* (Broglie et al., 1991; Vierheilig et al., 1993).

The segregation of selfed progeny at a ratio of 3:1 (resistant to susceptible) is consistent with a single locus of an active chitinase gene. The level of resistance expressed in most segregating resistant progeny (T_1) is comparable with or similar to that observed in the transgenic mother plant, although some expressed a much higher resistance. Progeny that showed higher levels of resistance than the mother plant were selected for further seed production and evaluation of T_2 and T_3 generations.

Several studies have been reported on disease resistance of transgenic plants carrying chitinase genes. Our results are compatible with those reported by others in various plants. For example, tobacco and canola transgenic plants expressing a bean-chitinase gene showed resistance to *R. solani* (Broglie et al., 1991); rice plants transformed with a rice-chitinase gene under the 35S promoter were resistant to sheath blight disease caused by *R. solani* (Lin et al., 1995); transgenic cucumber plants containing a rice-chitinase gene expressed an enhanced resistance to gray mold disease caused by *Botrytis cinerea* (Tabei et al., 1998); rose plants transformed with a rice-chitinase gene had reduced blackspot disease caused by the fungus *Diplocarpon rosae* (Marchant et al., 1998) . However, our work is the first to report on a transgenic tropical forage legume *S. guianensis* containing a rice-chitinase gene showing resistance to *Rhizoctonia* foliar blight disease.

This study indicates that benefits can result from introducing naturally existing defense-related genes that are automatically triggered in plants when attacked by pathogens or injured by abiotic stresses. The transfer of a gene from rice to a legume forage plant like *Stylosanthes* is not possible with traditional breeding methods. Recombinant DNA techniques can make this possible, thus generating genetic diversity for disease resistance. As more cloned plant-defense genes and effective promoters become available, more disease-resistant and environmentally friendly plants are likely to be created. The challenge ahead would be to educate the general public and media on the ever-growing debate of transgenics, their potential benefits and potential unknown drawbacks.

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