Transgenic Plants Expressing Antimicrobial Lactoferrin Protein are Resistant to a Fungal Pathogen

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Abstract
Transgenic tobacco (Nicotiana tabacum var Xanthi) and Arabidopsis (A. thaliana) plants expressing an antimicrobial bovine lactoferrin (BLF) gene were developed and evaluated for resistance against an economically important fungal pathogen Rhizoctonia solani, the causal agent of damping off diseases. The plants were transformed with the Agrobacterium strain C58C1 containing a plasmid construction carrying a modified BLF cDNA. The introgression of BLF cDNA into susceptible tobacco and Arabidopsis lines was confirmed by Southern blot and the expression of full-length lactoferrin transcript and protein was also detected by Northern and Western blots, respectively. Transgenic lines segregating for a single locus insertion were identified and used for disease resistance assays. Transgenic tobacco plants exhibited high levels of Rhizoctonia resistance in detached leaf assays. Similarly, transgenic Arabidopsis seedlings were resistant to the fungus and prevented damping off symptoms. Use of lactoferrin gene is a potential new approach to consider for control of diseases caused by fungal pathogens.

Key words: Arabidopsis, disease resistance, transformation, Rhizoctonia.

INTRODUCTION

About 90% of the 2000 major diseases of principle crops in the United States are caused by soil borne plant pathogens (Lewis and Papavizas 1991) resulting in losses in excess of $4 billion/year (Lumsden et al. 1995). Root rot diseases account for the largest percentage of loss in commercial ornamental production. The basidiomycetous soil borne fungus Rhizoctonia solani, sensu lato (Tele: Thanatephorus cucumeris, T. praticolor, etc) is known to attack 188 species of higher plants in 32 families, including various staple crops, ornamentals and turf grasses (Manibushanrao et al. 1981; Anderson 1982). Also, some R. solani isolates infect distinct tissues on the same plant causing multiple diseases.

There are at least 13 hyphal anastomosis groups (AGs) of R. solani (Carlimg et al. 2002). AGs are considered as genetically isolated groups with distinct host specializations, epidemiology and sensitivity to pesticides (Ogoshi 1987; Kataria and Gishi 1996). Isolates belonging to AG-4 have wide host range, are most often pathogenically associated with ornamental plants (Benson and Cartwright 1996) and is the focus of this investigation.

R. solani is a major cause of root diseases causing damping off and blights of ornamentals, vegetables, cereals and patches of turfgrasses (Burpee and Martin 1996; Tu et al. 1996; Daughtrey and Benson 2005; Wu et al. 2006). Since Rhizoctonia has a propensity for attacking juvenile tissues, bedding plants grown from seeds are especially vulnerable to pre-emergence damping-off (Benson and Cartwright 1996; Lewis and Lumsden 2001). Rhizoctonia root rots are generally managed by seed treatment with fungicides, soil fumigation, biological and
cultural practices (Agrios 2005). However, control with fungicides alone could be unreliable at times (Kataria and Gisi 1996; Cotterill 1991). Some pesticides used in commercial vegetable and fruit production to control soil borne diseases could be highly toxic and deleterious to the environment and pathogens often develop resistance to pesticides (Crop Protection Handbook 2003; Disease Control in Crops 2009). For example, methyl bromide, once used extensively for commercial vegetable, ornamental and fruit productions has been banned worldwide since 2005 due to high toxicity and stratospheric ozone depletion (http://www.epa.gov/). There only very limited fungicides registered for horticultural use and no suitable alternatives exist to control pathogens in organic production systems. As a result, alternative management practices have been a top research priority for controlling soil borne pathogens.

There are no reports of true resistance against *R. solani* within cultivable species and there are only very few commercial agronomic cultivars partially resistant to the pathogen. In absence of natural genetic resistance, transgenic introduction of resistance may be a sustainable alternative to chemical approaches to disease management (Hammond et al. 2006). Recently, there are a number of reports describing transgenically induced resistance with various genes against *R. solani* (Broglie et al. 1991; Logemann et al. 1992; Lorito et al. 1998; Datta et al. 1999; O’Brien et al. 2001; Wang and Fristensky 2001; Dong et al. 2007; Almasia et al. 2008) emphasizing both the importance of this pathogen as well as its suitability as model system to test transgenic resistance against plant pathogens. Most of the reports did not specifically identify the AG group of *R. solani* being evaluated against transgenic constructs (O’Brien et al. 2001). Only in a few report, transgenic resistance have been evaluated against commonly occurring AG-4 isolates. O’Brien et al. (2001) also stated that transgenic resistance against AG-4 isolates in most instances may be ineffective against AG-8 isolates accentuating the genetic heterogeneity of AG groups. In fact it has been proposed that the isolates of AG-4 belong to *T. pratensis* whereas most other AGs belong to *T. cucumeris* (Sneh et al. 1991). Similarly, Perl-Treves et al. (2004) reported that a stress response promoter of *Arabidopsis thaliana* responded differently between *R. solani* strains and the response was not observed with aggressive strains that caused death of seedlings. Such variations in resistance to *Rhizoctonia* are also not uncommon among agronomic cultivars. For example, Zhao et al (2005) reported that naturally occurring moderate resistance of soybean line PI 442031 against AG-4 and AG-5 isolates is completely ineffective against AG-2 IIIB isolates. Nevertheless, many of the early studies of introduced plant resistance dealt with foreign genes against a narrow group of pathogens and at times of questionable safety for human consumption. Introduction of resistance gene against a broad range of plant pathogens is also anticipated for disease management. Moreover, any introduced resistance gene with additional nutritional benefit for human consumption could be a desirable feature.

Lactoferrin (Lf), a cationic iron-binding glycoprotein of 77 kDa belonging to the transferring family (Fei et al. 2005), is present in milk, tears, saliva, and mucous secretions of most mammals and plays a major role in the immune system of newborns by modulating immune functions. The N-terminal peptide of Lf (about 25 to 47 amino acids), which can be released by proteolytic cleavage, is highly bactericidal (Yamauchi et al. 1993). This peptide, lactoferricin, is the shortest active amino acid sequence that is resistant to further enzymatic cleavage (Bellamy et al. 1992). In the present investigation, we evaluated if the bacterial wilt inhibiting properties of Lf in transgenic plants can be extended to controlling damping off caused by a soil borne fungal pathogen *R. solani*. We utilized transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* as model systems to evaluate possible resistance conferred by expressed bovine lactoferrin (BLF) against *R. solani*.

![Figure 1](image.png)

**Figure 1** Schematic diagram of the Transfer-DNA segment of the binary plasmid pAM4212. The plasmid contains an antibiotic resistance gene neomycin phosphotransferase (*NPT II*) for the selection of transformants and an antimicrobial bovine lactoferrin (BLF) gene for resistance against *R. solani*. BL & BR, T-DNA left and right borders; 35S, Cauliflower Mosaic Virus 35S promoter; NT & T7, T-DNA Nos and T7 gene terminators; NOS, promoter from nopaline synthase gene. Probe: DNA sequence used for Northern blot.

**MATERIAL AND METHODS**

**Construction of vector and development of transgenic plants**

A binary vector containing a bovine lactoferrin gene (Mitra and Zhang 1994) was used for transformation of tobacco (*Xanthi cultivar*) and *Arabidopsis thaliana* ecotype RLD. The lactoferrin gene was driven by the promoter of cauliflower mosaic virus 35S promoter (Cooke and Penon 1990) and a DNA sequence from *Agrobacterium* T-DNA gene 7 was used as a transcription termination signal creating a binary plasmid pAM4212 (Figure 1). The binary vector also contained a selectable marker gene *nptII* driven by the nopaline synthase gene promoter of *Agrobacterium* (strain C58Cl) Ti plasmid T-DNA. The plasmid was transferred to *Agrobacterium* strain EHA 105 (Hood 1991) and used for plant transformation. Routine *Agrobacterium* leaf-disk
cocultivation method was used for tobacco transformation (Klee et al. 1987). *Arabidopsis* plants were transformed following the in-planta floral-dip method of transformation (Bechtold and Pelletier 1998).

### Culturing and inoculum preparation of a pathogenic *R. solani* isolate

*R. solani* AG-4 was maintained in potato dextrose agar medium (Difco, Detroit, MI). Inoculum of *R. solani* for soil bioassay was prepared by adding 10 growing plugs (10 mm diameter) of actively growing fungus in 200 g of autoclaved cracked wheat containing 15 to 20% moisture and incubating for two weeks. The colonized wheat was air dried, blended and passed through 2 mm pore sieve and stored at 4 C in a sterile plastic bottle for soil inoculations.

### Analysis of lactoferrin expression in transgenic plants

Transgenic wheat and *Arabidopsis* plants were examined for the expression of the transgene by Northern blot assay. Subsequently, the presence of lactoferrin protein was also detected by Western blot.

**Northern blot analysis:** Young leaf tissues (100 mg) were collected from each transgenic seedling and ground in a mortar with liquid nitrogen. Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA using the manufacturer’s instructions. Fifteen µg of total RNA was separated on a formaldehyde agarose gel (1%) at 85 volts for 2 hours, the gel was pre-soaked in 20XSSC solution for 15 min followed by transfer to a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) using a TurboBlotter (Schleicher & Schuell, Inc., Keene, NH) and cross-linked by an UV crosstlinker (Stratagene Strataglinker, La Jolla, CA). Gel eluted bovine lactoferrin fragment (202 bp) (Fig. 1) was used for the 32P-labeled probe. Hybridization was performed at 65C overnight and the membrane was washed three times each with the washing solution at 65C. The washed membranes were exposed to Kodak X-OMAT film in a cassette at -80C for 24 hours.

**Western Blot assay and Enzyme-Linked Immunosorbent Assay (ELISA):** Total proteins from transgenic plants were extracted according to Mitra and Zhang (1994). Extracts containing 50 µg of total soluble proteins were separated on 12.5% (w/v) acrylamide gels (Laemmli 1970) along with a 200 ng of commercially available lactoferrin (Sigma, St. Louis, MO) as a standard. Blotting to membrane, immuno-hybridization and color development followed the protocol of Mitra and Zhang (1994). Transgenic tobacco and *Arabidopsis* seedlings were screened to determine the levels of lactoferrin expression. Commercially available polyclonal antibodies (Sigma) were used following the manufacturer’s instructions.

Bioassays for in vitro and in vivo resistance of transgenic plants expressing lactoferrin

**Agar-gel diffusion assay:** An agar-gel diffusion assay was set up to test in vitro tolerance of *R. solani* to lactoferrin expressed in transgenic tobacco and *Arabidopsis* plants (Broglie et al. 1991; Dhingra and Sinclair 1995). Total soluble protein extracted from transgenic and control leaves were used in the assay. A 5 mm diameter agar plug of one-day old *R. solani* is placed in the middle and samples were added to each peripheral wells of potato dextrose agar plate and incubated for 4 days at room temperature in dark. There were three replicates of each treatment and the whole experiment was repeated one more time. Effect of transgenic protein extracts on radial fungal growth was observed and photographed when the fungus in control plate reached the periphery of the plates.

**Detached whole leaf assay:** For infectivity of *R. solani*, third to 4th leaves from young transgenic tobacco were used. Detached leaves were placed on sterile moist filter papers inside sterile petri dishes. A 5 mm mycelial disc from the periphery of 1 day old *R. solani* was placed in the center of each leaf (Rancé et al. 1998; Reuveni et al. 2006). The leaves were photographed on 3, 4, 5, 7, and 9 days following inoculation with mycelial plug. The area (in cm²) of necrotic symptoms developed around the discs was determined from the images using Scion Image 4.03 for Windows software. All inoculations were performed in 3 replicates and the leaves were incubated in a humid chamber under fluorescent light at room temperature in a completely randomized manner.

**Seed germination assay for damping off of seedlings**

Damping off bioassay followed a modified protocol of Lewis and Lumsden (2001). *R. solani* inoculum was mixed 0.75% (wt/wt) to autoclaved soil-less potting mix containing 30% moisture. For control, soil was amended only without inoculated, but autoclaved cracked wheat at the same ratio. Plastic pots (5” X 6”) were filled with either inoculated soil or not inoculated soil and transgenic *Arabidopsis* seeds were sown. Pots were incubated at 22 C, 75-80% relative humidity, and 16 h (7350 lx) illumination with daily irrigation with sterile water. Observations were made 12 days after sowing for relative emergence and symptoms of seedlings.

### RESULTS

#### Development of transgenic lines

*Agrobacterium*-mediated leaf-disc and in-planta transformation generated many tobacco and *Arabidopsis* transformants, respectively. These plants were characterized for transgene copy number by Southern blot, mRNA level and size by Northern blot and the expression of transgene protein by Western blot. Five transgenic lines each from
tobacco and *Arabidopsis* carrying a single copy of BLF gene with highest BLF protein expression levels were selected. Kanamycin resistant T2 progeny of these lines were tested for resistance against *R. solani*.

**Figure 2** Expression of lactoferrin in transgenic tobacco (T) and *Arabidopsis* (A) detected by Western (I) and Northern (II) blot analyses. LF, Lactoferrin protein; C, control tobacco plant; M, molecular weight marker (kD), 28S RNA is shown as loading control for Northern blot.

**Expression of BLF in transgenic plants**

Transgenic tobacco and *Arabidopsis* were tested for the expression of lactoferrin mRNA and protein. Northern blot assay detected full length BLF transcript in all kanamycin resistant tobacco plants, and in all but three kanamycin resistant *Arabidopsis* plants. Among these three, two *Arabidopsis* lines showed truncated transcripts and the remainder line had no detectable BLF transcript. Results of the immuno-blotting experiment with BLF-specific antibody demonstrated that BLF is expressed in both tobacco and *Arabidopsis* lines. Transgenic tobacco and *Arabidopsis* plants expressing lactoferrin protein demonstrated the presence of a band at 77 kDa, the predicted molecular weight of lactoferrin. The bands co-migrated with a band from purified lactoferrin protein. Only one representative plant from each species with full length BLF transcripts are shown in Fig. 2.

**In vitro Agar-Gel diffusion inhibition assay**

In Fig. 3, wells B & D contained extracts from two transgenic tobacco lines not expressing BLF protein and wells A & C contained extracts from two transgenic tobacco lines expressing BLF. Inhibitory effect of transgenic extracts containing BLF was clearly noticeable in both the wells A and C, whereas no inhibition was detected in wells B and D containing protein extracts from control transgenic plants not expressing BLF and in wells containing only extraction buffer (not shown).

**N. tabaccum detached whole leaf-bioassay:**

A detached leaf assay was used to quickly determine the effect of plant expressed lactoferrin on *R. solani* infection. Transgenic tobacco leaves were excised from aseptically grown young seedlings, placed on sterile moist filter paper in a large Petri dish, and inoculated with fresh *R. solani* inoculum.

**Figure 3** In vitro inhibition of *R. solani* by protein extracts of transgenic tobacco and *Arabidopsis* plants expressing lactoferrin protein 9 days after inoculation. 35 μg total protein was used in each well. Well A and Well C: Protein extracts from two transgenic tobacco and *Arabidopsis* lines respectively. Well B and Well D: Protein extracts from two control vector only transgenic tobacco and *Arabidopsis* lines.

Necrotic areas developed around the mycelial plug in control leaves quickly which rapidly increased in size every day until the entire leaf surface became necrotic at 9 days after inoculation. In contrast, the transgenic leaves did not show any visible necrosis for 5-6 days. A small necrotic area was visible in transgenic leaves on the day 7 and only increased slightly on day nine (Fig. 4). However, the necrotic area increased quickly after 10 days and covered the entire leaf during the next two days. This eventual overcome of lactoferrin induced disease resistance is most likely due to the rapid senescence of detached leaves and associated protein degradation toward the end.

**Damping off bioassay of Arabidopsis seedlings expressing BLF:**

Seeds from two transgenic T2 generation of *Arabidopsis* lines were germinated in pots containing *Rhizoctonia* corn-meal mycelial inoculums. In control pots no inoculums was added. Although germination of seeds was comparable for both transgenic and control *Arabidopsis* plants, the establishment of control seedlings was severely affected by the presence of *Rhizoctonia*. Most seedlings in the control pots died out soon after germination. Most transgenic seedlings, in contrast, grew normally indicating resistance against *Rhizoctonia* damping off (Fig. 5). As the transgenic *Arabidopsis* lines were not homozygous, some seedlings are expected to succumb to *Rhizoctonia*. Resistance to *Rhizoctonia* was confirmed in all transgenic *Arabidopsis* lines expressing lactoferrin.
Figure 4 Detached leaf assays. Fourth leaf of transgenic tobacco seedlings were used to determine infectivity of *R. solani*. A 5 mm mycelial disc was placed in the center of each leaf. The leaves were incubated in a humid chamber under fluorescent light at room temperature. The diameter of necrotic symptom developed around the disc was measured on 3, 4, 5, 7, and 9 days after inoculation. All inoculations were performed in 3 replicates in a completely randomized manner. Disease progress is shown in the graph (necrotic area (cm²)) using data from all three replicates. Scion Image 4.03 for Windows was used to analyze the images and determine necrotic areas.
DISCUSSION

In this paper, we demonstrated that BLF inhibits the growth of a soil-borne plant fungal pathogen *R. solani* in vitro. We also showed transgenic expression of BLF in *N. taccum* and *A. thaliana* imparts considerable resistance and also delays the onset and progress of disease development by the pathogen. Constitutive expression of this antimicrobial protein (AMP) does not seem to alter morphology and physiology of both these types of plants. Previous studies conducted by our group also demonstrated that BLF expressing tobacco calli inhibited several phytopathogenic bacteria in vitro (Mitra and Zhang 1994). Also, transgenic tobacco and tomato plants expressing Lf significantly delayed wilt symptom development caused by the bacterium *Ralstonia solanaceaum*, in a dose-dependent manner (Zhang et al. 1998; Lee et al. 2002).

A prominent property of lactoferrin is its potent activity against a wide range of microorganisms including both gram-negative and gram-positive bacteria, as well as fungi and viruses. In addition to its anti-microbial as well as anti-inflammatory properties, Lactoferrin may have role in iron absorption and/or excretion and in gastric health of newborns (van Berk et al. 2002). Mitra and Zhang (1994) showed that human lactoferrin expressed in tobacco calli inhibited several phytopathogenic bacteria in vitro. In addition, transgenic tobacco and tomato plants expressing lactoferrin significantly delayed wilt symptom development caused by *Ralstonia solanaceaum*, in a dose-dependent manner (Zhang et al. 1998; Lee et al. 2002). Similarly, expressed lactoferrin in transgenic pear showed an increased resistance against *Erwinia amylovora* (Malnoy et al. 2003). Takase et al. (2005) evaluated transgenically expressed human lactoferrin and lactoferricin in rice against disease-causing *Burkholderia plantarii* (causal agent of Bacterial Seedling Blight), Rice dwarf virus and *Pyricularia oryzae* (*Magnaporthe grisea*, causal agent of Rice Blast). However, they found significant resistance only against *B. plantarii*.

Since *Rhizoctonia* attacks mostly the juvenile tissues, bedding plants grown from seeds are especially vulnerable to pre-emergence damping-off (Benson and Cartwright 1996; Lewis and Lumsden 2001). Seedlings tend to develop resistance or tolerance to the pathogen with age (Bateman and Lumsden 1965; Kus et al. 2002). Thus, protecting the most vulnerable early developmental stage of seedling against *Rhizoctonia* seems to be more critical. Also, slowing down the onset and progress of *Rhizoctonia* disease by genetic means and complementing with reduced amount of pesticide applications should have an advantage to the host plants in terms of epidemiology of the disease. In this sense, our experiment showed near complete protection of *Arabidopsis* seedlings against pre- and post-emergence damping off caused by *R. solani* within the duration of experiment.

CONCLUSION

There has been a recent surge in research interest expressing AMPs in plant to control fungal, bacterial as well as viral diseases (Ousky et al. 2000; Malnoy et al. 2003; Peschen et al. 2004; Erika et al. 2006; Almasia et al. 2008). Our data suggest that transgenic resistance using lactoferrin gene confers broad-spectrum resistance against both fungal (this work) and bacterial plant diseases (Mitra and Zhang 1994; Zhang et al. 1998; Lee et al. 2002) and should be considered alone or in combination with other transgenes and
practices to manage soil borne plant pathogens. This together with the fact that lactoferrin is one of the safest and probably most unavoidable in the nutrition of human development; make this gene potentially a highly desirable candidate of introduced plant resistance against fungal and bacterial diseases. However, it will be important to demonstrate that the observed resistance against *Rhizoctonia* by lactoferrin will hold up under field conditions.

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