Transgenic Res (2008) 17:47–57
DOI 10.1007/s11248-007-9073-3

ORIGINAL PAPER

Expression of the bacteriophage T4 lysozyme gene in tall fescue confers resistance to gray leaf spot and brown patch diseases

Shujie Dong · H. David Shew · Lane P. Tredway · Jianli Lu · Elumalai Sivamani · Eric S. Miller · Rongda Qu

Received: 7 November 2006 / Accepted: 10 January 2007 / Published online: 2 February 2007

Abstract Tall fescue (*Festuca arundinacea* Schreb.) is an important turf and forage grass species worldwide. Fungal diseases present a major limitation in the maintenance of tall fescue lawns, landscapes, and forage fields. Two severe fungal diseases of tall fescue are brown patch, caused by *Rhizoctonia solani*, and gray leaf spot, caused by *Magnaporthe grisea*. These diseases are often major problems of other turfgrass species as well. In efforts to obtain tall fescue plants resistant to these diseases, we introduced the bacteriophage T4 lysozyme gene into tall fescue through *Agrobacterium*-mediated genetic transformation. In replicated experiments under controlled environments conducive to disease development, 6 of 13 transgenic events showed high resistance to inoculation of a mixture of two *M. grisea* isolates from tall fescue. Three of these six resistant plants also displayed significant resistance to an *R. solani* isolate from tall fescue. Thus, we have demonstrated that the bacteriophage T4 lysozyme gene confers resistance to both gray leaf spot and brown patch diseases in transgenic tall fescue plants. The gene may have wide applications in engineered fungal disease resistance in various crops.

Keywords Fungal resistance · *Magnaporthe grisea* · *Rhizoctonia solani* · T4 lysozyme · Tall fescue

S. Dong · J. Lu · E. Sivamani · R. Qu (✉)
Department of Crop Science, North Carolina State University, Raleigh, NC 27695, USA
e-mail: rongda_qu@ncsu.edu

H. D. Shew · L. P. Tredway
Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA

E. S. Miller
Department of Microbiology, North Carolina State University, Raleigh, NC 27695, USA

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season grass species that is widely used in temperate and subtropical regions as a forage and turfgrass (Buckner et al. 1979). It is a major turfgrass species for landscape and home lawns in the transition zone in the USA, mostly because of its tolerance to summer heat and drought.

Gray leaf spot disease is becoming an increasingly severe disease on turfgrasses in the USA. It is now a persistent problem for tall fescue in the Southeast, and for perennial ryegrass in many areas of the country (Tredway et al. 2003). The disease is caused by *Magnaporthe grisea* (T.T. Hebert) Yaegashi and Udagawa, an ascomycete fungus (Trevathan et al. 1994). The first symptoms of gray leaf spot are round or oval tan spots

with a dark brown border. Under high humidity conditions, the color of the spots becomes gray due to profuse spore production by the fungus. Expansion and coalescence of lesions may result in a foliar blight, where infected leaves are girdled and die back rapidly. Current research aims to develop resistant turfgrass cultivars through conventional breeding and transgenic approaches to control these fungal diseases. Partial resistance to gray leaf spot disease was observed in perennial ryegrass and the quantitative trait loci were mapped (Curley et al. 2005). The inheritance of the resistance to gray leaf spot in this species has also been investigated (Han et al. 2006).

More extensive knowledge about *M. grisea* comes from studies mainly from rice, since the pathogen is also the causal agent of rice blast, the most common and severe disease of cultivated rice. The biology of the fungal pathogen, the molecular process of its infection, and the rice resistance genes to the pathogen have been well studied (for a review, see Talbot 2003). *M. grisea* is considered a model pathogen for the molecular study of the fungal diseases of plants, and its genome was recently sequenced (Dean et al. 2005). Advances in genomic study of *M. grisea* would provide a powerful tool for further elucidating the interactions between the fungal pathogen and its host plants.

Brown patch disease is the most severe and frequently occurring disease on tall fescue lawns under warm and humid weather conditions. It is also a serious disease for other turfgrass species (Smiley et al. 2005). The disease is caused by members of multiple anastomosis groups (AG) of the soil-borne fungus *Rhizoctonia solani*, one of the most common and destructive fungal pathogens of higher plants. The early symptoms of brown patch are small, circular foliar lesions with a dark brown border. As the disease progresses, the pathogen spreads to neighboring plants through mycelial growth, forming infection centers that range from 6 in. to several feet in diameter. Lawns having brown patch disease appear droughty or wilted, even if sufficient water is provided (Anderson 1982).

Use of plant breeding to develop germplasm resistant to diseases has been an effective approach for improving disease control. Genetic engineering provides an additional means for development of resistant varieties by introducing resistance genes from other species or over-expressing relevant endogenous genes. In this approach, identifying the suitable genes against the target pathogens and expressing those genes to effective levels are critical to its success. Genes from various sources have been tested for fungal resistance in transgenic plants. The first report came from transgenic tobacco plants constitutively expressing a bean chitinase gene, which showed enhanced resistance to *R. solani* (Broglie et al. 1991). Co-transformation of a rice chitinase (*RCH10*) and an alfalfa glucanase gene (*AGLU1*) in tobacco and tomato had additive effects against the fungi *Cercospora nicotinae*, *R. solani*, and *Fusarium oxysporum* f. sp. *lycopersici* (Zhu et al. 1994; Jongedijk et al. 1995; Jach et al. 1995). Antimicrobial peptides can also confer fungal resistance in transgenic plants. For example, an analog of magainin 2, MSI-99, was expressed in tobacco plants through chloroplast transformation, resulting in resistance to fungal (*Aspergillus flavus*, *Fusarium moniliforme*, and *Verticillium dahliae*) and bacterial (*Pseudomonas syringae*) pathogens (DeGray et al. 2001). In addition, plant R genes could be expressed for plant protection against fungal pathogens. A rice R gene, *Pi9*, was recently cloned and conferred resistance in transgenic rice plants to many races of *M. grisea* (Qu et al. 2006).

Despite the severity of fungal diseases in turfgrasses, only a few reports have been published regarding engineered resistance to fungal pathogens in these species. Transgenic creeping bentgrass plants expressing PR5K, a receptor protein kinase, slowed the development of dollar spot symptom caused by *Sclerotinia homoeocarpa* F.T. Bennett (Guo et al. 2003). Transgenic creeping bentgrass over-expressing the rice thaumatin-like protein-encoding gene TLPD34 also showed enhanced dollar spot resistance (Fu et al. 2005). In the present research, we generated 13 independent transgenic tall fescue events via *Agrobacterium*-mediated transformation with a bacteriophage T4 lysozyme gene construct, and investigated the responses of these lines to turfgrass isolates of *M. grisea* or *R. solani* in a controlled environment in replicated experiments.

We observed that six transgenic lines were highly resistant to gray leaf spot, among which three lines also showed significant resistance to brown patch.

Materials and methods

Cloning of the lysozyme gene from T4 bacteriophage

Plate lysates of T4 bacteriophage B strain were obtained from growth of *Escherichia coli* B on EHA top agar with Luria-Bertani as bottom medium and purified by PEG precipitation (Carlson and Miller 1994). The genomic DNA of phage T4 was isolated using hexadecyltrimethylammonium bromide (Miller and Carlson 1994). The following primers were used to amplify the full-length coding sequence of the T4 gene *e* lysozyme (a murein hydrolase) from its genomic DNA through PCR:

- T4EF: 5′ATGAATATATTTGAAATGTTACGTAT3′
- T4ER: 5′TTATAGATTTTTATACGCGTCC3′

The PCR product was cloned into the TA cloning vector pCR® 2.1 from Invitrogen (Carlsbad, CA, USA) to generate pJLU1, and sequenced by the DNA Sequencing Facility at Iowa State University (Ames, IA, USA). The cloned T4 *e* gene is identical in nucleotide sequence to that described in Owen et al. (1983), but is slightly different from the NCBI entry (accession NC_000866). The differences in nucleotide sequences result in the substitutions of Arg-12 and Ile-137 with Gly and Arg, respectively, in NC_000866.

Agrobacterium transformation of tall fescue

The binary vector pSD9 (Fig. 1) was constructed based on pCAMBIA1300, which has a hygromycin B (hyg B) resistant *hph* gene under the control of the CaMV 35S promoter and its terminator (http://www.cambia.org.au). pSD9 contains a construct of the T4 lysozyme coding sequence from the plasmid pJLU1 under the control of the maize *Ubi1* gene promoter (Christensen et al. 1992). The freeze-thaw method (An et al. 1988) was used to mobilize plasmid pSD9 into *Agrobacterium* strain EHA105(pTOK47) as previously described (Dong and Qu 2005). The resulting *Agrobacterium* strain EHA105(pTOK47, pSD9) was designated as ETSD9.

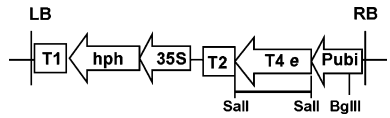

Fig. 1 Binary vector pSD9 used in the transformation experiments. The vector is a derivative of pCAMBIA1300. Only the T-DNA region is shown. RB right border, LB left border, 35S CaMV 35S promoter, *hph* hygromycin phosphotransferase gene coding region, T1 CaMV 35S terminator, T2 *A. tumefaciens nos* gene terminator, *Pubi* maize *Ubi1* gene promoter, T4 *e* T4 phage lysozyme gene *e* coding region. The *Bgl*II and *Sal*I restriction sites are marked. *Arrows* indicate directions of transcription. The probe used for Southern and Northern analyses is shown by a *bar*

Calli were induced from mature seeds of tall fescue cultivar ''Coronado'' (Rose-Fricker et al. 1999) and used for the transformation experiments. Embryogenic calli were transformed with *Agrobacterium tumefaciens* strain ETSD9 as described previously (Dong and Qu 2005). In brief, the infected calli were cultured under 250 mg l^{-1} hyg B selection at 25 °C in the dark for 8 weeks. Hyg B resistant calli were transferred to the selective regeneration medium (with hyg B at 50 mg l^{-1}) for shoot induction. Regenerated shoots were then cultured on a rooting medium (containing 50 mg l^{-1} hyg B) for root development. About 4 weeks later, rooted plants were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH, USA). The plants were subsequently grown in a greenhouse at 25°C. All chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise specified.

Southern blot analysis

Genomic DNA isolation, digestion, electrophoresis, membrane transfer, probe labeling, and hybridization were performed as described previously (Dong and Qu 2005). Twenty-five

micrograms of genomic DNA from each sample was digested overnight with *Bgl*II, which cut once within the construct (Fig. 1), and used for Southern analysis. The full-length coding region of the T4 lysozyme gene (0.55 kb *Sal*I fragment of the pSD9, Fig. 1) was used as a probe to detect the transgene. Restriction enzymes were purchased from Promega (Madison, WI, USA). The hybridized membranes were exposed to Kodak BioMax MS film (Kodak Eastman, Rochester, NY, USA) for autoradiography.

Northern blot analysis and RT-PCR assays

Total RNA was extracted from plant leaves using Trizol® Reagent following the manufacturer's instructions (Invitrogen). Twenty micrograms of total RNA from each plant was subjected to electrophoresis in 1% agarose gels prepared with MOP buffer (Huet et al. 1999). After electrophoresis, RNA was transferred to a Hybond N^+ hybridization membrane (Amersham Biosciences UK Limited, Little Chalfont, Bucks, UK) according to the manufacturer's instructions. The same probe described for Southern analysis was used for the Northern hybridization. The blotted membrane was probed in Denhart's hybridization solution and washed according to the manufacturer's instructions. Kodak BioMax MS film was used for autoradiography.

For RT-PCR analysis, total RNA was extracted from plant leaves using RNeasy® Plant Mini Kit (Qiagen Inc., CA, USA) following the manufacturer's instructions**.** During the process, RQ1 RNase-free DNase (Promega) was used to remove genomic DNA. Four hundred nanograms of total RNA from each plant was reverse transcribed with the reverse primer T4ER using AMV reverse transcriptase (Promega) at 42°C for 60 min. PCR was carried out with 2 μl of RT reaction product, and T4EF and T4ER primers for the T4 *e* gene. The expected size of the amplified fragment is 495 bp. Standard 35-cycle PCR reactions were performed using *AccuPower*® PCR Premix (Bioneer, Daejeon, Korea) with an annealing temperature of 50°C. PCR products were analyzed on a 0.9% agarose gel.

Plant inoculation test with *Magnaporthe grisea*

Leaf inoculation experiments were performed in a growth chamber in the NCSU Phytotron with *M. grisea*. Isolates 1213-59 and 533-78 of *M. grisea* from tall fescue (Tredway et al. 2003), were mixed and revived from –80°C storage by placing filter paper disks onto potato dextrose agar medium, amended with 50 mg l^{-1} each of tetracycline, streptomycin, and chloramphenicol. After 7 days culture at 25°C in the dark, each isolate was transferred to 1.5% Difco™ water agar medium overlaid with 12 sterilized alfalfa stem sections (6 cm long) for conidia production at room temperature under continuous fluorescence lighting. Two weeks later, conidia were harvested and the final concentration was adjusted to 2×10^5 conidia ml^{-1} using a hemacytometer under a light microscope (Tredway et al. 2003).

To avoid *R. solani* contamination, the fungicide Prostar® [*N*-{3-(1-methylethoxy)phenyl}-2-(trifluoromethyl) benzamide, Bayer Environmental Science, Montvale, NJ, USA] was applied to the test plants 4 days before inoculation, which has no activity against *M. grisea* infection (L.P. Tredway, personal observation). Each plant was inoculated with a spray of 7.5 ml conidia suspension using an airbrush sprayer (Badger Air-Brush Co., Franklin Park, IL, USA). For the first 24 h after inoculation, the plants were kept at 24°C, 100% relative humidity (RH), in the dark. Plants were subsequently incubated at 30°C with 70% RH at day time (12 h day^{-1}) in the lighted chamber with the same light intensity as described above, and at 24°C with 100% RH at night (12 h day^{-1}).

Disease development was evaluated 10–14 days after inoculation. Disease incidence was calculated as the percentage of the infected leaves in the number of the total leaves in a plant (Tredway et al. 2003). Lesion development was measured from ten representative leaves from each plant. The experiment was replicated two or three times.

Plant inoculation test with *Rhizoctonia solani*

Leaf inoculation experiments were performed in a growth chamber in the NCSU Phytotron with

R. solani Kuhn subgroup AG1 isolated from turfgrass. This is the most common AG of *R. solani* from tall fescue (Martin and Lucas 1984). The *R. solani* cultures were grown on potato dextrose agar (Becton, Dickinson and Company, Sparks, MD, USA) at room temperature for 3 days prior to use as inoculum. Four young leaves were arbitrarily chosen from each plant for inoculation. An agar plug (3 mm × 3 mm) infested with mycelium was placed on the midrib of each of the chosen leaves for each transgenic or control plant. After inoculation, plants were grown in a lighted growth chamber under a 14/10-h (day/night) photoperiod. The photosynthetic photon flux density was 600 μmol m^{-2} s^{-1} (400–700 nm) as measured at plant level from a combination of cool white fluorescent and incandescent lamps at an input wattage ratio of 10:3. The temperature and RH were 30°C and 70% during day time, and 24°C and 95% at night, respectively. After 14 days, disease was rated by measuring the total distance from the point of inoculation to the farthest point of the lesions extended. The inoculation experiment was replicated three times.

Statistical analysis

Analysis of variance for a randomized complete block design was carried out on the log-transformed lesion size data from brown patch inoculation test, and on the original lesion size and disease incidence data from gray leaf spot inoculation tests using SAS V. 9.2 (SAS Institute, Cary, NC, USA). CONTRAST statements were used to compare each of the transgenic plants to the non-transformed control plants or the mean of the control plants.

Results

The murein hydrolase (''lysozyme'') gene *e* was cloned from bacteriophage T4B by PCR. The nucleotide sequence of the coding region is identical to that published by Owen et al. (1983). A construct was made to have T4 gene *e* under control of the maize *Ubi1* gene promoter (Fig. 1). Four *Agrobacterium*-mediated transformation experiments were performed in which a total of 320 pieces of mature seed-derived calluses were infected (Dong and Qu 2005). Thirteen T_0 plants from independent transformation events were obtained using the construct (i.e., 4% of transformation efficiency). All of the plants were resistant to the selection agent hyg B in culture medium, looked normal, and grew vigorously in the greenhouse after transplanting.

Confirmation of T4 lysozyme transgenic tall fescue plants

Southern analysis was performed on the 13 putative transgenic plants and the transgenic nature of these plants was confirmed. Southern hybridization results of the six transgenic plants that showed resistance to the gray leaf spot in growth chamber tests, are shown in Fig. 2. The various sizes of the restricted gene *e* transgene fragments among the analyzed plants indicated stable integration of the transgenes at various loci in the tall fescue genome, and suggested they were from different transformation events. In contrast to transgenic tall fescue plants derived from microprojectile bombardment (Bai and Qu 2001), the transgenic plants obtained from *Agrobacterium*-mediated transformation in the experiments had simpler hybridization patterns and were estimated to have one to three transgene copies.

Inoculation test for gray leaf spot disease resistance

All T_0 transgenic plants were inoculated with the fungal pathogen *M. grisea* in a replicated growth chamber experiment. A conidia suspension of a mixture of two *M. grisea* isolates from tall fescue (1213-59 and 533-78, Tredway et al. 2003) was sprayed onto the plants, and plants were evaluated 10–14 days later. Six out of the 13 transgenic plants exhibited high resistance to gray leaf spot, as evidenced by greatly reduced lesion size and less disease incidence compared to the non-transformed control plants (Figs. 3a, 4). The six resistant plants are: Lys8a, 9a, 12b, 13a, 14a, and 18a. The lesion size on these plants were reduced by 74–90%. Statistical analysis indicated that the

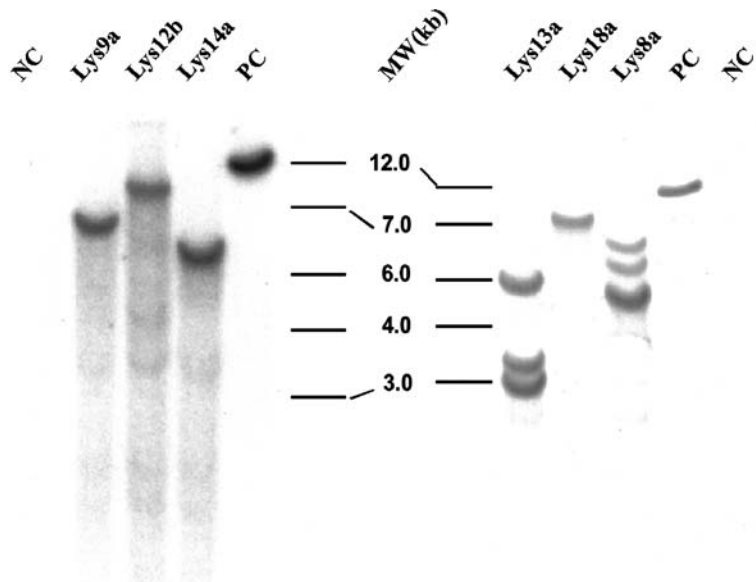

Fig. 2 Southern blot analysis of transgenic plants. T4 lysozyme coding sequence (gene *e*) was used as the probe. NC negative control, DNA from a non-transformed tall fescue plant; PC positive control and reconstruction: plasmid pSD9 DNA equivalent to one copy of T4 lysozyme gene in a 2C tall fescue genome, mixed with 25 μg non-transgenic plant DNA; putative transgenic plants transformed with the T4 phage lysozyme gene were labeled as Lys8a, 9a, 12b, 14a, 13a, and 18a; *MW* DNA molecular weight marker

lesion size reduction in all the six transgenic plants was highly significant ($P < 0.01$, Fig. 4a), suggesting that the invasion of tall fescue tissues by *M. grisea* was substantially suppressed in these transgenic plants. The disease incidence was also reduced in these plants, ranging from 15% (Lys18a) to 90% (Lys13a). The disease incidence reduction was highly significant ($P < 0.01$) in four transgenic plants (Lys8a, 9a, 13a, and 14a), indicating the infection process was inhibited as well in these plants (Fig. 4b).

Inoculation test for brown patch disease resistance

To test the effects of the T4 lysozyme transgene on brown patch development in tall fescue, T_0 transgenic plants were inoculated with agar plugs infested with mycelium of *R. solani*, subgroup AG-1-1a, obtained from an infected tall fescue (Martin and Lucas 1984). Experiments were conducted in a controlled environment chamber. Measurements of lesion size 2 weeks after inoculation revealed that lesion development was suppressed in three transgenic plants (Lys9a, 12b, and 14a) compared to the non-transformed control plants (Figs. 3b, 5). Lesion size was reduced in these plants by 68–84%. Based on the lesion size reduction, the resistance was scored as highly significant for transgenic plants Lys14a ($P = 0.0047$) and Lys9a ($P = 0.0063$), and significant for the transgenic plant Lys12b ($P = 0.0227$). These three plants were all resistant to gray leaf spot (*M. grisea*) infection (above), suggesting that these three plants (Lys9a, 12b, and 14a) were resistant to both fungal pathogens.

Expression analysis of the transgenic plants

Northern blot analyses were carried out to detect expression of the T4 lysozyme transgene among the resistant plants, and the results are shown in Fig. 6a. All five resistant transgenic plants (Lys8a was not tested) showed detectable transcript accumulation at the correct position (0.8 kb). However, unexpected RNAs higher and lower than the predicted transcript size also hybridized with the lysozyme gene probe in all the transgenic plants tested in an identical pattern. The control plant RNA did not hybridize with the probe (lane NC, Fig. 6a).

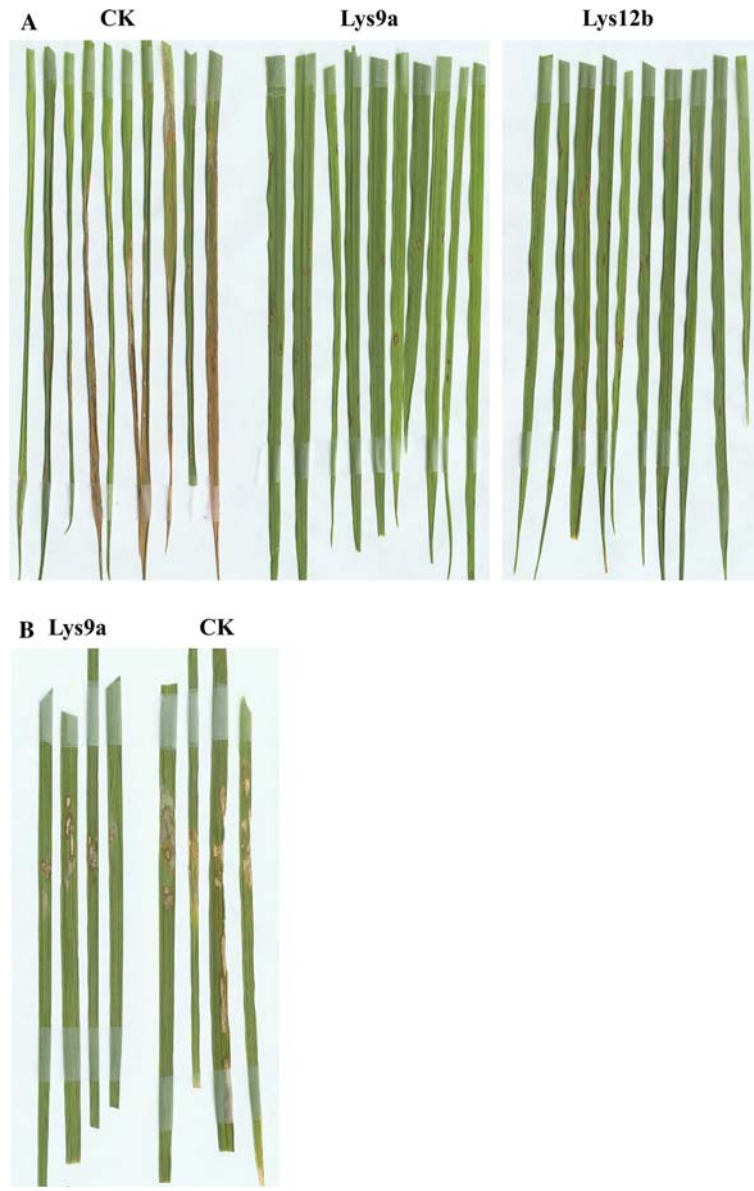

Fig. 3 Representative inoculation test results of the transgenic plants. **A** Leaves of two transgenic plants (Lys9a and 12b) and a control plant inoculated with *M. grisea*. **B** Leaves of Lys9a and a control plant (CK) inoculated with *R. solani*

To further confirm expression of T4 lysozyme gene *e* in transgenic plants, RT-PCR analysis was performed (Fig. 6b). The full coding sequence of T4 gene *e* (495 bp) was amplified using the RT cDNA product as template in all six resistant transgenic plants (Fig. 6b). No amplification was observed when PCR was carried out on the same set of RNA samples without reverse transcription (Fig. 6c), indicating no genomic DNA contamination in the RNA preparations.

Based on Fig. 6a, the expression level of T4 gene *e* among the transgenic plants varied a great deal. It seems that transgenic plants from a wide range of expression levels can be highly resistant to *M. grisea*. However, from Figs. 4, 5, and 6, no correlation between transgene expression and disease resistance can be established.

Discussion

Lysozymes exist widely in microorganisms and animals, and are generally considered to be anti-bacterial because of their 1,4-β-*N*-acetylmuramidase

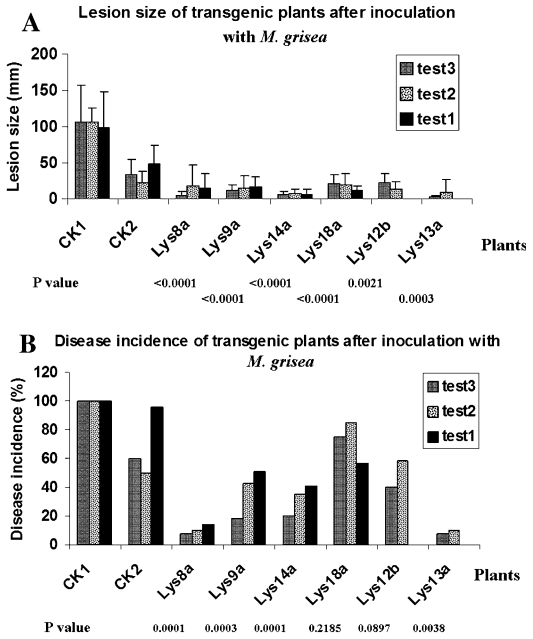

Fig. 4 Statistical analysis of *M. grisea* inoculation test on the transgenic plants. **A** Lesion size data of the six transgenic plants resistant to *M. grisea* in comparison with two non-transformed control plants (CK1 and CK2, cv. Coronado). **B** Disease incidence data of the above plants

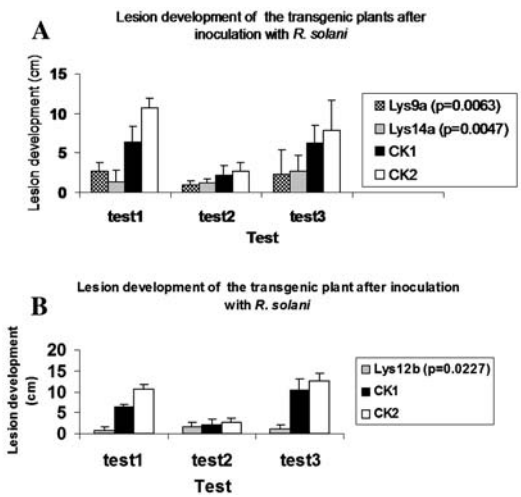

Fig. 5 Lesion development results of the transgenic plants (Lys9a, 12b, and 14a), and two non-transformed plants (CK1 and CK2, cv. Coronado) after inoculation with *R. solani*. Lesion size was determined 14 days after inoculation of leaves by measuring the total distance from the point of inoculation to the farthest point the lesions extended. Data in *panels A and B* were collected from separate experiments and thus analyzed separately

activities against peptidoglycan in bacterial cell walls. Based on their amino acid sequences, structures, and catalytic characters, distinct types of lysozymes were recognized (Jolles and Jolles 1984). Antifungal activity of T4 lysozyme was unexpectedly observed after completely abolishing the muramidase activity by heat treatment, and was attributed to the "membrane-disturbing" activity of its c-terminal domain (Düring et al. 1999). A similar phenomenon was observed with chicken egg white lysozyme although it does not share homology with the T4 lysozyme (Jolles and Jolles 1984). A couple of lysozyme genes have been used to engineer disease resistance in transgenic plants. For instance, the T4 lysozyme gene was reported to confer resistance to the phytopathogenic bacterium *Erwinia carotovora* in transgenic potato (Düring et al. 1993). Expression of T4 gene *e* in transgenic rice plants conferred resistance to rice blast, a fungal disease (Tian et al. 2002). Moreover, transgenic plants producing a human lysozyme, which shares homology with chicken egg white lysozyme (Jolles and Jolles 1984), were resistant to both fungal and bacterial diseases (Nakajima et al. 1997; Takaichi and Oeda 2000). In the experiments reported here, nearly half of the transgenic tall fescue plants showed greatly enhanced resistance to two turfgrass isolates of *M. grisea*. Moreover, three of these plants were also resistant to *R. solani*, implying that T4 lysozyme may have activity against a range of fungal pathogens. It remains to be seen how versatile T4 lysozyme is against fungal pathogens of plants. However, the results reported here clearly demonstrate that T4 lysozyme confers resistance to fungal diseases in transgenic plants, and may have a wide application on engineering fungal disease resistance in crops. Its resistance to *M. grisea* seems to be particularly potent. Although Düring et al. (1999) also reported the membrane-disturbing activity of T4 lysozyme against plant protoplasts, transgenic tall fescue plants with this gene did not show any abnormality in their growth.

Northern hybridization and RT-PCR analysis confirmed mRNA expression of the T4 lysozyme gene. Unexpected multiple bands were obtained in Northern hybridization assays, which may be attributed to transcription readthrough, RNA processing events, or alternative transcription start sites. Northern analysis does suggest that RNA expression levels varied among the trans-

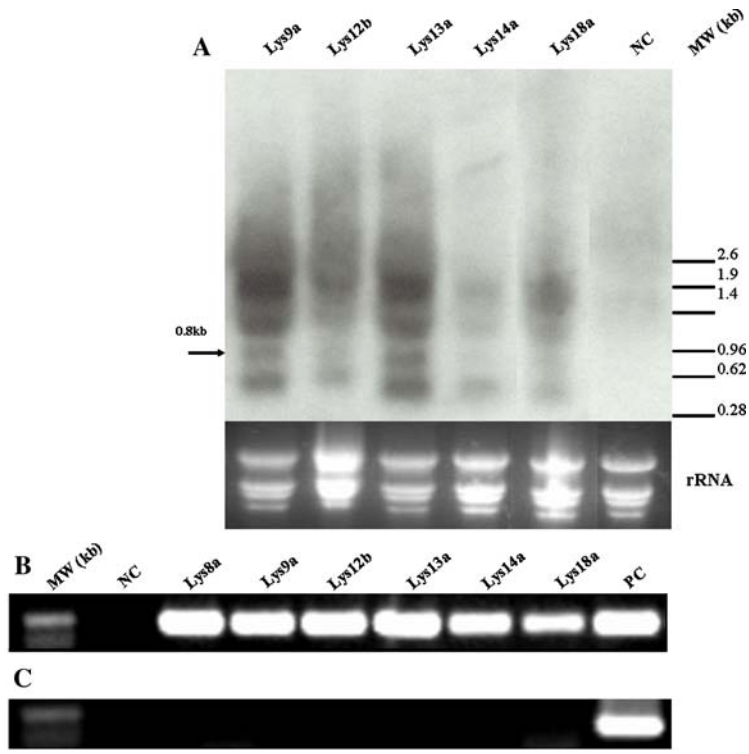

Fig. 6 Northern hybridization and RT-PCR analysis of transgenic plants. **A** Northern hybridization of total RNA from five transgenic plants using T4 lysozyme coding sequence as the probe. NC negative control, total RNA from a non-transformed tall fescue plant; MW RNA molecular weight marker. The expected 0.8-kb lysozyme mRNA was indicated. The rRNAs from EtBr stained gel is shown as a loading control in the *lower panel*. **B** RT-PCR analysis of six transgenic plants using T4EF and T4ER primers. RQ1 DNase was used to digest DNA; PC positive control, PCR using genomic DNA from transgenic plant Lys8a. MW DNA molecular weight marker. **C** Standard PCR analysis using plant RNAs after digestion with RQ1 DNase but without reverse transcription as a control to show no genomic DNA contamination of the RNA samples

genic plants analyzed, most likely due to position effects, even though these plants showed similarly reduced lesion sizes in gray leaf spot inoculation (Fig. 4a). There appears to be little correlation between the lysozyme RNA level and resistance to the two diseases. For example, Lys13a exhibited a relatively high expression level of the lysozyme transgene but did not show resistance to brown patch inoculation, whereas Lys12b and 14a showed apparently lower lysozyme gene expression, but still exhibited good resistance to both pathogens. In addition, the RNA expression level (Fig. 6a) does not seem to be correlated to the transgene copy number (Fig. 2).

It has been observed that some brown patch-susceptible cultivars, such as Coronado, exhibit improved resistance to gray leaf spot in the field (Fraser 1996). This inverse relationship was not obvious in our growth chamber inoculations. The control plants of Coronado developed severe symptoms in response to inoculation with either pathogen. The fact that three transgenic plants have resistance to both pathogens suggests that a biotechnological approach could be used to develop new cultivars with resistance to both brown patch and gray leaf spot, and possibly other fungal diseases.

No membrane-disturbing activity of T4 lysozyme was previously observed against mammalian cells (Düring et al. 1999), implying a level of safety with this gene in transgenic crops. The effects of T4 lysozyme-producing transgenic plants on the rhizosphere ecology of microorganisms were recently studied. In one study, the impact of transgenic plants on bacterial communities around the roots was found to be negligible

(Heuer et al. 2002). In another study, ''differences in the relative abundance of endophytic fungi colonizing the roots'' between transgenic plants and the control plants were observed (Gotz et al. 2006). More research may be needed to elucidate the impact that T4 lysozyme transgenic plants have on fungal rhizosphere communities. Nonetheless, resistance to important fungal pathogens in turfgrass is clearly demonstrated by this approach.

Acknowledgments The authors are grateful to CAMBIA (Canberra, ACT, Australia) for the binary vector used in the experiment, to Dr P. Quail for maize *Ubi1* promoter, C. Rose-Fricker for seed supply, and to the NCSU Phytotron for greenhouse and growth chamber facilities. This work was supported, in part, by the Center for Turfgrass Environmental Research and Education, NCSU, and North Carolina Turfgrass Foundation.

References

An G, Ebert R, Mitra A, Ha B (1988) Binary vectors. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, pp A3, 1–19

Anderson NA (1982) The genetics and pathology of *Rhizoctonia solani*. Annu Rev Phytopathol 20: 329–347

Bai Y, Qu R (2001) Genetic transformation of elite turf-type cultivar of tall fescue. Int Turfgrass Soc Res J 9:129–136

Broglie KE, Chet I, Holliday M, Cressman R, Biddle O, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Buckner R, Powell J, Frakes R (1979) Historical development. In: Buckner R, Bush LP (eds) Tall fescue. Agronomy monograph #20. American Society of Agronomy, Madison, pp 1–8

Carlson K, Miller ES (1994) General procedures. In: Karam JD (ed) Molecular biology of bacteriophage T4. American Society for Microbiology Press, Washington, pp 431–432

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes—structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Curley J, Sim SC, Warnke S, Leong S, Barker R (2005) QTL mapping of resistance to gray leaf spot in ryegrass. Theor Appl Genet 111:1107–1117

Dean RA, Talbot NJ, Ebbole DJ et al (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 134:980–986

DeGray G, Rajasekaran K, Smith F, Sanford J, Daniel H (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol 127:852–862

Dong S, Qu R (2005) High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. Plant Sci 186:1453–1458

Düring K, Porsch P, Fladung M, Lorz H (1993) Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. Plant J 3:587–598

Düring K, Porsch P, Mahn A, Brinkmann O, Gieffers W (1999) The non-enzymatic microbicidal activity of lysozymes. FEBS Lett 449:93–100

Fraser ML (1996) Susceptibility of tall fescue to gray leaf spot, 1995. Biol Cult Test 12:130

Fu AL, Tisserat NA, Xiao YM, Settle D, Muthukrishnan S, Liang GH (2005) Overexpression of rice TLPD34 enhances dollar-spot resistance in transgenic bentgrass. Plant Sci 168:671–680

Gotz M, Nirenberg H, Krause S, Wolters H, Draeger S, Buchner A, Lottmann J, Berg G, Smalla K (2006) Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods. FEMS Microbiol Ecol 58:404–413

Guo ZF, Bonos S, Meyer WA, Day PR, Belanger FC (2003) Transgenic creeping bentgrass with delayed dollar spot symptom. Mol Breed 11:95–101

Han YH, Bonos SA, Clarke BB, Meyer WA (2006) Inheritance of resistance to gray leaf spot disease in perennial ryegrass. Crop Sci 46:1143–1148

Heuer H, Kroppenstedt RM, Lottmann J, Berg G, Smalla K (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere relative to communities are negligible natural factors. Appl Environ Microbiol 68:1325–1335

Huet H, Mahendra S, Wang J, Sivamani E, Ong A, Chen L, deKochko A, Beachy RN, Fauquet C (1999) Near immunity to rice tungro spherical virus achieved in rice by a replicase-mediated resistance strategy. Phytopathology 89:1022–1027

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Mass C (1995) Enhanced quantitative resistance against fungal diseases by combinational expression of different barley antifungal proteins in transgenic tobacco. Plant J 8:97–109

Jolles P, Jolles J (1984) What is new in lysozyme research? Mol Cell Biochem 63:165–189

Jongedijk E, Tigelaar H, Van Roekel JSC, Bres-Vloemans SA, Dekker I, Van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173–180

Martin SB, Lucas LT (1984) Characterization and pathogenicity of *Rhizoctonia* spp. and binucleate *Rhizoctonia*-like fungi from turfgrasses in North Carolina. Phytopathology 74:170–175

Miller ES, Carlson K (1994) Characterization and analysis of T4 DNA. In: Karam JD (ed) Molecular biology of bacteriophage T4. American Society for Microbiology Press, Washington, p 455

Nakajima H, Muranaka T, Ishige F, Akutsu K, Oeda K (1997) Fungal and bacterial disease resistance in transgenic plants expressing human lysozyme. Plant Cell Rep 16:674–679

Owen JE, Schultz AW, Taylor A, Smith G (1983) Nucleotide sequence of the lysozyme gene of bacteriophage T4: analysis of mutations involving repeated sequences. J Mol Biol 165:229–248

Qu SH, Liu GF, Zhou B, Bellizzi M, Zeng L, Dai LY, Han B, Wang GL (2006) The broad-spectrum blast resistance gene *Pi9* encodes an NBS-LRR protein and is a member of a multigene family in rice. Genetics 172:1901–1914

Rose-Fricker CA, Fraser ML, Meyer WA, Funk CR (1999) Registration of 'Coronado' tall fescue. Crop Sci 39:288

Smiley RW, Dernoeden PH, Clarke BB (2005) Compendium of turfgrass diseases. The American Phytopathological Society Press, St Paul

Takaichi M, Oeda K (2000) Transgenic carrots with enhanced resistance against two major pathogens, *Erysiphe heraclei* and *Alternaria dauci*. Plant Sci 153:135–144

Talbot NJ (2003) On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. Annu Rev Microbiol 57:177–202

Tian WZ, Yang M, Ding YY, Ding L, Cao SY, Ye SQ, Tang ZS, Li LC, Ling ZZ, Zhang Q, Hain R (2002) Rice transformation with lysozyme gene and bioassay of the transgenic plants and their progenies. In: Future of agriculture—research on transgene technology (in Chinese). Hunan Science & Technology Press, Changsha, pp 191–197

Tredway LP, Stevenson KL, Burpee LL (2003) Components of resistance to *Magnaporthe grisea* in 'Coyote' and 'Coronado' tall fescue. Plant Dis 87:906–912

Trevathan LE, Moss MA, Blasingame D (1994) Ryegrass blast. Plant Dis 78:113–117

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotechnology 12:807–812