ABSTRACT

DONG, SHUJIE. Agrobacterium-mediated transformation of tall fescue (Festuca arundinacea Schreb.) for fungal disease resistance. (Under the direction of Dr. Rongda Qu).

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season turf and forage grass species of great economic importance. It is a major turfgrass species for home lawns in North Carolina and the transition zone states in the US mostly because of its tolerance to the summer heat. Brown patch disease is the most serious and frequently occurring disease of tall fescue, caused by a basidiomycete fungus, *Rhizoctonia solani* (Kuhn). Gray leaf spot, induced by *Pyricularia grisea* (Cooke) Sacc., the asexual stage of *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, is becoming another important disease of tall fescue. The objectives of this research were to establish an efficient transformation system for tall fescue using *Agrobacterium*; to introduce disease resistance genes into tall fescue; and to study the effects of the transgenes in resistance to the two major fungal diseases.

An efficient genetic transformation system, using *Agrobacterium tumefaciens*-mediated T-DNA delivery, was developed for tall fescue. Thirty four percent of the calli infected with *A. tumefaciens* were resistant to hygromycin B, the selection agent used to select/identify transformants, and the overall plant transformation frequency (the number of independently transformed plants over the number of calli infected) was about 8%. Southern analysis indicated the integration of transgenes into the plant nuclear genome and simple transgene copy patterns. The high efficiency observed was partly due to an elevated 2,4-D concentration (5 mg L^{-1}) in the culture medium used during callus culture

and co-cultivation. Inheritance studies revealed that the transgenes were transmitted to the progenies.

Four genes with potential for fungal disease resistance, including: alfalfa β-1,3 glucanase *AGLU1*, T4 phage lysozyme, frog dermaseptin, and rice *Pi9* genes, were introduced into two cultivars 'Coronado' and 'Matador' of tall fescue through *Agrobacterium*-mediated transformation. Of 29 T0 transgenic plants examined, six had a higher level of resistance to *R. solani* and 13 had enhanced resistance to *P. grisea*. The enhanced levels of resistance in most of these plants were highly significant. Five transgenic plants exhibited enhanced resistance to both fungal pathogens.

AGROBACTERIUM-MEDIATED TRANSFORMATION OF TALL FESCUE (FESTUCA ARUNDINACEA SCHREB.) FOR FUNGAL DISEASE RESISTANCE

By

Shujie Dong

A dissertation submitted to the Graduate faculty of
North Carolina State University
In partial fulfillment of the
Requirement for the Degree of
Doctor of Philosophy

CROP SCIENCE

Raleigh

2006

APPROVED BY

Dr. Rongda Qu
Chair of Advisory Committee

Dr. Richard J. Cooper **Dr. Ralph Dewey**

Dr. Melodee Fraser **Dr. David Shew**

BIOGRAPHY

Shujie Dong was born in Qingdao, a beautiful port city in eastern China. Since very young, she was fascinated with science, especially biology.

She obtained her Bachelor of Science degree with a major in biology education at Shandong Normal University in 1998. After realizing that her interest is in plant kingdom, she entered the Institute of Botany, Chinese Academy of Sciences, as a graduate student and received a Master of Science degree majoring in plant cell and molecular biology in 2001.

Anxious to learn more about crop science, she applied and was admitted as a Ph.D. student into Dr. Rongda Qu's laboratory at North Carolina State University in 2001, and started her study on turfgrass biotechnology.

ACKNOWLEDGEMENTS

I am very grateful to my major advisor Dr. Rongda Qu for his guidance throughout the last five years. I not only learned how to do research professionally, but also how to do right things in my life, which is never mentioned in any books or journals. I cannot imagine how hard it would be for me to successfully complete my degree without his leadership.

I would like to thank other committee members, Drs. Rich Cooper, Ralph Dewey, Melodee Fraser, and David Shew. Their support and encouragement were essential to the successful completion of this dissertation. I am grateful to Dr. Cooper for his help in turfgrass management study, to Dr. Dewey for his support in molecular biology throughout my Ph.D. study, to Dr. Fraser for supplying me with tall fescue seeds and discussions on turf breeding-related issues, and Dr. Shew for his guidance and help in brown patch inoculation test.

I would also like to thank Dr. Lane Tredway for his guidance in my gray leaf spot inoculation test, and Dr. Cavell Brownie for guidance and assistance in statistical analysis. I would like to thank Drs. E. Sivamani and K. Azhakanandam for technical discussions and generous help, Dr. Joe Burns for providing alfalfa stems, Ms. Penny Brenda for patiently setting the growth chamber conditions for inoculation tests. I would also like to thank Jianli Lu and Dr. G. L. Wang for the T4 lysozyme and the rice Pi9 gene clones, and NCSU Phytotron for the support in using the facilities.

I am also very thankful to all other members in our lab, especially Tan Tuong, who was there whenever I needed his help.

Finally, I would like to thank my family for their support and love. I would like to thank my 3 month old son, Andrew. His coming brought indefinite laughter and happiness to my family. I am profoundly grateful to my dear husband, Zhengyu Yang, for his love and support during the period of my Ph.D. study.

TABLE OF CONTENTS

LIST OF TABLES

Page

CHAPTER I

CHAPTER II

LIST OF FIGURES

Page

CHAPTER I

CHAPTER II

INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season grass species that is adapted to various soil and climatic conditions in temperate zones and has been widely used as a forage grass and a turfgrass worldwide (Buckner et al., 1979). It is a major turfgrass species for home lawns in North Carolina and the U.S. transition zone mostly because of its tolerance to summer heat and drought. A major problem in growing tall fescuce in this region is its susceptibility to fungal diseases. Traditional breeding has contributed a great deal during the past 30 years to improved color, texture, and disease tolerance for tall fescue and has produced many new commercially available cultivars in the market and continues to be the major means used for tall fescue improvement. Genetic engineering, on the other hand, will allow us to manipulate endogenous genes and to introduce gene fragments from other organisms, thus expanding the germplasm pool that may make conventional breeding efforts more efficient and fruitful. A major goal of this research project was to introduce disease resistance genes from various sources into tall fescue using a biotechnological approach.

Successful transformation of tall fescue was initially accomplished using protoplasts method. Either electroporation (Wang et al., 1992; Dalton et al., 1995) or polyethylene glycol (PEG) (Ha et al., 1992) was used to introduce DNA into tall fescue cells. Later, microprojectile bombardment was used to transform tall fescue using embryogenic calli or suspension cells (Spangenberg et al., 1995; Cho et al., 2000; Bai and Qu, 2001; Wang et al., 2001).

When we started this project, there were no reports regarding

Agrobacterium-mediated transformation of tall fescue. *Agrobacterium*-mediated transformation has some obvious advantages over other methods, including simplicity, lower cost, and lower transgene copies integrated. Therefore, establishment of a high frequency transformation system using *Agrobacterium* would greatly facilitate the efforts for tall fescue improvement through genetic engineering. The objectives of this project were to develop an efficient *Agrobacterium*-mediated transformation system for tall fescue; to introduce disease resistance genes; and to investigate the effects of the transgenes in resistance to two major fungal diseases: brown patch caused by *Rhizoctonia solani* Kuhn and gray leaf spot caused by *Pyricularia grisea* (Cooke) Sacc.

LITERATURE REVIEW

Use of *Agrobacterium tumefaciens* for plant transformation

The phytopathogenic soilborne bacterium *Agrobacterium tumefaciens* causes crown gall in a wide range of broad-leaf plants (Nester et al., 1984). *Agrobacterium tumefaciens* has the unique ability to genetically transfer a defined fragment of its Ti (Tumor inducing) plasmid, the T-DNA (transfer DNA), into plant nuclear genomes. *A. tumefaciens* naturally infects many dicot plant species but only a very limited number of monocot species. *Agrobacterium* infects plants through wounds. Chemical signals released from the wounded tissue activate a series of *Vir* genes and, in turn, initiate the infection process. When used as a vector to deliver DNA into plant cells, the efficiency of *Agrobacterium*-mediated transformation varies a great deal and could be affected by various factors, including plant species, explants, medium, antibiotics, *Agrobacterium* strains, co-cultivation, and selection.

Agrobacterium strains LBA4404, EHA101, EHA105, and AGL1 are the most widely used strains for plant transformation. LBA4404 harbors the disarmed Ti plasmid pAL4404 with chromosomal background TiAch5 (Hellens et al., 2000). Supervirulent strain EHA101 is an A281 derivative harboring pEHA101. Supervirulent strain EHA105 is a derivative of EHA101, containing plasmid pEHA105, a T-DNA deletion derivative of the supervirulent plasmid pTiBo542. *Agrobacterium* strain AGL1 with C58 background also carries plasmid pTiBo542, on which the T-DNA is deleted (Lazo et al., 1991).

Significant enhancement of the T-DNA transfer and integration frequency has been achieved by an increase in the expression level of *VirG* and *VirE* genes (Hellens et

al., 2000). Plasmid pTOK47 increased the virulence of *Agrobacterium* strains due to the possession of an additional copy of the *VirB*, *VirC*, and *VirG* genes on the Ti plasmid (Le et al., 2001; Tang, 2003).

Various methods have been developed to overcome the barriers of *Agrobacterium*-mediated plant transformation involving host specificity, such as sonication, addition of acetosyringone (a naturally occurring phenolic compound to induce expression of the *Vir* genes), or the addition of antioxidants to the co-culture medium. Trick and Finer (1997) enhanced T-DNA transfer using sonication–assisted *Agrobacterium*-mediated transformation (SAAT). It was also reported that antioxidants, such as polyvinylpyrrolidone, dithiothreitol, or L-cysteine favored stable transgenic events during explant germination, preculture, and infection of rice (Enriquez-Obregon et al., 1999), or during and after co-cultivation of grape (Perl et al., 1996).

Monocotyledonous plants are generally difficult to transform using *Agrobacterium*, because most monocot species are not natural hosts of the bacterium. Efficient *Agrobacterium*-mediated transformation of monocot plants was first demonstrated in rice (Hiei et al., 1994). The key factors for this success were a 'super-binary' vector, pTOK233, which included an extra copy of the *VirB, VirC, and VirG* genes, the addition of acetosyringone to the co-cultivation medium, and the use of actively dividing embryonic cells. A*grobacterium*-mediated transformation was also reported in other important monocot crops. Maize immature embryos were efficiently transformed using *Agrobacterium* strains containing the super-binary vector, pTOK233 (Ishida et al., 1996). Zhao et al. (2002) optimized the transformation conditions based on Ishida's protocol and obtained an average of 40% stable callus transformation frequency.

The optimized conditions included the use of N6 medium for infection and co-cultivation, introduction of a resting step before selection, the use of AgNO3 in key steps, optimized *Agrobacterium* concentration for infection, and the choice of the antibiotics for suppressing *Agrobacterium* growth afterwards. Cheng et al. (1997) developed a rapid *Agrobacterium*-mediated transformation system for wheat, in which various factors were evaluated like explants and addition of surfactant in the inoculation medium. Years later, a more efficient (average transformation frequency of 4.4 %) and large-scale *Agrobacterium*-mediated wheat transformation system was developed through the use of the super-binary vector, addition of polyamine in the regeneration medium, and glyphosate selection (Hu et al., 2003; Khanna et al., 2003). Polyamine-supplemented medium helped the recovery of high numbers of transformants. Efficient *Agrobacterium*-mediated transformation of barley using the strain AGL1 was reported in 1997 (Tingay et al., 1997). Zhao et al. (2000) developed an efficient *Agrobacterium*-mediated transformation system for sorghum and showed that the embryos from the field had higher transformation frequency than those from the greenhouse.

Some monosaccharides, such as L-arabinose and D-xylose, can greatly increase acetosyringone-dependent expression of the *Vir* genes when acetosyringone is not sufficient to induce the expression of the genes (Shimoda et al., 1990). The addition of antioxidants also enhanced *Agrobacterium* mediated transformation frequencies of some important monocot species. For example, addition of L-cysteine to the co-cultivation medium resulted in maize transformation by *Agrobacterium* using a standard binary vector without the addition of a super-binary vector (Frame et al., 2002). The presence of

L-cysteine was thought to reduce cell death caused by the hypersensitive reaction of maize cells to *Agrobacterium* infection (Olhoft and Somers, 2001; Frame et al., 2002**).**

Recently, *Agrobacterium*-mediated transformation of turfgrasses, such as creeping bentgrass (Yu et al., 2000), Italian ryegrass (Bettany et al., 2003), and tall fescue (Wang and Ge, 2005; Dong and Qu, 2005) were reported. We developed a high-frequency *Agrobacterium*-mediated transformation system for tall fescue. A stable callus transformation frequency of 34% and an overall plant transformation frequency (the number of independently transformed plants over the number of calli infected) of 8% were observed. Our results indicated that a higher 2,4-D (5 mg L^{-1}) concentration in the culture medium played an important role in the high transformation efficiency.

Engineered disease resistance in plants through biotechnology

Crop losses caused by plant pathogens, insect pests, and weeds, have reached 42% (of total crop losses by all factors) worldwide and $26 billion annually are spent for pest management (Oerke et al., 1994).

Host resistance is the most economical and potentially the most effective way to manage diseases in plants. Two types of resistance have been observed: vertical (or single dominant resistance) and horizontal resistance (or multiple gene resistance). Vertical resistance is a complete resistance, which places strong selection pressure on a pathogen. The pathogen often adapts by developing new races through DNA mutation; therefore, a complete resistance usually is not stable. Horizontal resistance is more stable because a pathogen is only exposed to a weak selection pressure, but the resistance is partial. The long-sought goal of plant breeding is to have durable disease resistance in crops, which remains effective over a long period of time and in a large growing area (Johnson, 1993).

During evolution, plants developed defense systems, which involve various genes and pathways, for their survival upon pathogen attacks. However, to many pathogens, host plants do not have the corresponding genes to provide high levels of resistance. Thus, introduction of disease resistance genes from external sources into susceptible plant species by biotechnology may become an efficient way to manage disease, and could be of great economic importance. The potential genes for disease resistance could include plant defense-related genes, pathogen-derived genes, genes coding for antimicrobial proteins/peptides, and genes of plant-expressed antibodies (plantibodies).

Use of plant defense genes for disease resistance

Plants have specific resistance (R) genes for certain pathogens. These genes usually confer vertical resistance and play an important role in plant defense against pathogens. Flor (1955, 1971) was the first to propose the gene-for-gene hypothesis and existence of the plant R genes. The hypothesis stated that, for each gene conferring resistance in the host, there is a corresponding avirulence gene in the parasite with which it specifically interacts. This concept is fundamental to understanding the interation between plants and pathogens, and provides a useful theory for disease management. Resistance occurs only if there is a specific recognition between the resistance gene product and an avirulence gene product, which triggers the cascade of events in plant cells leading to the active defense response. Isolation and characterization of many R genes and their ligands (elicitors from the pathogens) greatly help our understanding of the plant defense mechanism. So far, based on common structural motifs, five classes of plant R proteins have been discovered (Martin et al., 2003). Most R genes encode intracellular proteins with leucine-rich repeats (LRR) (Gregory et al., 2003). The LRR

motifs were demonstrated to be involved in protein-protein or receptor-ligand interactions, and are considered to determine R gene specificity. Another group of R proteins have a TM (transmembrane) domain and an extracellular LRR domain (Dixon et al., 1996; Jones et al., 1994), such as the rice Xa21 gene product (Song et al., 1995). Some R proteins do not belong to any of these classes; an example is the toxin reductase *Hm1* gene product from maize (Johal et al., 1992).

Sometimes, in response to pathogen invasion, an R gene triggers a rapid cell death, which is called the hypersensitive response or programmed cell death (Dangl et al., 1996; Pennell et al., 1997). The rapid cell death slows down or completely stops the growth of the pathogen.

R genes have been used in plant biotechnology-based strategies for engineering disease resistance. Expression of the *Xa-21* gene in rice showed increased resistance to *Xanthomonas oryzae pv. oryzae (Xoo*) (Century et al., 1999). The tomato *Cf-9* R gene was reported to confer resistance to *Cladosporium fulvum* (Jones et al., 1994). Functional expression of *cf-9* and *AvrCf-9* genes in *Brassica napus* resulted in enhanced resistance to *Leptosphaeria maculans* (Hennin et al., 2001).

In addition to R genes, plants have other defense-associated genes, including PR (pathogenesis related) genes, the *NPR1* (Non-expresser of PR proteins) gene, genes encoding plant defensins, and genes related to the biosynthesis of phytoalexins, compounds plants produce for its defense.

The PR proteins are plant proteins that accumulate after pathogen attacks. They were first described in the 1970s in tobacco leaves infected with tobacco mosaic virus (TMV). Several PR proteins including PR1, β-1,3-glucanase (PR2), chitinase (PR3), PR4,

and osmotin, possess antimicrobial activities *in vitro* (Honee et al., 1999).

Glucans are a major component of fungal cell walls. The most abundant plant glucanases for host defenses are the β-1, 3-glucanases, which play a role in plant defense by attacking fungal cell walls, and are referred to as PR2 proteins. Plant chitinases catalyze degradation of chitin, another major cell wall component of pathogens, such as fungi and bacteria, and are classified as PR3 proteins (Theis et al., 2004). The inhibitory mechanisms of chitinase on pathogen growth are not always due to degradation of chitin, but probably also by its binding to the chitin of the fungal cell wall (Theis et al., 2004). Chitin-binding proteins interfere with fungal growth because of their affinity to nascent chitin, which leads to severe morphological changes in fungi (Bormann et al., 1999; Nielsen et al., 1997). Plant PR genes have been used to transform plants for improved disease resistance. A bean vacuolar chitinase gene enhanced resistance against *R. solani* in tobacco and *Brasica napus* (Broglie et al., 1991). An endochitinase from the mycoparasitic fungus *Trichoderma* conferred improved resistance in tobacco and potato to various pathogens, including *Alternaria alternate*, *A. solani*, *Botrytis cinerea*, and *R. solani* (Lorito et al., 1998). Co-expression of a rice chitinase and an alfalfa glucanase gene in transgenic tobacco showed additive protective effects against fungal attack (Zhu et al., 1994).

The *NPR1* gene was identified in *Arabidopsis* through genetic screening for SAR (systemic acquired resistance)-compromised mutants (Cao et al., 1997). The *NPR1* gene encodes a protein with a BTB/BOZ domain and an ankyrin-repeat domain, both of which mediate protein-protein interactions. *NPR1* mutants do not respond well to various SAR treatments. Over-expression of *NPR1* in transgenic *Arabidopsis thaliana* enhanced the

resistance level of plants against various pathogens by activating the SAR pathway (Cao et al., 1998).

Phytoalexins are low-molecular weight antimicrobial compounds that accumulate in plants in response to pathogen infections. The expression of stilbene synthase from grapevine in tobacco resulted in production of a phytoalexin, resveratrol, which inhibited growth of the gray mold pathogen *B. cinerea* (Hain et al., 1993).

Plant defensins are small, basic peptides with a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines (Thomma et al., 2002). Most plant defensins were found to have antifungal activity. Experiments indicated that there is a very specific recognition mechanism between the plant defensin and its fungal target site (Thomma et al., 2002). The precise mode of action of plant defensins is not well understood. Studies showed that introduction of defensin genes into plants enhanced protection against fungal attack. Constitutive expression of a radish defensin increased resistance of tobacco and tomato plants against some fungal pathogens (Terras et al., 1995; Parashina et al., 2000). Protection against the fungus *Verticillium dahliae* was demonstrated in field conditions by constitutive expression of an alfalfa defensin in potato (Gao et al., 2000).

Recently, mechanisms related to non-host resistance in plants to pathogens have become better understood. Some of the genes involved in the mechanisms may be very valuable for engineering pathogen resistance in plants in the future (Lipka et al., 2005).

Use of pathogen derived genes for plant disease resistance

Diseases caused by plant viruses are generally difficult to manage. Plant-derived virus resistance genes are often unavailable. Sanford and Johnston (1985) proposed the

use of pathogen genes for disease resistance. The rationale was: modification of the host genes may interfere with the development and reproduction of the plants. But expression of pathogen genes would not be expected to be detrimental to the host plant. Pathogen-derived resistance is mediated either by the protein encoded by the transgene (protein-mediated) or by the transcript produced from the transgene (RNA-mediated). The explanation for the resistance is co-suppression: overproducing one of the important virus genes blocks the reproduction of virus.

Virus coat protein genes, replicase genes, and movement protein genes are often used to achieve pathogen-derived resistance through genetic engineering. Coat protein-mediated resistance has been reported for tobacco mosaic virus (Abel et al., 1986), tomato mosaic virus (Sanders et al., 1992), and many other viruses. Transgenic papaya resistant to papaya ringspot potyvirus (PRSV) was commercialized in 1998, and saved the papaya industry in Hawaii (Lius et al., 1997). RNA interference (RNAi) is a novel cellular mechanism mediated by double-stranded small interfering RNA (siRNA) (Watson et al., 2005), and is proposed as the principal mechanism of engineered virus resistance in plants. This phenomenon is also referred to as virus induced gene silencing (VIGS).

Use of other antimicrobial proteins for disease resistance

Fungi, insects, animals, and humans all possess genes encoding antimicrobial proteins or peptides. Many antimicrobial proteins/peptides have been identified and some have been introduced into plants for enhanced disease resistance.

Small antimicrobial peptides can be engineered into plants for disease resistance. Typically, these antimicrobial peptides permeabilize the cell membrane or cause osmotic

shock to the pathogens (Nissen-Meyer et al., 1997). Transgenic poplar (*Populus tremula* L. *x P. alba* L.) plants expressing a synthetic antimicrobial peptide D4E1 showed a significant reduction in symptoms caused by *A. tumefaciens* and *X. populi* (Mentag et al., 2003). Temporins and dermaseptins are small antimicrobial peptides originally isolated from frog skin. Temporins (Simmaco et al., 1996) are active mostly against Gram-positive bacteria, but also show activities against some Gram-negative bacteria and the fungi *Candida albicans* and *Batrachochytrium dendrobatidis* (Wade et al., 2001; Rollins-Smith et al., 2003). Dermaseptins and their truncated analogs were cytolytic to bacteria, yeast, filamentous fungi, and protozoa (Mor and Nicolas, 1994) although the mode of action is not understood.

Ribosome-inactivating proteins (RIPs) from various sources are also used to transform plants for enhanced disease resistance. RIPs are toxic N-glycosidases that cleave a highly conserved sequence of the 28S rRNA (Endo et al., 1982; Theis et al., 2004). Ectopic expression of a barley type I RIP in tobacco conferred resistance to *R. solani* (Logemann et al., 1992)

Lysozymes exist widely in microorganisms, animals, and plants, and function by lysing of bacterial pathogens. Transgenic potato and apple plants expressing a T4 phage lysozyme gene were resistant to the bacterium *Erwinia carotovora* (During et al., 1993; Ko et al., 2002). Engineered resistance of lysozyme genes against fungal pathogens have also been reported. Expression of a human lysozyme gene in tobacco increased resistance against the fungus *Erysiphe cichoracearum* (Nakajima et al., 1997). The T4 lysozyme gene was introduced into rice and conferred enhanced resistance against the fungal pathogen causing rice blast disease (*Magnaporthe grisea*) and a bacterial pathogen

(*Xanthomonas oryzae pv. oryzae*) causing bacterial blight disease (Tian et al., 2002).

The use of plantibodies for disease resistance

Plantibody refers to an antibody produced by a genetically modified plant. The expression of viral- or nematode-specific antibodies *in planta* provides a new avenue for pest management. This method relies on cloning of the coding sequences of the light (VL) and heavy (VH) chains of an antibody molecule. Plantibodies are accumulated and assembled inside cells of transgenic plants, and specifically interact with the invading pathogen and interfere with its biological function (Zhang and Wu, 1998). This strategy has been used for control of tomato spotted wilt virus (TSWV) and root-knot nematodes *Meloidogyne* spp (Baum et al., 1996).

Future perspectives

Engineering defense-related genes into plants is an efficient way to obtain or enhance disease resistance in plants. Many such genes have been isolated, characterized, and introduced into plants. But, so far, no transgenic crops resistant to bacterial and fungal diseases have been commercialized, mainly due to an insufficient expression level of the transgenes or a high physiological cost to the plants (Hulbert et al., 2001). Therefore, enhancing transgene expression levels is an important factor for engineered plant disease resistance to succeed in a field environment.

In addition, single gene mediated disease resistance is usually unstable and tends to be overcome by more adapted races of the pathogen. Development of durable disease resistance against a pathogen is one of the major goals for plant breeders. Since durable resistance is usually a quantitative trait and controlled by multiple genes, a possible

approach is to introduce several different resistance genes into plants through genetic engineering. The resultant plants are expected not only to acquire a longer-lasting resistance, but also to have additive or synergistic effects on disease resistance.

References

Abel, P.P., Nelson R.S., De B., Hoffman N., Rogers S.G., Fraley R.T., Beachy R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-43.

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

Baum, T.J., Hiatt, A, Parrott, W.A., Pratt, L.H., Hussey R.S. 1996. Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode. Mol. Plant Microbe. In., 9:382-387.

Bettany A.J.E., Dalton S.J., Timms E., Manderyck B., Dhanoa M.S. Morris P. 2003. *Agrobacterium tumefaciens*-mediated transformation of *Festuca arundinacea* (Schreb.) and *Lolium multiflorum* (Lam.). Plant Cell Rep. 21 (5): 437-444.

Bormann C., Baier D., Horr I., Raps C., Berger J., Jung G. et al. 1999. Characterization of a novel , antifungal, chitin-binding protein from Streptomyces tendae Tu901 that interferes with growth polarity. J. Bacteriol. 181: 7421-7429.

Broglie K., Dhet I., Holliday M. et al. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science. 254: 1194-1197.

Buckner R.C., Powell J.B., Frakes R.V. 1979. Historical development p.1-8. In: Buckner R.C., and L.P.Bush (ed) Tall fescue. Agronomy monograph # 20, Am. Soc of Agronomy, WI.

Cao H., Glazebrook J., Clarke J.D., Volko S., Dong X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell. 88(1): 57-63.

Cao, H., Li X., Dong X., 1998. Generation of broad-spectrum disease resistance by overepression of an essential regulatory gene in systemic acquired resistance. Proc. of the Nat. Acad. of Sci., USA, 95:6531-6 536.

Century K.S., Lagman R.A., Adkisson M., Morlan J., Tobias R., Schwartz K., Smith A., Love J., Ronald P.C., Whalen M.C. 1999. Developmental control of Xa21-mediated disease resistance in rice. Plant J. 20 (2): 231-236.

Cheng M., Fry J.E., Pang S.Z., Zhou H.P., Hironaka C.M., Duncan D.R., Conner T.W., Wan Y.C. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115 (3): 971-980.

Cho M.J., Ha C.D., Lemaux P.G. 2000. Production of transgenic tall fescue and red fescue plants by particle bombardment of highly regenerated tissues. Plant Cell Rep. 19: 1084-1089.

Dalton S.J., Bettany A.J.E., Timms E., Morris P. 1995. The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca-arundinacea* Schreb). Plant Sci. 108 (1): 63-70.

Dangl J.L., Dietrich R.A., Richberg M.H. 1996. Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell. 8: 1793-1807.

Dixon M.S., Jones D.A., Keddie J.S., Thomas C.M., Harrison K., Jone J.D.G. 1996. The tomato *cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell. 84: 451-459.

Dong S.J., Qu R.D. 2005 High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. Plant Sci. 168(6): 1453-1458.

During K., Porsch P., Fladung M., Lorz H. 1993. Transgenic potato plants resistant to the phytopathogenic bacterium Erwinia-carotovora. Plant J. 3(4): 587-598.

Endo Y., Wool I.G. 1982. The site of action of α-sarcin cleavage site in 28S ribosomal ribonucleic acid. J. Biol. Chem. 257: 9054-9060.

Enriquez-Obregon G.A., Prieto-Samsonov D.L., de la Riva G.A., Perez M., Selman-Housein G., Vazquez-Padron R.I. 1999. Agrobacterium mediated Japonica rice transformation: a procedure assisted by an anti-necrotic treatment. Plant Cell Tissue Organ Cult. 59: 159-168.

Flor H.H. 1955. Host-parasite interactions in flax – its genetics and other implications. Phytopathology 45: 680-685.

Flor H.H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Plant Pathol. 9: 275-196.

Frame B.R., Shou H.X., Chikwamba R.K., Zhang Z., Xiang C., Fonger T.M., Pegg S.E.K., Li B., Nettleton D.S., Pei D.Q., Wang K. 2002. *Agrobacterium tumefaciens* -mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129: 13-22.

Gao A.G., Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CM. 2000. Fungal pathogen protection in potato by expression of a

plant defensin peptide. Nat. Biotechnol. 18: 1307-1310.

Gould J., Devey M., Hasegawa O., Ulian E.C., Peterson G., Smith R.H. 1991. Transformation of Zea-mays L using Agrobacterium-tumefaciens and the shoot apex. Plant Physiol. 95 (2): 426-434.

Ha S.B., Wu F.S., Thorne T.K. 1992. Transgenic turf-type tall fescue (*Festuca-arundinacea* Schreb) plants regenerated from protoplasts. Plant Cell Rep. 11 (12): 601-604.

Hain R., Reif H.J., Krause E., Langebartels R., Kindl H., Vornam B., Wiese W., Schmelzer E., Schreier P.H., Stocker R.H., Stenzel K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. Nature 361 (6408): 153-156.

Hellens R., Mullineaux P., Klee H. 2000. Technical Focus: A guide to Agrobacterium binary Ti vectors, Trends in Plant Science 5(10): 446-451.

Hennin C., Hofte M., Diederichsen E. 2001. Functional expression of Cf9 and and Avr9 genes in Brassica napus induces enhanced resistance to Leptosphaeria maculans. Mol. Plant-Microbe Interactions 14 (9): 1075-1085.

Hiei Y., Ohta S., Komari T., Kumashiro T. 1994. Efficient transformation of rice (*Oryza-Sativa* L) mediated by *Agrobacterium* and sequence-analysis of the boundaries of the T-DNA. Plant J. 6 (2): 271-282.

Honèe G. 1999. Engineered resistance against fungal plant pathogens. Eur. J. Plant Path. 105:319-326.

Hain R., Reif H.J., Krausse E., Langebartels R., Kindl H., Vornam B., Wiese W., Schmelzer E., Schreier P.H., Stocker S.K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature*, 361:153-156.

Hu T., Metz S., Chay C., Zhou H.P., Biest N., Chen G., Cheng M., Feng X., Radionenko M., Lu F., Fry J. 2003. Agrobacterium-mediated large-scale transformation of wheat (Triticum aestivum L.) using glyphosate selection Plant Cell. Rep. 21: 1010-1019.

Hulbert S.H., Webb C.A., Smith S.M., Sun Q. 2001. Resistance gene complexes; evolution and utilization. Annu. Rev. Phytopathol.. 39: 285-312.

Ishida Y., Saito H., Ohta S., Hiei Y., Komari T. and Kumashiro T. 1996. High efficiency transformation of maize (*Zea mays* L) mediated by *Agrobacterium tumefaciens*. Nat. Biotechnol. 14 (6): 745-750.

Johal G.S., Briggs S.P. 1992. Reductase activity encoded by the *HM1* disease resistance gene in maize. Science 258: 958-987.

Johnson R. 1993. Durability of disease resistance in crops: some closing remarks about the topic and the symposium. Dordrecht: Kluwer. Pp.283-300.

Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti P.J., Jones J.D.G. 1994. Isolation of the tomato *cf-9* gene for resistance of *Cladosporium fulvum* by transposon tagging. Science 266: 789-793.

Khanna H.K., Daggard G.E. 2003. Agrobacterium tumefaciens -mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Rep. 21: 429-4436.

Ko K.S., Norelli J.L., Reynoird J.P., Aldwinckle H.S., Brown S.K. 2002. T4 lysozyme

and attacin genes enhance resistance of transgenic 'Galaxy' apple against Erwinia amylovora. J. American Society Horticulture Sci. 127 (4): 515-519.

Lazo G.R., Stein P.A., Ludwig R.A. 1991. A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Bio/Technology 9:963-967.

Le V.Q., Belles-Isles J., Dusabenyagasani M. and Tremblay F.M. 2001. An improved procedure for production of white spruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. J. Exp. Bot. 52 (364): 2089-2095.

Lipka V., Dittgen J., Bednarek P., Bhat R., Wiermer M., Stein M., Landtag J., Brandt W., Rosahl S., Scheel D., Llorente F., Molina A., Parker J., Somerville S., Schulze-Lefert P. 2005. Pre-and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. 310: 1180-1183.

Lius, S., Manshardt, R. M., Fitch, M. M. M., Slightom, J. L., Sanford, J. C. and Gonsalves, D. 1997. Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus. Mol. Breed. 3: 161-168.

Logemann J., Jach G., Tommerup H., Mundy J., Schell J. 1992. Expressio of a barley RIP leads to increased fungal protection in transgenic tobaccoplants. BioTechnology 10: 305-308.

Lorito M., Woo S.L., Fernandez I.G., Colucci G., Harman G.E., Pintor-Toro J.A., Filippone E., Muccifora S., Lawrence C.B., Zoina A., Tuzun S., Scala F. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA 95: 7860-7865.

Martin G.B., BogdanoveA.J., SessaG. 2003. Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 54: 23-61.

Mentag R., Luckevich M., Morency M.J., Seguin A. 2003. Bacterial disease resistance of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1 Tree Physiol. 23 (6): 405-411.

Mor A., Nicolas P. 1994. The NH2-terminal a-helical domain 1-18 of Dermaseptin is responsible for antimicrobial activity. J. Biological Chem. 269(3): 1934-1939.

Nakajima H., Muranaka T., Ishige F., et al. 1997. Fungal and bacterial disease resistance in transgenic plants expressing human lysozyme. Plant Cell Rep. 16: 674-679.

Nester E.W., Gordon M.P., Amasino R.M., Yanofsky M.F. 1984. Crown gall: a molecular and physiological analysis. Ann. Rev. Plant Physiol. 35: 387-413.

Nielsen K.K., Nielsen J.E., Madrid S.M., Mikkelsen J.D. 1997. Characterization of a new antifungal chitin-binding peptide fro sugar beet leaves. Plant Physiol. 113: 83-91.

Nissen-Meyer J., Nes I.F. 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis and mechanism of action. Arch. Microbiol. 167: 67-77.

Olhoft P.M., Somers D.A. 2001. L-Cystein increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep. 20:706-711.

Parashina E.V., Serdobinskii L.A., Kalle E.G., Lavorova N.V., Avetisov V.A., Lunin V.G., Naroditskii B.S. 2000. Genetic engineering of oilseed rape and tomato palnts expressing a radish defensin gene. Rus. J. Plant Physiol. 47: 417-423.

Perl A., Lotan O., Abu-Abied M., Holland D. 1996. Establishment of an Agrobacterium mediated transformation system fro grape(Vitis vinifera L.) : the role of antioxidant during grape-Agrobacterium interactions. Nature Biotechnol 14: 624-628.

Pennell R.L., Lamb C. 1997. Programmed cell death in plants. Plant Cell. 9: 125-135.

Rollins-Smith L., Carey C., Conlon J.M., Reinert L.K., Doersam J.K., Bergman T., Silberring J., Lankinen H., Wade D. 2003. Activities of temporin family peptides against the Chytrid fungus (Batrachochytrium dendrobatidis) associated with global amphibian declines. Antimicrobial Agents and Chemotherapy. 47(3): 1157-1160.

Sanders P.R., Sammons B., Kaniewski W., Haley L., Layton J., Lavallee B.J., Delannay X., Tumer N.E. 1992. Field-resistance of transgenic tomatoes expresing the tobacco mosaic virus or tomato mosaic virus coat protein genes. Phytopathology 82 (6): 683-690.

Sanford, J.C., Johnston, S.A. 1985. The concept of parasite-derived resistance–deriving reistance genes from the parasite's own genome. J. Theor. Biol. 113: 395-405.

Shimoda N., Toyoda-Yamamoto A., Nagamine J., Usami S., Katayama M., Sakagami Y., Machida Y. 1990. Control of expression of Agrobacterium Vir genes by synergistic actions of phenolic signals molecules and monosaccharides. Proc.Natl. Acad. Sci. USA 887: 6684-6688.

Simmaco M., Mignogna G., Canofeni S., Miele R., Mangoni M.L., Barra D. 1996. Eur. I. Biochem. 242:788.

Song W.Y., Wang G.L., Chen L.L., Kim H.S., Pi L.Y. et al. 1995. A receptore kinase-like protein encoded by the rice disease reisstnace gene, *Xa21*. Science. 270: 1804-1806.

Spangenberg G., Wang Z.Y., Wu X.L., Nagel J., Potrykus I. 1995. Transgenic tall fescue (Festuca-arundinacea)and red fescue (f-rubra) plants from microprojectile

bombardment of embryogenic suspension cells. J. Plant Physiol. 145 (5-6): 693-701.

Tang W. 2003. Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. Plant Cell Rep. 21 (6): 555-562.

Terras F.R., Eggermont K., Kovaleva V., Raikhel N.V., Osborn R.W., Kester A., Rees S.B., Torrekens S., Van Leuven F., Vanderleyden J. 1995. Small cysteine-rich antifungal protein from radish: their role in host defense. Plant Cell 7 (5): 573-588.

Theis T., Stahl U. 2004. Antifungal proteins: targets, mechanisms and prospective applications. Cell. Mol. Life Sci. 61: 437-455.

Thomma B.P.H.G., Cammue B.P.A., Thevissen K. 2002. Plant defensins. Planta 216: 193-202.

Tian W.Z., Yang M., Ding Y.Y., Ding L., Cao S.Y., Ye S.Q., Tang Z.S., Li L.C., Ling Z.Z., Zhang Q., Hain R. 2002. Rice transformation with lysozyme gene and bioassay of the transgenic plants and their progenies. In: "Futuer of Agriculture – Gene Transfer Technology", pp. 191-197 (in Chinese)

Tingay S., McElroy D., Kalla R., Fieg S., Wang M.B., Thornton S. and Brettell R. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11 (6): 1369-1376.

Trick H., Finer J.J. 1997. SAAT: sonication-assisted Agrobacterium -mediated transformaiton. Transgenic Res. 6: 329-336.

Wade D., Bergman T., Silberring J., Lankinen H. 2001. Synthesis and characterization of

new temporin A analogs and a hybrid peptide. Pro. Pep. Lett. 8(6): 443-450.

Wang Z.Y., Ge Y.X. Agrobacterium-mediated high efficiency transformation of tall fescue (Festuca arundinacea) 2005. J. Plant Physiol. 162 (1): 103-113.

Wang Z.Y., Ye X.D., Nagel J., Potrykus I., Spangenberg G. 2001. Expression of a sulphur-rich sunflower albumin gene in transgenic tall fescue (*Festuca arundinacea* Schreb.) plants. Plant Cell Rep. 20 (3): 213-219.

Wang Z.Y., Takamizo T., Iglesias V.A., Osusky M., Nagel J., Potrykus I. and Spangenberg G. 1992. Transgenic plants of tall fescue (*Festuca-arundinacea* Schreb) obtained by direct gene-transfer to protoplasts. Bio/Technology 10 (6): 691-699.

Watson J.M., Fusaro A.F., Wang M.B., Waterhouse P.M. 2005. RNA silencing platforms in plants. Febs Letters 579 (26): 5982-5987.

Yu T.T., Skinner D.Z., Liang G.H., Trick H.N., Huang B., Muthukrishnan S. 2000. *Agrobacterium*-mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas 133 (3): 229-233.

Zhang Z.H., Wu, L.P. 1998. Research and development of expressing antibodies in plants. *Prog. Biochem. Biophys.*, 25:136-139.

Zhao Z.Y., Gu W.N., Cai T.S., Tagliani L., Hondred D., Bond D., Schroeder S., Rudert M. and Pierce D. 2002. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Mol. Breeding 8 (4): 323-333.

Zhao Z.Y., Cai T.S., Tagliani L., Miller M., Wang N., Pang H., Rudert M., Schroeder S., Hondred D., Seltzer J. and Pierce D. 2000. *Agrobacterium*-mediated sorghum transformation. Plant Mol. Biol. 44 (6): 789-798.

Zhu Q., Maher E.A., Masoud S., Dixon R.A., Lamb C.J. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/technol. 12: 807-812.

Chapter I

High Efficiency Transformation of Tall Fescue with *Agrobacterium tumefaciens*

(This chapter was published as an article in Plant Science, 2005, 168: 1453-1458, and is in the format of the journal)

Shujie Dong and Rongda Qu

Department of Crop Science, North Carolina State University, Raleigh, NC 27695-7620, USA.

* Corresponding author, e-mail: rongda_qu@ncsu.edu, Tel: (919) 515-7616, Fax: (919) 515-7959

Abstract

An efficient genetic transformation system for tall fescue (*Festuca arundinacea* Schreb.), using *Agrobacterium tumefaciens*-mediated T-DNA delivery, is described. Seed-derived embryogenic calli were infected with *Agrobacterium tumefaciens* strain EHA105 harboring plasmids pTOK47 and pCAMBIA1301. Infected calli were selected at 250 mg L^{-1} hyg B and the regenerated plantlets at 50 mg L^{-1}. Using the protocol developed, 34% of the calli infected were hyg B resistant, and the overall plant transformation frequency (number of independently transformed plants over number of calli infected) was 8%. Stable integration of transgene into plant genome and GUS reporter gene expression were confirmed. Low transgene copies (1 or 2 as estimated) were observed in all the transgenic plants analyzed, and the transgene was transmitted to the progenies. Further experiments suggest an elevated 2,4-D concentration (5 mg L^{-1}) used during callus culture and co-cultivation was partially attributable to the observed high transformation efficiency, while the inclusion of plasmid pTOK47 in *Agrobacterium* was not. The whole process from callus induction to transplanting green plantlets to the soil takes about six months, significantly shorter than the suspension culture approach often used in tall fescue transformation.

Key words: 2,4-D, *Agrobacterium tumefaciens*, genetic engineering, tall fescue, transgenic plants

Abbreviations: GUS – β-glucuronidase; *hph* – hygromycin phosphotransferase gene; hyg B – hygromycin B; X-Gluc – 5-bromo-4-chloro-3-indolyl-β-d-glucuronide; 2,4-D –

2,4-dichlorophenoxyacetic acid; BAP – 6-benzylaminopurine; NAA – α-naphthalene acetic acid; ABA – abscisic acid

1. Introduction

Tall fescue is a widely used open-pollinated, perennial, cool-season turf and forage grass species [1]. Improvement of important agronomic traits, such as disease resistance and abiotic stress tolerance, would be very helpful for tall fescue as a grass crop. Genetic engineering has opened new avenues to the modification of turf and forage grasses, and provides us an alternative approach to meet specific breeding goals [2-4]. Establishment of a high frequency transformation system would greatly facilitate the efforts for grass improvement via genetic engineering.

First attempts to introduce foreign DNA into tall fescue were through the protoplast [5-7]. Microprojectile bombardment of embryogenic calli or suspensions was later used [8-10]. A recent correspondence reported *Agrobacterium tumefaciens*-mediated transformation of tall fescue [11]. In the report, the authors used the 'super-binary' vector system to infect tall fescue suspension cells and recovered two transgenic plants.

Plant transformation mediated by the soil-borne pathogen *Agrobacterium tumefaciens* was first reported in the 80's [12]. Since then *Agrobacterium tumefaciens*-mediated transformation has been the standard method to genetically modify dicotyledonous plants. Repeatable and efficient *Agrobacterium*-mediated transformation of monocotyledonous plants was first demonstrated in rice a decade ago [13]. The key factors in the method were a 'super-binary' vector and the addition of acetosyringone to the co-cultivation medium. Subsequently, *Agrobacterium*-mediated transformation of maize [14, 15], wheat [16], barley [17], sorghum [18], creeping bentgrass [19], and

Italian ryegrass [11] were reported using similar approaches. *Agrobacterium*-mediated transformation is often preferred over other plant transformation systems because of the simplicity, the low cost, and lower transgene copies integrated into plant genome [14]. Although in some cases, relatively high transformation frequencies were reported [13, 15, 16, 18], *Agrobacterium*-mediated transformation of monocot plants often suffers from its inefficiency.

In this correspondence, we report establishment of an efficient *Agrobacterium*-mediated transformation system for tall fescue. In our experiments, average frequency of stable callus transformation was 34%, and the overall plant transformation frequency (number of independently transformed plants over number of calli infected) was 8%.

2. Materials and Methods

2.1. Plant materials and tissue culture conditions

Approximately 5 g mature seeds of tall fescue cultivar "Matador" [20] or "Coronado" [21] were dehusked by stirring in 50% sulfuric acid for 30 min [22]. The dehusked seeds were rinsed with distilled water followed by 95% ethanol, and then surface-sterilized with stirring in full strength Clorox® (containing 6% sodium hypochlorite, Clorox, Oakland, CA) plus 0.1% of Tween–20 (Fisher, Fairlawn, NJ) for 30 min. After 10 times rinsing with distilled water, the seeds were sliced longitudinally and plated on callus induction medium which contains MS basal medium ingredients (Caisson Laboratories, Sugar City, Idaho) supplemented with 30 g L^{-1} sucrose, 5 mg L^{-1} 2,4-D, 0.05 mg L^{-1}

BAP, and 3.2 g L^{-1} phytagel [23]. After 4 weeks, the induced calli were sub-cultured on the same medium. Four weeks later, light yellowish and compact embryogenic calli were chosen for *Agrobacterium tumefaciens*-mediated transformation.

2.2. Agrobacterium strains and vectors

The binary vector pCAMBIA1301 (http://www.cambia.org.au) was used in the transformation experiment. The T-DNA of pCAMBIA1301 includes a selectable marker gene construct for hyg B resistance and a construct of a GUS reporter gene containing the first intron of castor bean CAT-1 gene in GUS coding region [24] (Fig. I-1A). The freeze-thaw method [25] was used to mobilize pCAMBIA1301 and another plasmid, pTOK47, into *Agrobacterium* strain EHA105 [25a]. Plasmid pTOK47 carries a 20 kb *Kpn*I fragment [26] of Ti plasmid from pTiBo542, which contains *virB*, *virC,* and *virG* virulence genes [27]. The resulted *Agrobacterium* strain, EHA105 (pTOK47, pCAMBIA1301), designated as ET1301, was grown in 50 ml YEP medium [25] in the presence of 20 mg L^{-1} rifampicin, 5 mg L^{-1} tetracycline, and 50 mg L^{-1} kanamycin until OD_{595} reached above 1.0. The bacteria were diluted with liquid MS medium to OD_{595} = 0.4 for co-cultivation with tall fescue calli.

2.3. Transformation procedure

Embryogenic calli were immersed in *Agrobacterium* suspension for 15 min, then transferred to callus induction medium supplemented with 100 µM acetosyringone (Aldrich, Milwaukee, WI), in the dark at 25°C for 3 d co-cultivation. The calli were then transferred to the "resting medium" [callus induction medium containing 200 mg L^{-1}

carbenicillin (Apollo Scientific, Stockpork, UK) and 150 mg L^{-1} Timentin (GlaxoSmithKline, Research Triangle Park, NC)] in the dark at 25°C to inhibit *Agrobacterium* growth as well as to have the calli recovered from co-cultivation "shock" [15]. The calli were then incubated on selection medium containing 150 mg L^{-1} hyg B in dark at 25°C for 4 weeks. Surviving calli were transferred to the same medium containing 250 mg L^{-1} hyg B [9] for two more rounds of selection for a total of 4 additional weeks. Selection at 250 mg L^{-1} hyg B is very tight for tall fescue and no escapes were obtained [9]. Hyg B resistant calli were then cultured on pre-regeneration medium, which is the basal MS medium supplemented with 1 mg L^{-1} NAA, 1 mg L^{-1} BAP, 5 mg L^{-1} ABA, 30 g L^{-1} sucrose, and 3.2 g L^{-1} phytagel, for 1 to 2 weeks [31]. The calli were then transferred onto selective regeneration medium [MS basal medium, 30 g L^{-1} maltose (Spectrum Chemical, Gardena, CA), 2.5 mg L^{-1} BAP, 7.5 g L^{-1} phytagar (GIBCO, Langley, OK) and 50 mg L^{-1} hyg B] in a lighted growth chamber at 25 °C under a 16/8 h (day/night) photoperiod (140 μmol m^{-2} s^{-1} of cool white fluorescent light). Regenerated shoots were transferred for rooting onto a half strength MS rooting medium containing 50 mg L^{-1} hyg B and 0.5 mg L^{-1} NAA in Magenta boxes (Magenta Corp., Chicago, IL). About 4 weeks later, rooted plants were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH), covered for the first few days with transparent lids for fast recovery. The plants were subsequently grown in a greenhouse at 25°C. All the chemicals used in the experiments were purchased from Sigma (St. Louis, MO) unless otherwise specified.

2.4. GUS histochemical assay and Southern analysis

GUS gene expression was confirmed by histochemical assays with X-gluc [28] as the substrate for the enzyme. Putative transgenic calli and leaves were immersed in the GUS assay buffer overnight at 37°C and examined under a dissection microscope. For Southern analysis, genomic DNA were isolated from plant leaves based on the protocol of Dellaporta et al. [29] with the addition of DNase-free RNase A treatment (Sigma, 0.5 mg mL^{-1}, 37°C, 10 min). Twenty-five μg of genomic DNA from each sample was digested with *Bam*HI (Promega, Madison, WI) overnight and was subjected to electrophoresis in a 0.8% agarose gel. Plasmid DNA equivalent to one copy of *hph* gene in a 2C tall fescue genome [30], mixed with 25 μg non-transgenic plant DNA was used as a transgene reconstruction/positive control. The fractionated DNA was transferred to a Hybond N^+ hybridization membrane (Amersham, Little Chalfont, Bucks, England) according to the manufacturer's instructions. A 1.1 Kb *Xho*I fragment of the pCAMBIA1301 containing the full length coding region of the *hph* gene was used as a probe for Southern hybridization. The probe was labeled with [α-^{32}P]-dCTP (Amersham) using the Primer-It® II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX) and hybridized with the blotted membrane in MiracleHyb™ hybridization solution (Stratagene) according to the manufacturer's instructions.

2.5. Inheritance study of the transgenic plant

Transgenic plant ETR3-6 was naturally vernalized in the field during winter and the flowers were pollinated with pollen grains from non-transgenic 'Coronado' plants in the following summer inside a greenhouse. The seeds from the crossing were collected from

ETR3-6 and germinated in half MS medium without addition of phytohormones. To test the hyg B resistant phenotype, the plantlets were transferred to the same medium containing hyg B (50 mg L^{-1}).

In PCR assays of *hph* gene, genomic DNA was extracted from the offspring seedlings based on the protocol of Dellaporta et al. [29]. The two primers used were as previously described [31] and the expected size of the amplified fragment is 592 bp. HotM Taq DNA polymerase (Eppendorf, Westbury, NY) was used in a standard 35-cycle reaction with annealing temperature of 58°C. The positive control for PCR used 1 ng plasmid CAMBIA1301 as the template while the negative control used genomic DNA extracted from a non-transgenic plant.

3. Results and Discussion

3.1. Establishment of an efficient Agrobacterium-mediated transformation system of tall fescue

Two-month-old, light yellowish, compact calli were used as explants for transformation experiments. A total of 300 pieces of calli of cv. "Matador" was co-cultivated with *Agrobacterium* strain ET1301 in the three transformation experiments reported here (Table I-1). Spotted transient GUS expression was observed on calli after co-cultivation (Fig. I-2A). Since "intron-GUS" gene is used in the construct, the observed expression indicated the T-DNA delivery into the plant cells. During the selection period on hyg B (150 mg L^{-1}), a majority of calli gradually turned brown whereas some yellowish hyg B resistant calli were observed after three to four wks selection (Fig. I-2B). These calli were

subjected to a higher-level selection (250 mg L^{-1} hyg B). The resistant calli were then subjected to the regeneration process. In each of the three transformation experiments, 30-40% calli showed resistance to the hyg B selection (Table I-1). A total of 24 such resistant calli regenerated into green plantlets (Fig. I-2D, Table 1). No albino plantlets were observed in the experiments. All the plantlets developed good root system in the selective rooting medium (Fig. I-2E) and survived transplantation (Fig. I-2G).

3.2. Analyses of transgenic plants

GUS expression was observed from resistant calli and plants (Fig. I-2C, 2F). All the 24 resistant plants were GUS positive (Table I-1) although the GUS expression levels varied among the plants (Fig. I- 2F).

Southern analysis was performed on 23 putative transgenic plants and the transgenic nature of these plants was confirmed, suggesting the tightness of the selection. The hybridization results of 8 such plants were shown in Figure 1B. The various positions of the hybridized *hph* gene among the analyzed plants indicated stable integration of the transgene into plant genome. Comparing to the transgenic tall fescue plants obtained from microprojectile bombardment [9], transgenic plants recovered from *Agrobacterium*-mediated transformation had simpler hybridization patterns and were estimated to have one or two transgene copies. Because of this, it is sometimes difficult to judge whether two plants with similar hybridization patterns were from the same or different transformation events, such as in the case of lanes 4 and 6. To solve the confusion, the same blot was re-hybridized with GUS gene probe. Distinct hybridization

patterns between the two lanes were observed suggesting the two plants derive from two independent transformation events (data not shown).

Agrobacterium-mediated transformation in monocots is often genotype dependent [14]. To see whether our protocol also applies to other cultivars, we used the protocol to transform cultivar 'Coronado'. Among 1846 pieces of calli infected, 31.5% developed into resistant calli under 250 mg L^{-1} hyg B selection, indicating the high transformation efficiency was also achieved for 'Coronado', and the protocol may apply to other cultivars.

In the inheritance study, among the ten germinated plants from the offspring seeds obtained from crossing on transgenic plant ETR3-6, six were resistant to hyg B (Fig. I-2H-a) and GUS positive (data not shown), while the other four were hyg B sensitive (Fig. I-2H-b) and GUS negative. The observation and PCR analysis of the offspring plants for *hph* gene (data not shown), suggest the transgene be inherited to the offspring plants. The segregation ratio was near 1:1, an indication that the transgene(s) was inserted at a single genetic locus.

3.3 Analyses of factors that may affect tall fescue transformation efficiency

Plasmid pTOK47, containing an extra copy of *virB*, *virC,* and *virG* virulence genes [27], respectively, was shown to enhance *Agrobacterium*-mediated transformation in white spruce [27] and loblolly pine [32]. Although the *Agrobacterium* strain used in the experiments contains pTOK47, its role in the observed high transformation frequency

was not clear. To address this question, the strain EHA105 (pCAMBIA1301), designated as E1301, was constructed. E1301 is the same as ET1301 except that it does not contain pTOK47. The two were compared in an experiment for transformation efficiency, where 240 pieces of calli (cv. "Matador") were infected by each strain, respectively. Almost identical numbers of hyg B resistant calli were obtained from the two treatments (102 from E1301 vs. 101 from ET1301), suggesting that pTOK47 do not play a substantial role in the observed high transformation frequency, and E1301 is sufficient and equally efficient to ET1301 in tall fescue transformation. However, inclusion of pTOK47 does not have a detrimental effect either.

In addition, experiments were performed to evaluate the role of 2,4-D concentration on the observed high transformation efficiency. In the replicated experiments, 647 pieces of callus (cv. "Matador") were cultured, co-cultivated, and selected on media containing 5mg L^{-1} 2,4-D, while 640 pieces of callus were on media containing 2 mg L^{-1} 2, 4-D throughout. In 5 mg L^{-1} treatment, 25.2% calli were resistant to hyg B whereas only 17.3% were hyg B-resistant for the 2 mg L^{-1} treatment. ANOVA and F test [33] indicated that the difference between the two treatments was significant ($p=0.0363$), suggesting that 2,4-D concentration in the medium play an important role in the efficient transformation. 2,4-D stimulates cell division and is the most commonly used growth regulator to induce callus and maintain callus growth in grass tissue culture [34]. It is possible that at 5 mg L^{-1} 2,4-D, more cells are actively dividing and are thus more

competent for *Agrobacterium* infection [35]. Alternatively, elevated 2,4-D may somehow make the host cells more competent for transformation [36], other than cell division. The possibility that the elevated 2,4-D may help the transformed cells recover [37], or even facilitate the infection process by *Agrobacterium*, cannot be excluded either. To our knowledge, this is the first report regarding an effect of 2,4-D concentration on *Agrobacterium*-mediated transformation efficiency. It remains to be seen whether this approach can be applied to other plant species to improve their transformation efficiencies. Since long-term exposure to high level of 2,4-D may have adverse effects on callus regeneration, the period of high-level 2,4-D treatment will need to be optimized.

It seems the callus transformation frequency was more consistent from batch to batch using this protocol than the plant transformation frequency in the experiments. Thus more attention will be needed to maintain the callus regeneration ability during selection. Overall, our system yields 34% hyg B resistant calli and has 8 % overall transformation efficiency. That is comparable to the rice transformation system [13] and is among the best of *Agrobacterium*-mediated transformation systems in monocot species. The whole process, spared from the need of time-consuming establishment of suspension cultures, only takes approximately six months from callus induction from mature seeds to transplantation of transgenic plantlets to the soil.

In conclusion, this correspondence reports an efficient and reliable *Agrobacterium*–mediated transformation system using 2 elite tall fescue cultivars.

Southern analysis confirmed that the transgenes were integrated into the genome of the hyg B-resistant plants, and that the individual plants were from independent transformation events. This system has significant advantages over the previous reported ones on tall fescue transformation, including high transformation efficiency, low transgene copy number in plants, simple procedure, and a shorter period to recover transgenic plants. The low transgene copies could help reduce the possibility of gene silencing and increase the stability of the transgenes [38]. The efficiency and reliability of the reported transformation system make it possible to generate a large number of transgenic tall fescue plants in a relatively short period, and to test the performance of various transgenes for tall fescue improvement.

Acknowledgements

The authors are grateful to CAMBIA (Canberra, ACT, Australia) for the binary vector used in the experiment, to Drs. K. Azhakanandam, and E. Sivamani for technical discussions and critical reading of the manuscript, to Dr. L. Li for technical discussions, to C. Rose-Fricker for seed supply, and to NCSU Phytotron for the greenhouse facilities. This work was supported, in part, by HybriGene, LLC. (West Kingston, RI).

References

[1] R.C. Buckner, J.B. Powell, R.V. Frakes, Historical development p.1-8. In: Buckner R.C., and L.P.Bush (ed) tall fescue. Agronomy monograph # 20, Am. Soc. of Agronomy, Madison, WI. 1979, pp. 1-8.

[2] J.J. Estruch, N.B. Carozzi, N. Desai, N.B. Duck, G.W. Warren, M.G. Koziel, Transgenic plants: An emerging approach to pest control. Nat. Biotechnol. 15 (1997) 137-141.

[3] Z.Y. Wang, A. Hopkins, R. Mian, Forage and turf grass biotechnology. Critic. Rev. Plant Sci. 20 (2001) 573-619.

[4] L. Chen, C.K. Auh, P. Dowling, J. Bell, F. Chen, A. Hopkins, R.A. Dixon, Z.Y. Wang, Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. Plant Biotechnol. J. 1 (2003) 437-449.

[5] S.B. Ha, F.S. Wu, T.K. Thorne, Transgenic turf-type tall fescue (*Festuca-arundinacea* Schreb) plants regenerated from protoplasts. Plant Cell Rep. 11 (1992) 601-604.

[6] Z.Y. Wang, T. Takamizo, V.A. Iglesias, M. Osusky, J. Nagel, I. Potrykus, G. Spangenberg, Transgenic plants of tall fescue (*Festuca-arundinacea* Schreb) obtained by direct gene-transfer to protoplasts. Bio-Technol. 10 (1992) 691-699.

[7] S.J. Dalton, A.J.E. Bettany, E. Timms, P. Morris, The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca-arundinacea* Schreb). Plant Sci. 108 (1995) 63-70.

[8] M.J. Cho, C.D. Ha, P.G. Lemaux, Production of transgenic tall fescue and red fescue plants by particle bombardment of highly regenerated tissues. Plant Cell Rep. 35 (1999) 60-A.

[9] Y. Bai, R. Qu, Genetic transformation of elite turf-type cultivar of tall fescue. Intl. Turfgrass Soc. Res. J. 9 (2001a) 129-136.

[10] Z.Y. Wang, X.D. Ye, J. Nagel, I. Potrykus, G. Spangenberg, Expression of a sulphur-rich sunflower albumin gene in transgenic tall fescue (*Festuca arundinacea* Schreb.) plants. Plant Cell Rep. 20 (2001b) 213-219.

[11] A.J.E. Bettany, S.J. Dalton, E. Timms, B. Manderyck, M.S. Dhanoa, P. Morris, *Agrobacterium tumefaciens*-mediated transformation of *Festuca arundinacea* (Schreb.) and *Lolium multiflorum* (Lam.). Plant Cell Rep. 21 (2003) 437-444.

[12] M. De Block, L. Herrera-Estrella, M. Vanmontagu, J. Schell, P. Zambryski, Expression of foreign genes in regenerated plants and in their progeny. EMBO J. 3 (1984) 1681-1689.

[13] Y. Hiei, S. Ohta, T. Komari, T. Kumashiro, Efficient transformation of rice (*Oryza-sativa* L) mediated by *Agrobacterium* and sequence-analysis of the boundaries of the T-DNA. Plant J. 6 (1994) 271-282.

[14] Y. Ishida, H. Saito, S. Ohta, Y. Hiei, T. Komari, T. Kumashiro, High efficiency transformation of maize (*zea mays* l) mediated by *Agrobacterium tumefaciens.* Nat. Biotechnology. 14 (1996) 745-750.

[15] Z.Y. Zhao, W.N. Gu, T.S. Cai, L. Tagliani, D. Hondred, D. Bond, S. Schroeder, M. Rudert, D. Pierce, High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Mol. Breed. 8 (2002) 323-333.

[16] M. Cheng, J.E. Fry, S.Z. Pang, H.P. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Conner, Y.C. Wan, Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115 (1997) 971-980.

[17] S. Tingay, D. McElroy, R. Kalla, S. Fieg, M.B. Wang, S. Thornton, R. Brettell, *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11 (1997) 1369-1376.

[18] Z.Y. Zhao, T.S. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer, D. Pierce, *Agrobacterium*-mediated sorghum transformation. Plant Mol. Bio. 44 (2000) 789-798.

[19] T.T. Yu, D.Z. Skinner, G.H. Liang, H.N. Trick, B. Huang, S. Muthukrishnan, *Agrobacterium*-mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas. 133 (2000) 229-233.

[20] C.A.Rose-Fricker, M.L. Fraser, W.A. Meyer, C.R. Funk, Registration of 'Coronado' tall fescue. Crop Science. 39 (1999) 288.

[21] M.L. Fraser, C.A. Rose-Fricker, W.A. Meyer, Registration of 'Matador' tall fescue. Crop Science. 39 (1999) 871-872.

[22] Y. Bai, R. Qu, Factors influencing tissue culture responses of mature seeds and immature embryos in turf-type tall fescue. Plant Breed. 120 (2001b) 239-242.

[23] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 (1962) 473-497.

[24] S. Ohta, S. Mita, T. Hattori, K. Nakamura, Construction and expression in tobacco of a beta-glucoronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol. 31 (1990) 805-813.

[25] G. An, P.R. Ebert, A. Mitra, S.B. Ha, Binary vectors, in: S.B. Galvin, R.A. Schilperoort (Eds.) Plant Molecular Biology Manual. Kluwer, Dordrecht, 1988, pp. A3: 1-19.

[25a] E. E. Hood, S. B. Gelvin, L. S. Melchers, A. Hoekema, New *Agrobacterium* helper plasmids for gene-transfer to plants. Transgenic Res. 2 (1993) 208-218.

[26] S.G. Jin, T. Komari, M.P. Gordon, E.W. Nester, Genes responsible for the supervirulence phenotype of *Agrobacterium-tumefaciens* A281. J. Bacteriol. 169 (1987) 4417-4425.

[27] V.Q. Le, J. Belles-Isles, M. Dusabenyagasani, F.M. Tremblay, An improved procedure for production of white spruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens.* J. Exp. Bot. 52 (2001) 2089-2095.

[28] R.A. Jefferson, Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5 (1987) 387-405.

[29] S.L. Dellaporta, J. Wood, J.B. Hicks, A plant DNA minipreparation: Version ii. Plant Mol. Bio. Rep. 14 (1983) 19-21.

[30] K. Arumuganathan, S.P. Tallury, M.L. Fraser, A.H. Bruneau, R.D. Qu, Nuclear DNA content of thirteen turfgrass species by flow cytometry. Crop Sci. 39 (1999) 1518-1521.

[31] L.C. Li, R.D. Qu, A. De Kochko, C. Fauquet, R.N. Beachy, An improved rice transformation system using the biolistic method. Plant Cell Rep. 12 (1993) 250-255.

[32] W. Tang, Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. Plant Cell Rep. 21 (2003) 555-562.

[33] R.G. D. Steel, J. H. Torrie, D. H. Dicky, Principles and procedures of statistics: a biometrical approach. (3rd edition) McGraw-Hill, New York, 1997.

[34] I. K. Vasil, Developing cell and tissue culture systems for the improvement of cereal and grass crops. J. Plant Physiol. 128 (1987) 193-218.

[35] Y. Hiei, T. Komari, T. Kubo, Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. 35 (1997) 205-218.

[36] S. B. Gelvin, Improving plant genetic engineering by manipulating the host. Trends in Biotech. 21 (2003) 95-98.

[37] B. R. Frame, H. Shou, R. K. Chikwanba et al., *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129 (2002) 13-22.

[38] M. Matzke, A. J. M. Matzke, J. M. Kooter, RNA: guiding gene silencing. Science 293 (2001) 1080-1083.

Chapter II

Engineered resistance of tall fescue to brown patch and gray leaf spot diseases

Introduction

Brown patch disease is the most severe and frequently occurring disease on tall fescue lawns in the summer in North Carolina. It is also one of the most serious disease problems for other turfgrass species (Smiley, 1983). The disease is caused by the soilborne basidiomycete fungus, *Rhizoctonia solani.* The pathogen usually stays in the form of mycelium or sclerotia in soil as well as on the infected perennial plants. Under favorable conditions, sclerotia or mycelium germinate to produce hyphae that then attack the host plant. Hyphae penetrate the plant cells after forming an infection cushion or an appresorrium and then colonize the plant tissues (Anderson, 1982).

Brown patch occurs during warm and humid conditions. The early symptoms of brown patch are small, circular lesions with a dark brown border. The infected plant clusters form brown-colored patches, which often coalesce to blight large sections of the turf, ranging from 6 inches to several feet in diameter. Lawns having brown patch disease appear droughty or wilted, even if sufficient water is provided. The fungus stops spreading as the grass dries, and the ring disappears (Anderson, 1982).

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. The disease is caused by *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, an ascomycete fungus (Tredway et al., 2003). 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, as fungal spores are produced. As the disease develops, the foliar blight phase becomes evident. Components of resistance to *M. grisea* in two tall fescue cultivars were studied by Tredway et al. (2003).

Much knowledge about the gray leaf spot pathogen and disease come from studies in rice, since *M. grisea* is also the causal agent of rice blast, the most common and severe disease of rice. More than 20 resistance (R) genes have been mapped on the rice genome and three have been cloned, *Pib* (Wang et al., 1999), *Pi-ta* (Bryan et al., 2000), and *Pi9* (Qu et al., 2006).

Management strategies for fungal diseases of tall fescue include fungicide treatments, cultural, biological controls, and use of resistant cultivars. Cultural control of brown patch involves modifying turfgrass management practices such as fertilizer application and irrigation scheduling. Concerns about the economics and environmental contamination associated with fungicides have increased interest in the use of biological disease control (Smiley, 1983).

Development of plant germplasm resistant to the diseases would be an effective approach for disease control through breeding efforts. Conventional breeding has made great progress in developing brown patch resistant cultivars in tall fescue. As a result,

several new cultivars resistant to the brown patch disease, such as 'Tar Heel', 'Wolfpack', 'Olympic Gold', and 'Endeavor', were released over the past several years (Fraser et al., 1998; Fraser et al., 1999; Fraser et al., 2001; Fraser and Rose-Fricker, 2001).

Genetic engineering provides a new approach obtaining fungal resistance by introducing genes from other species or overexpressing the relevant endogenous genes, and thus would enrich the germplasm pool for breeding efforts. 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 the fungal pathogen *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 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 introduced into tobacco plants through chloroplast transformation, resulting in resistance to fungal (*Aspergillus flavus*, *Fusarium moniliforme*, and *Verticillium dahlige*) and bacterial (*Pseudomonas syringae*) pathogens (DeGray et al., 2001). Moreover, 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 caused by *Sclerotinia homoeocarpa* F.T. Bennett (Guo et al., 2003). Transgenic creeping bentgrass overexpressing the rice thaumatin-like protein-encoding gene TLPD34 also showed enhanced dollar spot resistance (Fu et al., 2005). In this study, we obtained 29 transgenic tall fescue lines via *Agrobacterium*-mediated transformation, which constitutively expressed the T4 bacterial phage lysozyme gene, the alfalfa glucanase *AGLU1* gene, a frog dermaseptin gene, or the rice *Pi9* gene, respectively, and investigated the responses of these lines to inoculation with *R. solani* and/or *M. grisea*. We observed that 6 of the 29 transgenic lines showed significant improvement in brown patch resistance, while 13 of the transgenic lines were highly resistant to gray leaf spot. Among these lines, five conferred resistance to both the pathogens.

Materials and Methods

Plant materials

Calli were induced from mature seeds of tall fescue cultivars 'Matador' (Fraser et al., 1999) or 'Coronado' (Rose-Fricker et al., 1999) and used for the transformation experiments. Seed treatment and tissue culture conditions were as described in Chapter 1.

Gene construction and Agrobacterium strains

Three binary vectors, pSD7, pSD9, and pSD12, were constructed and a fourth vector, pNBS2, was kindly provided by Dr. G.-L. Wang. All the vectors were derivatives of the binary vector pCAMBIA1300 which has a hygromycin B resistant *hph* gene under

control of the CaMV 35S promoter and the 35S terminator (http://www.cambia.org.au). The pSD9 vector contains a construct of the T4 phage lysozyme gene (Owen et al., 1983) driven by the maize *Ubi1* gene promoter (Figure II-1A). The gene clone was kindly provided by J. Lu (NCSU). The pSD7 vector has a construct of the alfalfa (*Medicago sativa*) glucanase *AGLU1* cDNA (Maher et al., 1994) under control of the maize *Ubi1* gene promoter (Figure II-1B). pSD12 contains a truncated dermaseptin SI gene of 18 AA from the South American arboreal frog *Phyllomedusa sauvagei* (Mor and Nicolas, 1994; Mohamed et al., 1994). The 54 nt coding sequence was fused in-frame at its 5'-terminus with the ubiquitin monomer coding sequence of the rice *rubi3* gene, and the fusion gene was under control of the *rubi3* gene promoter with its 5' UTR intron (Figure II-1C, Sivamani & Qu, 2006). Primers (Forward primer sequence: 5'-*GTATTGTATCTGGCTCTTTGCC3'*; reverse primer sequence:5'TCGAGCTCTTAGCCAGCGTGCAGAGCCATGGTGCCGAGCTTCTTCA GCATGGTCTTCCAGAGGGCGCCTCCACGAAGGCGG3') were designed and used for PCR to obtain the fusion gene from the plasmid pRESQ38 (Sivamani & Qu, 2006). The pNBS2 vector has the 13.5 kb fragment of rice genomic DNA containing the *Pi9* gene plus a GUS reporter gene construct (Figure II-1D) (Qu et al., 2006). The freeze-thaw method (An et al., 1988) was used to mobilize plasmids pSD7, pSD9, pSD12, and pNBS2, respectively, into *Agrobacterium* strain EHA105 (pTOK47) as described in Chapter 1. The resulting *Agrobacterium* strains were designated as ETSD7, ETSD9, ETSD12, and ETNBS2, respectively.

Tall fescue transformation

Embryogenic calli induced from mature tall fescue seeds were transformed by *A. tumefaciens* as described in Chapter 1. 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 for shoot induction. Regenerated shoots were then cultured on a rooting medium for root development. About 4 weeks later, rooted plants were transplanted into potting soil (Metro-Mix 200, Scotts, Marysville, OH). The plants were subsequently grown in a greenhouse at 25°C. All the chemicals used were purchased from Sigma (St. Louis, MO), unless otherwise specified.

Southern blot analysis

Genomic DNA isolation, digestion, electrophoresis, membrane transfer, probe labeling, and hybridization were performed as described in Chapter 1. Twenty-five μg of genomic DNA from each sample was digested overnight and used for Southern analysis. DNA of transgenic plants from pSD7 and pSD9 was digested with *Bgl*II, while DNA of transgenic plants from pSD12 and pNBS2 was digested with *Bam*HI, or *Pst*I, respectively (Fig. II-1). All of the restriction enzymes were purchased from Promega (Madison, WI). The full-length coding region of *AGLU1* (1.3 Kb *Eco*RI fragment of the pSD7) or T4 lysozyme gene (0.55 Kb *Sal*I fragment of the pSD9) was used as a probe to detect the two transgenes, respectively. A 0.3 Kb PCR fragment of pSD12 containing the dermaseptin SI coding sequence and the *nos* terminator sequence, or a 3 kb *Pst*I fragment of pNBS2 containing a segment of the *Pi9* genomic DNA was employed to probe these two

transgenes, respectively. The membranes were exposed to Kodak BioMax MS film for autoradiography (Kodak Eastman, Rochester, NY).

Northern blot analysis

Total RNA was extracted from plant leaves using Trizol® Reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty μg of total RNA from each plant was subjected to electrophoresis in 1% agarose gels prepared with 1X MOP (Huet et al., 1999). After electrophoresis, RNA was transferred to a Hybond N^+ hybridization membrane (Amersham) according to the manufacturer's instructions. The same probes described for Southern analysis were used for the Northern hybridizations. The blotted membranes were probed in Denhart's hybridization solution and washed according to the manufacture's instructions. Kodak BioMax MS film was used for autoradiography.

***Plant inoculation test with* R. solani**

Leaf inoculation experiments were performed in a growth chamber in the NCSU Phytotron with *R. solani* Kuhn subgroup AG1, isolated from turfgrass, kindly provided by Dr. D. Shew. The *R. solani* cultures were grown on potato dextrose agar (PDA) (Difco) medium at room temperature for 3 days prior to use as inoculum. One agar plug (3X3 mm) with mycelium from the fungal culture was placed on the midrib of each of young leaves for each transgenic or control plant. Six leaves were arbitrarily chosen from each plant. After inoculation, plants were grown in a lighted growth chamber under a 14/10 h (day/night) photoperiod with controlled environment. The temperature and relative

humidity (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 where the lesions extended. The inoculation experiment was repeated three times.

***Plant inoculation test using* M. grisea**

Leaf inoculation experiments were performed in the NCSU Phytotron with *M. grisea*. Turfgrass isolates 1213-59 and 533-78 of *M. grisea* (Tredway et al., 2003), kindly provided by Dr. L. Tredway, were mixed and revived from -80°C storage by placing filter paper discs onto PDA medium, amended with 50 mg L^{-1} each of tetracycline, streptomycin, and chloramphenicol. After seven days culture at 25°C in the dark, the mixed isolates were transferred to 1.5% water agar (WA, Difco) 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 X 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) was applied to test plants 3 days before inoculation, which did not interfere with *M. grisea* infection. Each plant was inoculated with 7.5 ml conidia suspension using an airbrush sprayer (Badger Air-Brush Co., Franklin Park, IL). For the first 24 hrs after inoculation, the plants were kept at 24°C, 100% RH, in the dark. The plants were subsequently incubated at 30°C

with 70% RH at day time (12 h/d) in the lighted chamber, and at 24°C with 100% RH at night (12 h/d).

Disease development was evaluated 10 to 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 repeated two or three times.

***In vitro growth inhibition assay of* R. solani**

To further evaluate the resistance exhibited by the transgenic plants to brown patch, an *in vitro* assay was performed to investigate whether leaf sap of the transgenic plants inhibited the growth of *R. solani* hyphae. One gram of leaf tissue from a transgenic plant was ground with a pestle and mortar in 10 ml grinding buffer (0.05 M KPO4, pH 7.2) and filter sterilized with a syringe filter (0.4 micron, Fisher Scientific). One ml of the extracted sap was added to a well in a 24-well culture plate (Corning Incorporated, Corning, NY). Five replicates were analyzed for each sample. *R. solani* (subgroup AG-1) mycelium from water agar plate (5 days culture) was inoculated into each well containing the extracted sap. The culture plate was incubated at room temperature in the dark. The growth (length of hypha) of mycelia was evaluated 24 hrs later under a microscope.

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). 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

A total of 29 T0 transgenic plants were obtained through *Agrobacterium*-mediated transformation from the disease resistance gene constructs. Among them, 12 contained the *AGLU1* gene (Glu plants), 14 had the T4 lysozyme gene (Lys plants), two expressed the dermaseptin fusion gene (Derm plants), and one had the *Pi9* gene (Pi9 plant). All the plants looked normal and grew vigorously. The trucated dermaseptin SI gene used in the experiments encodes a peptide of 18 AA residues (ALWKTMLKKLGTMALHAG) which was shown to have active antimicrobial activity (Mor & Nicolas, 1994). To increase the peptide expression, plant preferred codons were used in the synthetic gene (Campbell and Gowri, 1990). It was demonstrated that the N-terminal fusion of the rice *rubi3* ubiquitin monomer coding sequence in frame with a transgene coding sequence enhances transgene expression by about four fold and the original protein encoded by the transgene is released after excision of the ubiquitin monomer by a ubiquitin cleavage mechanism inside the cells (Sivamani & Qu, 2006). To enhance the dermaseptin gene expression in transgenic tall fescue, such a fusion gene construct was made under control of the rice *rubi3* gene promoter and its 5' UTR intron.

Southern blot analysis of transgenic tall fescue plants

Southern analysis was performed on the 29 putative transgenic plants and the transgenic nature of these plants was confirmed. Among them, 8 Lys transgenic plants, 4 Glu transgenic plants, 2 Derm plants, and 1 *Pi9* plant are shown in Fig. II-2, 3, 4, and 5, respectively. The various sizes of the restricted transgene bands among the analyzed plants indicated stable integration of the transgenes at different loci in the tall fescue genome. In contrast to transgenic tall fescue plants derived from microprojectile bombardment (Bai and Qu, 2001), transgenic plants obtained from *Agrobacterium*-mediated transformation had simpler hybridization patterns and were estimated to have 1 to 3 transgene copies.

Inoculation test for brown patch disease resistance

To test the effects of *AGLU1*, T4 lysozyme, or dermaseptin transgene expression on brown patch resistance for tall fescue, T0 transgenic plants were inoculated with the fungal pathogen *R. solani*, subgroup AG-1, which was isolated from infected turfgrass plants. Lesion development was suppressed in six transgenic plants compared to the non-transformed control plants; the rest of them didn't show resistnace to brown patch. The resistance was highly significant for transgenic plants Lys14a, Lys9a, and Derm1 ($P<0.01$), and significant for transgenic plants Glu24a and Lys12b ($P<0.05$). A slight improvement in the resistance in transgenic plant Glu3a was also observed ($P<0.1$) (Table II-1).

Preliminary *in vitro* assay data showed that the growth of *R. solani* hyphae was

inhibited by the sap extracted from leaves of the transgenic plant Lys9a, but not by the sap from a non-transformed plant (Fig. II-16), further confirming that the expressed T4 lysozyme had an inhibitory effect on the pathogen growth.

Inoculation test for gray leaf spot disease resistance

All T0 transgenic plants were inoculated with the fungal pathogen *M. grisea*. Thirteen out of the 29 transgenic plants exhibited improved resistance with smaller lesion size and reduced disease incidence compared to the non-transformed control plants (Fig. II-12 to 15); the rest of them didn't show resistance to gray leaf spot. Statistical analysis indicated that the lesion size reduction in 13 transgenic plants was highly significant ($P<0.01$), suggesting that the growth of *M. grisea* would be substantially restricted. Among them, seven were Lys plants, three were Glu plants, two were Derm plants, and one was Pi9-1. Nine transgenic plants also had much lower disease incidence ($P<0.01$), indicating the infection process was inhibited (Table II-2). Moreover, five transgenic plants showed resistance to both *R. solani* and *M. grisea* (Table II-3).

Northern analysis of the transgenic plants

Northern blot analyses were carried out to detect the expression of the transgenes among the resistant plants, and the results are shown in Fig II-6 to 8. All the resistant transgenic plants obtained from pSD7, pSD9 and pSD12 constructs showed detectable transcript accumulation. Similarly unexpected multiple bands were observed in Lys and Glu plants. The reason for the unusual expression pattern was unknown but can be traced to a plasmid used for the construction of both pSD7 and pSD9 vectors. Northern analysis

failed to detect the *Pi9* transcript in the Pi9-1 plant probably due to its low natural expression (Qu et al., 2006). However, since the gene is in between the hph gene and gus gene, and both genes were expressed in the selected callus (data not shown), it is most likely the Pi9 gene is expressed in the transgenic plants.

Discussion

We successfully integrated the T4 lysozyme gene, the alfalfa *AGLU1* gene, and a frog dermaseptin fusion gene into tall fescue and obtained multiple plants that have enhanced resistance to *R. solani* and *M. grisea*. We also integrated the *Pi9* gene and obtained resistance to *M. grisea*.

Lysozymes exist widely in microorganisms and animals, and are generally considered to be anti-bacterial because of their 1,4-β-N-acetylmuramidase activity against peptidoglycan in bacterial cells (Jolles & Jolles, 1984). Although some lysozymes can hydrolyze chitin and thus also have anti-fungal activities, T4 lysozyme was not considered to be one of them. T4 lysozyme was reported to confer resistance to the phytopathogenic bacterium *Erwinia carotovora* in transgenic potato (During et al., 1993). The first report of T4 lysozyme activity against a fungal disease was in transgenic rice, where over 80% of the transgenic plants were resistant to four isolates of the rice blast pathogen *M. grisea* (Tian et al., 2002). In our experiments, seven out of the 14 Lys transgenic plants showed highly significant resistance (Table II-2b) to *M. grisea*. Moreover, three of these plants were also resistant to *R. solani*. The results clearly

demonstrate that T4 lysozyme does have activity against some fungi, and can be used for enhancing fungal disease resistance in plants. Its resistance to *M. grisea* seems to be highly potent, and can be widely used in rice and various turfgrasses against the pathogen although the mechanism of suppression is yet to be understood.

β-1,3-glucan is a major component of fungal cell walls. Plants have evolved to have β-1,3-glucanases as one of the major PR proteins in their defense systems (De Lucca et al., 2005). It is believed that β-1,3-glucanases can directly digest the β-1,3-glucans of fungal cell walls, resulting in cell lysis, and/or release of bioactive cell wall fragments as elicitors for inducing plant defense reactions (Ryan et al., 1991). It was reported that expression of the alfalfa *AGLU1* β-1,3-glucanase gene in transgenic tobacco conferred resistance to a fungal pathogen *C. nicotianae* (Zhu et al., 1994). Wang et al. (2003) attempted to introduce the alfalfa *AGLU1* gene into creeping bentgrass but could not observe its expression, and concluded that the dicot gene may not be able to express in monocot plants. In our study, among 12 tall fescue plants containing the *AGLU1* gene, two were resistant to *R. solani* and three were highly resistant to *M. grisea*, with one being resistant to both pathogens. Northern analysis detected *AGLU1* expression in these plants. The results suggest that the *AGLU1* gene did express in tall fescue, and can be used for fungal disease resistance in monocot plants.

The original dermaseptin SI gene encodes a 34 AA peptide. The peptide is cytolytic to bacteria, yeast, filamentous fungi, and protozoa, but has not shown cytolytic activity to mammalian cells (Mor & Nicolas, 1994). Immunofluorescence, electron

microscopic, and electrophysiological studies indicated that the interactions between the peptide and the lipid bilayer of cells caused changes in membrane functions, which resulted in the imbalance of the osmotic pressure and cell death (Pouny et al., 1992; Hernandez et al., 1992). Research also demonstrated that the N-terminal 18 AA of the dermaseptin SI is sufficient for and actually has improved antimicrobial activity (Mor & Nicolas, 1994). Considering the small size of the truncated dermaseptin B gene (54 nt), we thought an ubiquitin fusion would enhance its expression. The ubiquitin monomer should be precisely cleaved co- or post-translationally by the endogenous ubiquitin-specific proteases (Hondred et al., 1999; Sivamani & Qu, 2006) inside the cell to release the 18 AA dermaseptin peptide. In our experiment, we only obtained two transgenic tall fescue plants from this gene construct. However, both transgenic plants expressed the fusion gene and exhibited good resistance to *M. grisea*. Derm1 was also resistant to *R. solani.* (Derm2 was not available at the time when the *R. solani* tests were performed.) This may suggest a high potentency of the peptide against fungal infection. Moreover, the transgenic plants looked normal, indicating the peptide was not harmful to plant cells. Recently, resistance using a derivative of a dermaseptin B gene to various fungal pathogens from *Alternaria, Cercospora, Fusarium, Phytophthora, Pythium, Rhizoctonia and Verticillium* genera in transgenic potato was reported (Osusky et al., 2005), suggesting that dermaseptins may have good potential for conferring resistance to many plant fungal pathogens, and have wide application in engineering fungal resistance in plants.

The isolated rice blast R gene, *Pi9*, belongs to a NBS-LRR R gene class. In general, an R gene has strong resistance to only one or a few closely-related races of a pathogen species. The *Pi9* gene is unusual in that it exhibits high resistance to all the rice blast-causing races of *M. grisea* tested so far (Qu et al., 2006). The *Pi9* gene used in the experiments is a 13.5 kb fragment of rice genomic DNA which contains its own promoter, exons, introns, and terminator (Qu et al., 2006). Although we only obtained one transgenic tall fescue plant with the *Pi9* construct, it did have a high level of resistance to the turfgrass isolates of *M. grisea* tested. The first attempt to detect its transcript failed, probably due to its low expression level (Qu et al., 2006). An RT-PCR experiment will be performed to assay the expression of the *Pi9* transcript in the transgenic plant.

It has been observed that some brown patch-susceptible cultivars, such as Coronado and Matador, are resistant, to some extent, to gray leaf spot (Fraser, 1996). The negative correlation was not obvious in our inoculation tests. The control plants from both cultivars developed severe symptoms to inoculation of either pathogen. The fact that five transgenic plants have resistance to both pathogens suggests that the negative correlation of the two traits, if present, could be uncoupled by the biotechnological approach, and these plants could serve as good germplasm to breed new cultivars resistant to both diseases, and maybe even other fungal diseases.

To demonstrate inheritance of a transgene and its associated trait(s) was very important to further support the observation from the T0 generation plants and to utilize the improved traits in the breeding efforts. We have obtained a permit from USDA to

vernalize the resistant transgenic plants in the field during the winter of 2005-2006. The transgenic plants will be crossed with non-transgenic plants in the spring and the T1 generation plants will be studied for transgene transmission, as well as the association between the transgene and the disease resistance traits.

The resistance conferred by a single transgene may be lost quickly due to the strong selection pressure placed on the pathogen. To obtain more durable resistance, a promising way to obtain durable resistance may be to introduce more than one disease resistance gene into a plant. The "pyramid" or "stacking" effects of the transgenes would make the resistance much more difficult for a pathogen to overcome, as demonstrated in an insect case involving the Bt genes and plant resistant QTL (Walker et al., 2004). It would be very possible to have additive or even synergistic effects between the transgenes and to obtain even better resistance when these transgenes are stacked together, which can be achieved by crossing transgenic plants with different trangenes.

References

An G., Ebert R., Mitra A., Ha B. 1988. Binary vectors. Plant Mol. Biol. Manual. A 1-19.

Anderson, N.A. 1982. The genetics and pathology of *Rhizoctonia solani*. Annu. Rev. Phytopathol. 20:329-347.

Bai Y.Y., Qu R.D. 2001. Genetic transformation of elite turf-type cultivar of tall fescue. Intl. Turfgrass Soc. Res. J. 9 129-136.

Bryan G.T., Wu K.S., Farrall L., Jia Y., Hershey H.P., et al. 2000. A single amino acid

difference distinguishes resistant and susceptible alleles of the rice blast resistance gene Pi-ta. Plant Cell 12: 2033-2046.

Broglie K.E., Chet I., Holliday M., Cressman R., Biddle O., Knowlton S., Mauvais C.J., Broglie R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194-1197

Campbell W.H., and Gowri G. 1990. Codon usage in higher plants, green algae, and cyanobacteria. Plant Physiol. 92: 1-11.

De Gray 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.

De Lucca A.J., Cleveland T.E., Wedge D.E. 2005. Plant-derived antifungal proteins and peptides. Canadian J. Microbiol. 51 (12): 1001-1014.

During K., Porsch P., Fladung M., Lorz H. 1993. Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. Plant J. 3 (4): 587-598.

Fraser M.L. 1996. Susceptibility of tall fescue to gray leaf spot, 1995 Biol. Cultural Test 12:130.

Fraser M.L., Rose-Fricker C.A., Meyer W.A. 1999. Registration of 'Matador' tall fescue. Crop Sci. 39: 871-872.

Fraser M.L., Rose-Fricker C.A. 2001. Registration of 'Endeavor' tall fescue. Crop Sci 41: 1997.

Fraser M.L., Rose-Fricker C.A., Meyer W.A. 2001. Registration of 'Olympic Gold' tall

fescue. Crop Sci. 1997-1998.

Fraser M.L., Rose-Fricker C.A., Meyer W.A., Funk C.R. 1998. Registration of 'TarHeel' tall fescue. Crop Sci. 38:539.

Fraser, M.L., Rose-Fricker C.A., Meyer W.A., Funk C.R. 1999. Registration of 'Wolfpack' tall fescue. Crop Sci. 39:872.

Fu A.L., Tisserat N.A., Xiao Y.M., Settle D., Muthukrishnan S., Liang G.H. 2005. Overexpression of rice TLPD34 enhances dollar-spot resistance in transgenic bentgrass. Plant Sci. 168: 671-680.

Guo Z.F., Bonos S., Meyer W.A., Day P.R., Belanger F.C. 2003. Transgenic creeping bentgrass with delayed dollar spot symptom. Mol. Breed. 95-101.

Hondred D., Walker J.M., Mattew D.E., Vierstra R.D. 1999. Use of ubiquitin fusions to augment protein expression in transgenic plants. Plant Physiol. 119: 713-723

Huet, H., Mahendra, S., Wang, J., Sivamani, E., Ong, C. A., Chen, L., deKochko, A., Beachy, R. N., 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 J.S.C., Bres-Vloemans S.A., Dekker I., Van den Elzen P.J.M., Cornelissen B.J.C., Melchers L.S. 1995. Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173-180.

Maher E.A., Lamb C.J., Dixon R.A. 1994. Stress responses in alfalfa (*Medicago sativa* L). XVII. Identification of multiple hydrolases and molecular characterization of an acidic glucanase. Physiol. Mol. Plant Pathol. 43: 329−342.

Amiche M., Ducancel F., Mor A., Boulain J.C., Menez A., Nicolas P. 1994. Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. J. Biol. Chem. 269(27): 17847-17852.

Mor A., Nguyen V.H., Delfour A., Miglioresamour D., Nicolas P. 1991. Isolation, amino acid sequence, and synthesis of dermaseptin, a novel antimicrobial peptide of amphibian skin. Biochemistry. 30 (36): 8824-8830.

Mor A. and Nicolas P. 1994. The NH2-terminal a-helical domain 1-18 of Dermaseptin is responsible for antimicrobial activity. J. Biological Chem. 269(3): 1934-1939.

Osusky M., Osuska L., Kay W., Misra S. 2005. Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2. Theor. Appl. Genet. 111 (4): 711-722.

Owen J.E., Schultz A.W., 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.

Pouny Y., Rapaport D., Mor A., Nicolas P., Shai Y. 1992**.** Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. Biochemistry. 31: 12416-124223.

Qu S.H., Liu G.F., Zhou B., Bellizzi M., Zeng L., Dai L.Y., Han B., Wang G.L. 2006. The broad- spectrum blast resistance gene Pi9 encodes an NBS-LRR protein and isa member of a multigene family in rice. Genetics. (in print)

Ryan C.A., Farmer E.E. 1991. Oligosaccharide signals in plant: a current assessment. Annu. Rew. Plant Physiol. Plant Mol. Biol. 42: 651-674.

Rose-Fricker C.A., Fraser M.L., Meyer W.A., Funk C.R., 1999. Registration of 'Coronado' tall fescue. Crop Science. 39: 288.

Sivamani E., and Qu R.D. 2006. Expression enhancement of a rice polyubiquitin gene promoter. Plant Mol. Bio. 60: 225-239.

Smiley R.W., in M.N. St. Paul (ed.) 1983. Compendium of turfgrass diseases, The American Phytopathological Society, APS Press, St. Paul, Minnesota, pp11-72

Tian W.Z., Yang M., Ding Y.Y., Ding L., Cao S.Y., Ye S.Q., Tang Z.S., Li L.C., Ling Z.Z., Zhang Q., Hain R. 2002 Rice transformation with lysozyme gene and bioassay of the transgenic plants and their progenies. In: “Future of Agriculture – Gene Transfer Technology”, pp. 191-197 (in Chinese)

Tredway L.P., Stevenson K.L., Burpee L.L. 2003. Components of resistance to Magnaporthe grisea in 'Coyote' and 'Coronado' tall fescue Plant Dis. 87 (8): 906-912.

Wang Y.X., Kausch A.P., Chandlee J.M., Luo H., Ruemmele B.A., Browning M., Jackson N., Goldsmith M.R. 2003. Co-transfer and expression of chitinase, glucanase, and bar genes in creeping bentgrass for conferring fungal disease resistance Plant Sci. 165 (3): 497-506.

Wang Z.X., Yano M., Yamanouchi U., Iwamoto M., Monna et al. 1999. The *Pib* for rice blast resistance belongs to the nucleotide biding and leucine-rich repeat class of plant disease resistance genes. Plant J. 19: 55-64.

Walker D.R., Narvel J.M., Boerma H.R., All J.N., Parrott W.A. 2004. A QTL that enhances and broadens Bt insect resistance in soybean. Theor. Appl. genet. 109 (5): 1051-1057.

Zhu Q., Maher E.A, Masoud S., Dixon R.A., Lamb C.J., 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio/Technology 12: 807-812.

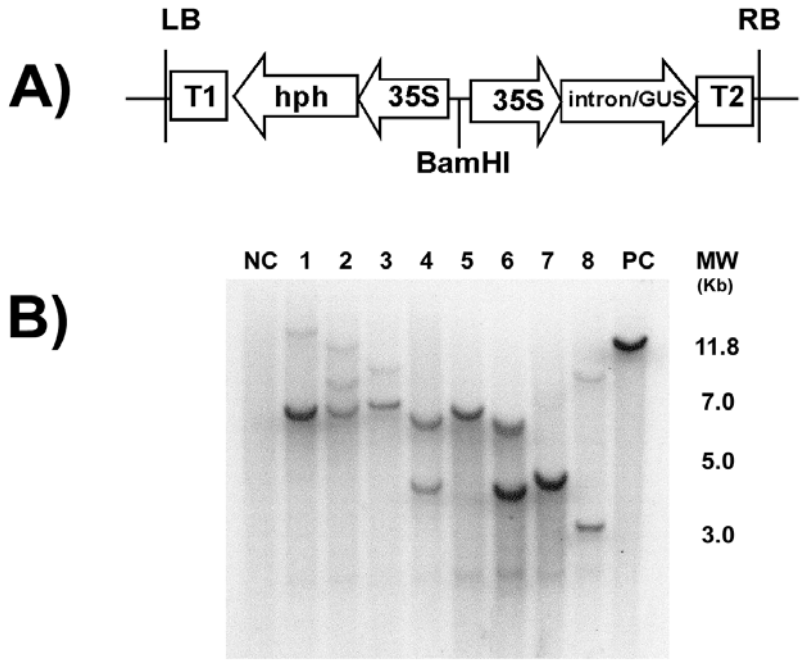


Figure I-1. Map of plant expression vector and southern blot analysis of transgenic plants
A) Map of the T-DNA region containing intron-*gus* and *hph* plant expression cassettes in pCAMBIA1301. RB: right border, LB: left border, 35S: CaMV 35S promoter, intron/GUS: GUS coding region with intron insertion, *hph*: hygromycin phosphotransferase gene coding region, T1: CaMV35S terminator, T2: *Agrobacterium tumefaciens nos* gene terminator. Arrows indicate direction of transcription. The location of restriction site *Bam*HI used in Southern analysis is also indicated.
B) Southern blot analysis of transgenic plants using *hph* coding sequence as the probes. Genomic DNA was digested with *Bam*HI. NC, negative control, DNA from a non-transformed tall fescue plant; 1—8, DNA from 8 putative transgenic plants, PC, positive control and reconstruction: plasmid DNA equivalent to one copy of *hph* gene in a 2C tall fescue genome [30], mixed with 25 mg non-transgenic plant DNA; MW, molecular markers in kb.

Figure I-2. *Agrobacterium tumefaciens*-mediated transformation of tall fescue with hyg B selection and inheritance of hyg B resistance to T1 offspring plants.
A. Transient GUS expression in the infected cells. B. A resistant callus growing on hyg B selection. C. Stable GUS gene expression in an hyg B resistant callus. D. Plantlet regeneration under hyg B selection. E. Root development of an hyg B resistant plantlet in the selection rooting medium. F. Various levels of GUS gene expression in different transgenic plants. G. Transgenic plants growing in soil. H. Response of progenies of transgenic plant ETR3-6 to hyg B (a: an offspring plant resistant to hyg B; b: an offspring plant susceptible to hyg B); the photo was taken after two-month selection.

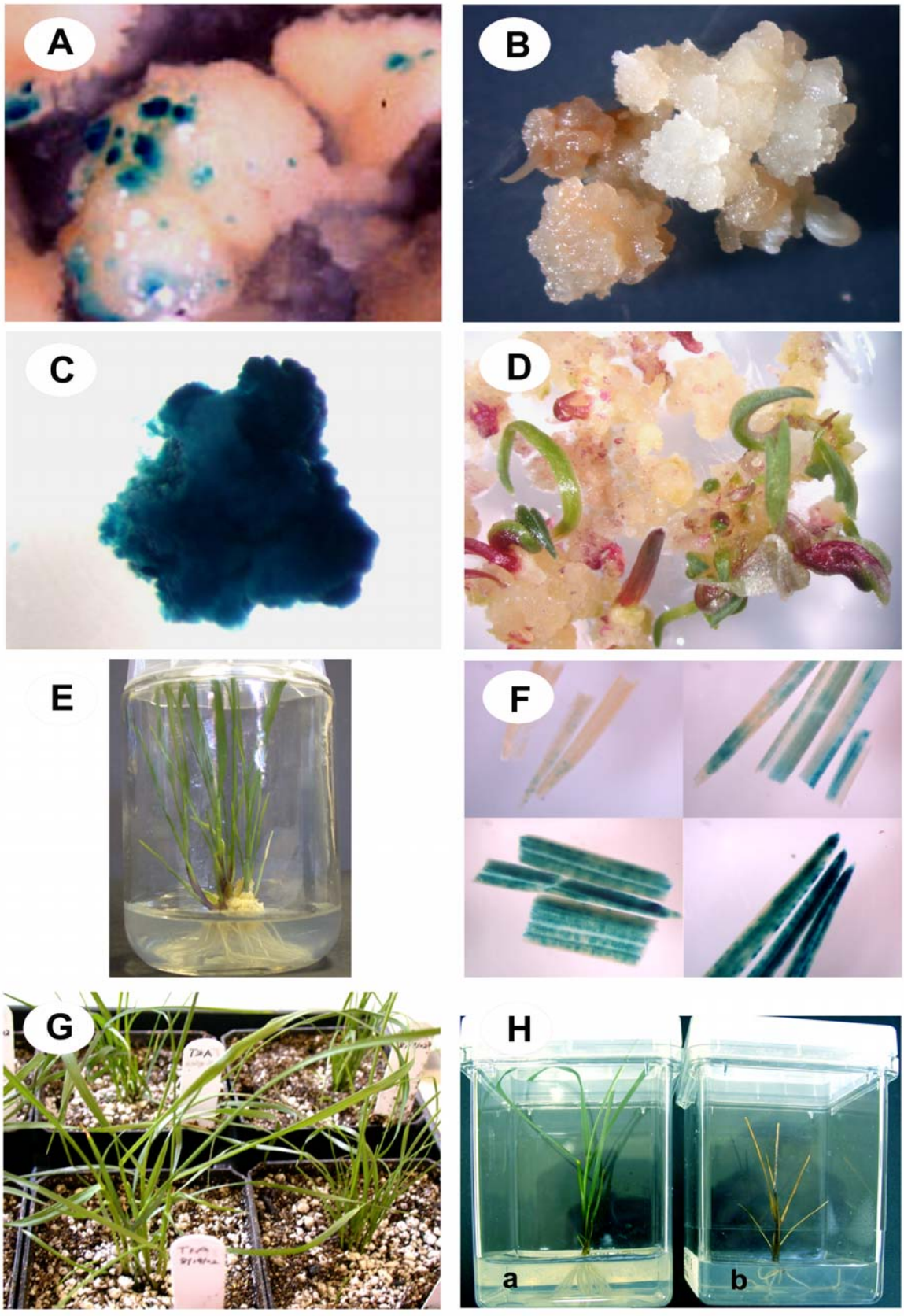

A
B
C
D
E
F
G
H
a
b

Table I-1. Results of three experiments of *Agrobacterium*-mediated tall fescue transformation (cv. "Matador")

Experiment	Total no. of callus infected	Hyg B resistant callus	Regenerated plants	GUS+ plants	Overall transformation frequency
1	120	33.30%	7	7	5.83%
2	90	33.30%	1	1	1.10%
3	90	36.70%	16	16	17.78%
Total	300	34.30%	24	24	8.00%

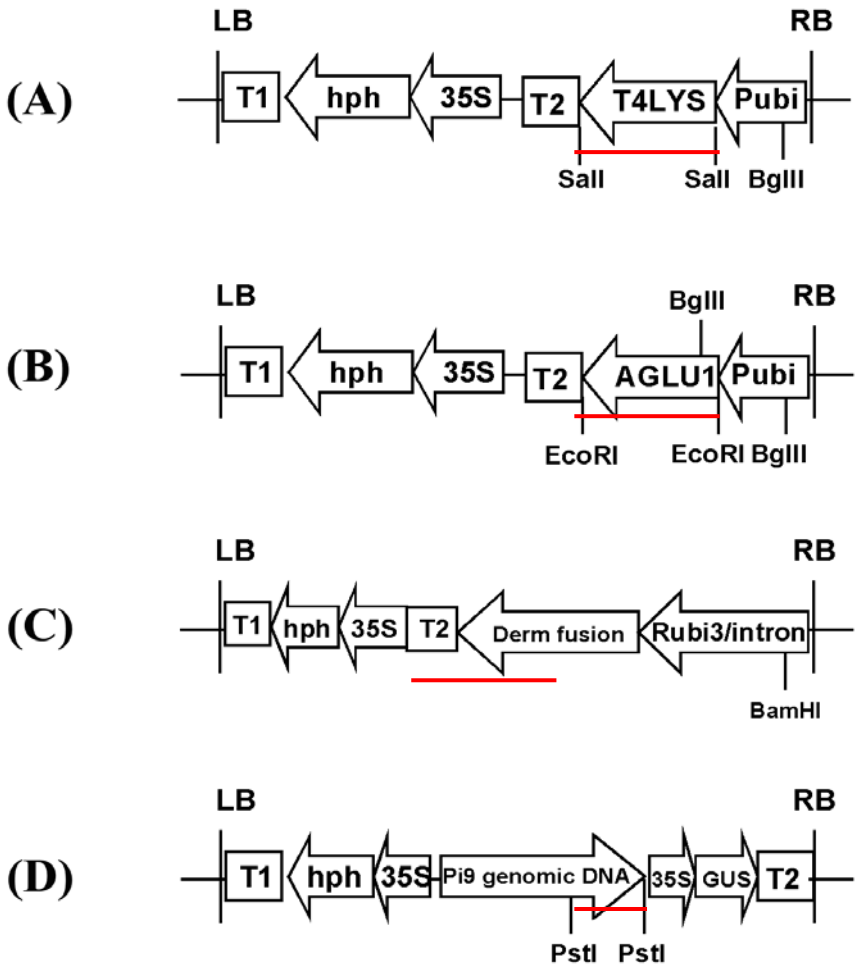


Figure II-1. Diagrams of the binary vectors used in the transformation experiments. All the vectors are the derivatives of pCAMBIA1300. Only T-DNA regions are shown here. RB: right border, LB: left border, 35S: CaMV 35S promoter, *hph*: hygromycin phosphotransferase gene coding region, T1: CaMV 35S terminator. Arrows indicate directions of transcription. Bars were used to indicate the probes regions.
(A) T-DNA region of pSD9. Pubi: maize *ubi1* gene promoter, T4LYS: T4 phage lysozyme gene coding region, T2: *Agrobacterium tumefaciens nos* gene terminator. The *Bgl*II and *Sal*I restriction sites are indicated.
(B) T-DNA region of pSD7. AGLU1: alfalfa *AGLU1* glucanase gene coding region. The *Bgl*II and *EcoR*I restriction sites are indicated.
(C) T-DNA region of pSD12. Rubi3/intron: rice *rubi3* gene promoter with its 5' UTR intron, Derm fusion: in frame fusion of *rubi3* ubiquitin monomer coding sequence with the truncated 18 AA dermaseptin SI gene. The *BamH*I site is indicated.
(D) T-DNA region of pNBS2 containing the rice Pi9 genomic sequence and *a GUS* reporter gene construct. The *Pst*I sites are indicated. GUS: GUS coding region with an intron insertion

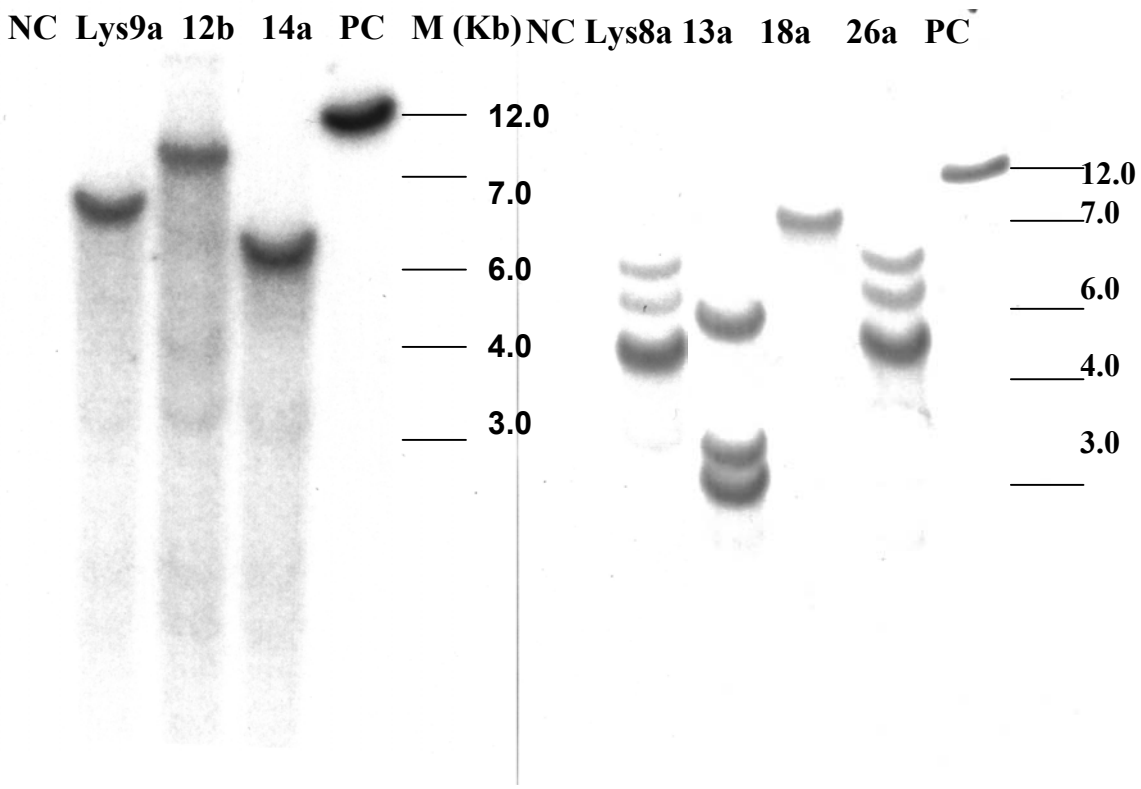


Fig. II-2. Southern blot analysis of Lys transgenic plants using T4 lysozyme coding sequence as the probe. NC, negative control, DNA from a non-transformed tall fescue plant; PC, positive control and reconstruction: plasmid pSD9 DNA equivalent to one copy of T4 lysozyme gene in a 2C tall fescue genome, mixed with 25 μg non-transgenic plant DNA; Lys refers to transgenic plants transformed with the T4 phage lysozyme gene. M, DNA molecular marker.

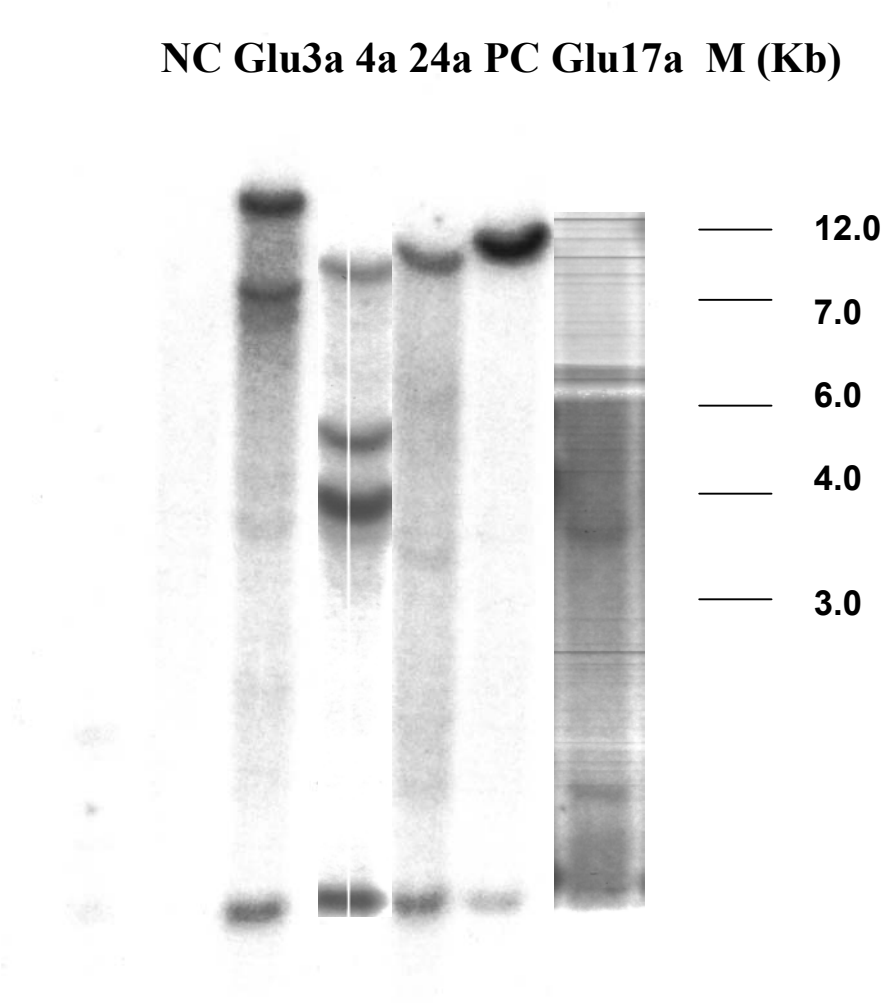


Fig. II-3. Southern blot analysis of Glu transgenic plants using *AGLU1* coding sequence as the probe. NC, negative control, DNA from a non-transformed tall fescue plant; PC, positive control and reconstruction: plasmid pSD7 DNA equivalent to one copy of *AGLU1* gene in a 2C tall fescue genome, mixed with 25 mg non-transgenic plant DNA; Glu refers to transgenic plants. M, DNA molecular marker.

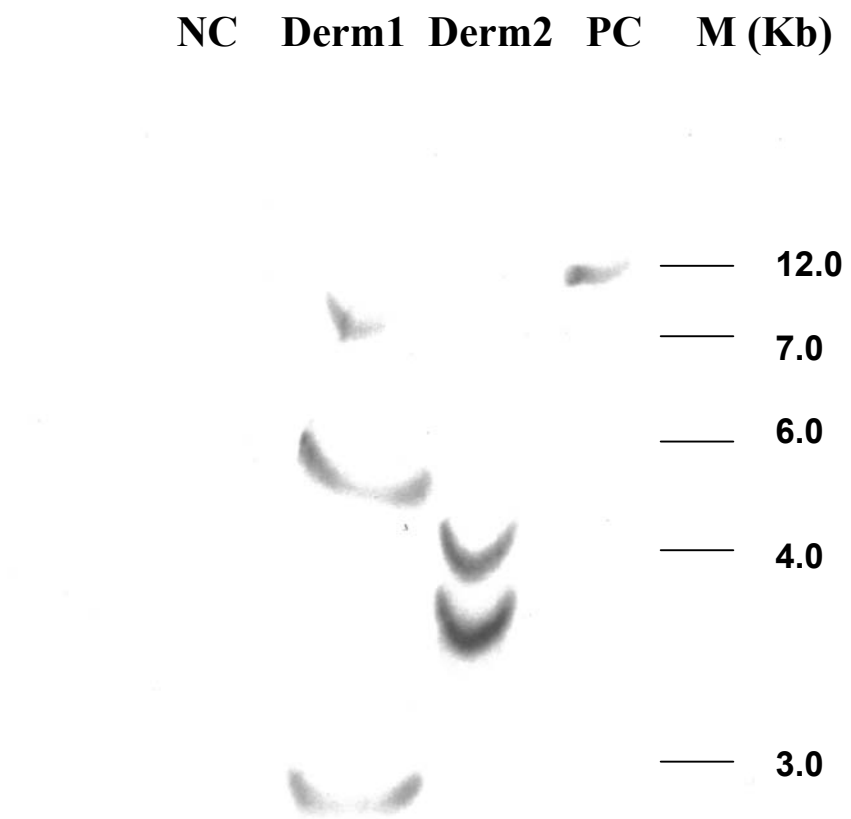


Fig. II-4 Southern blot analysis of Derm transgenic plants containing Dermaseptin SI fusion gene, using Dermaseptin SI coding sequence and the *nos* terminator sequence as the probe. NC, negative control, DNA from a non-transformed tall fescue plant; PC, positive control and reconstruction: plasmid pSD12 DNA equivalent to one copy of Dermaseptin SI gene in a 2C tall fescue genome, mixed with 25 mg non-transgenic plant DNA; M, DNA molecular marker.

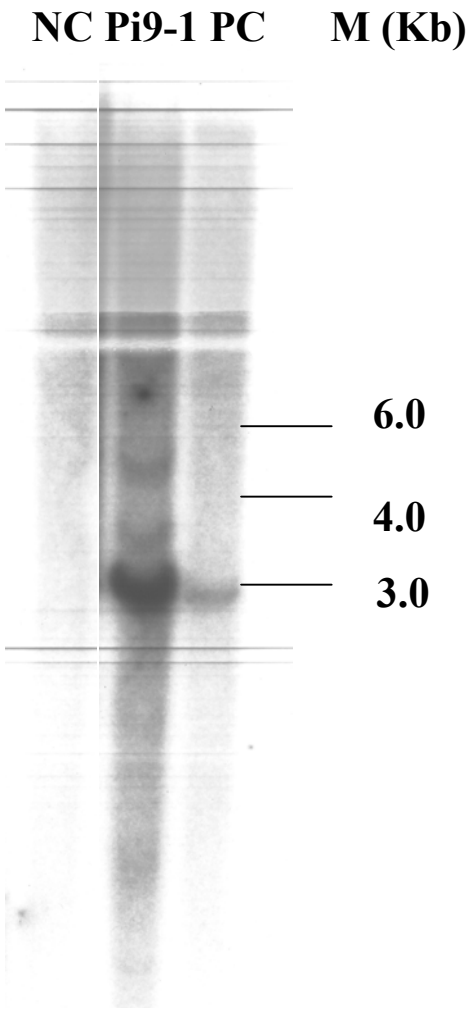


Fig II-5 Southern blot analysis of the Pi9-1 transgenic plant. NC, negative control, DNA from a non-transformed tall fescue plant; PC, positive control and reconstruction: plasmid pNBS2 DNA equivalent to one copy of the Pi9 gene in a 2C tall fescue genome, mixed with 25 mg non-transgenic plant DNA; M, DNA molecular marker.

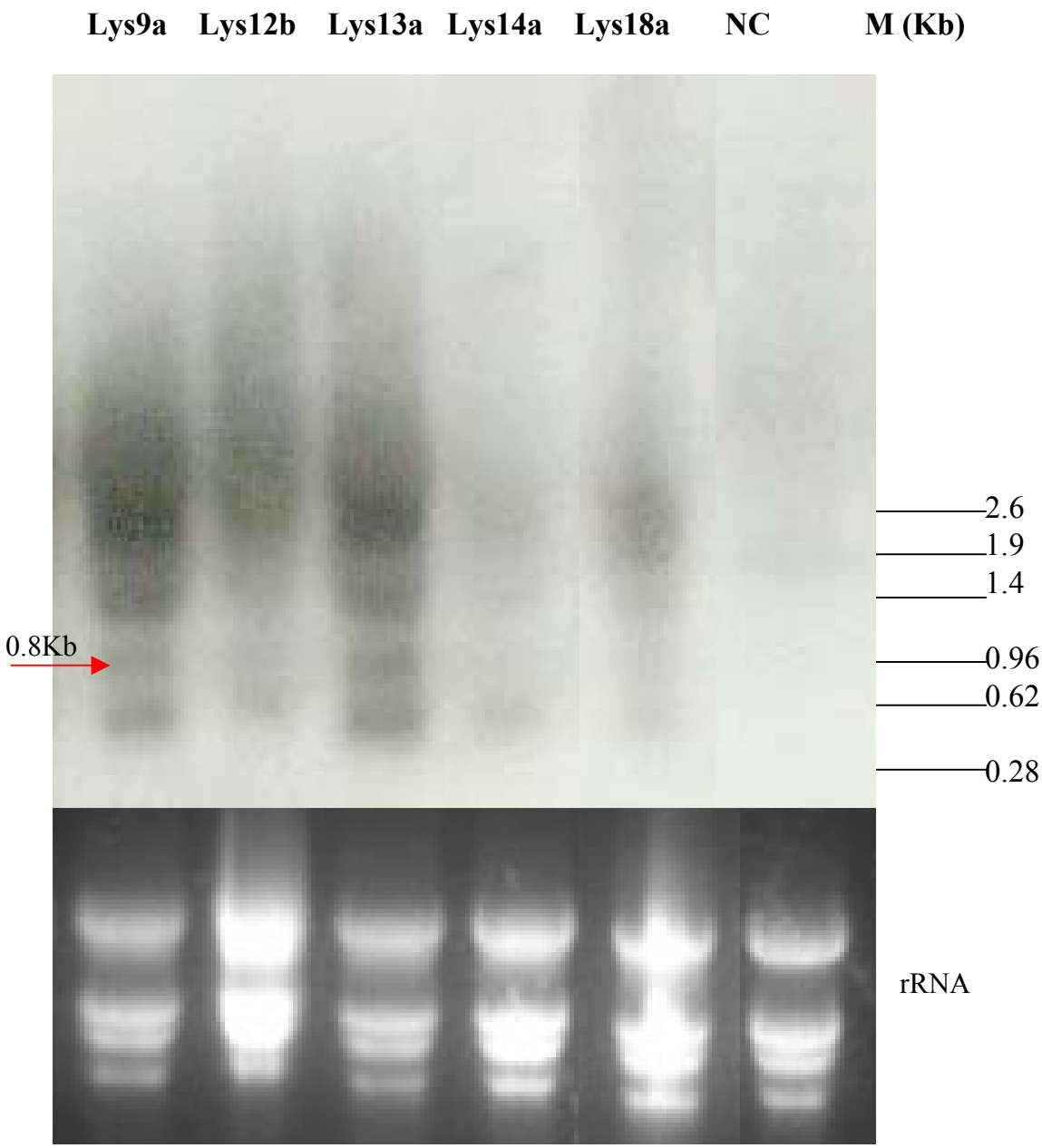


Fig. II-6 Northern blot analysis of total RNA from 5 Lys transgenic plants using T4 lysozyme coding sequence as the probes. NC, negative control, total RNA from a non-transformed tall fescue plant. Lys refers to transgenic plants having T4 lysozyme gene. M, RNA molecular marker. The expected 0.8 kb lysozyme mRNA was indicated. Photo of rRNAs from EtBr stained gel is shown as a loading control.

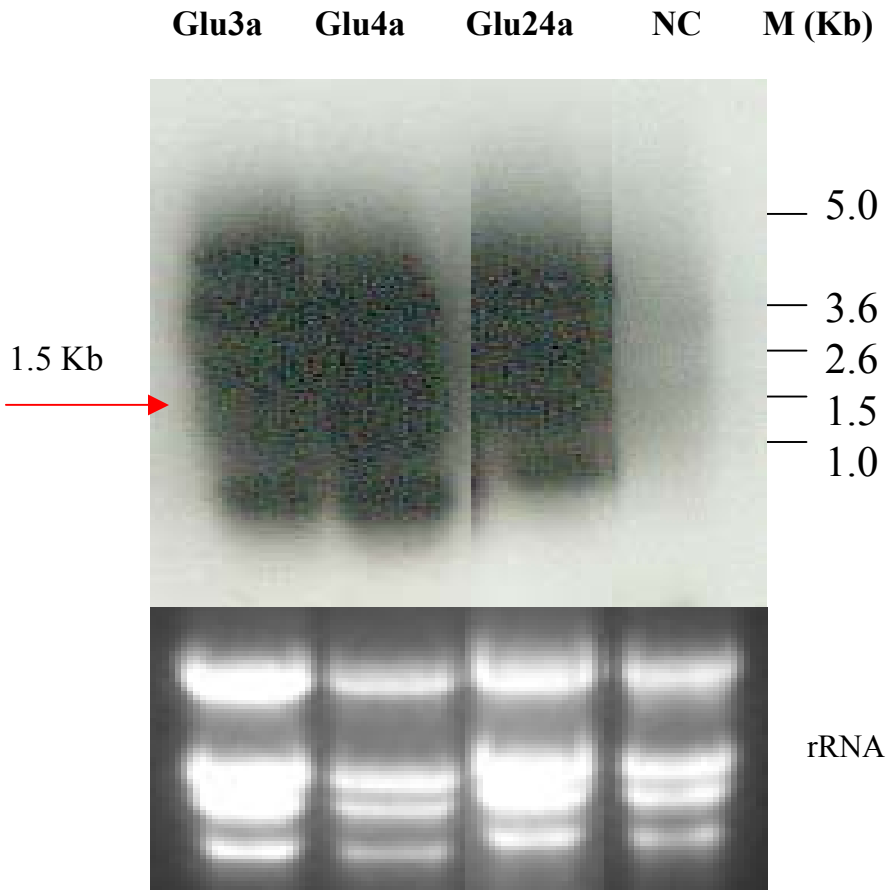


Fig. II-7 Northern blot analysis of Glu transgenic plants using *AGLU1* coding sequence as the probe. NC, negative control, RNA from a non-transformed tall fescue plant. Glu refers to transgenic plants containing AGLU1 gene; M, RNA molecular marker. The expected 1.5 kb *AGLU1* mRNA was indicated. Photo of rRNAs from EtBr stained gel is shown as a loading control.

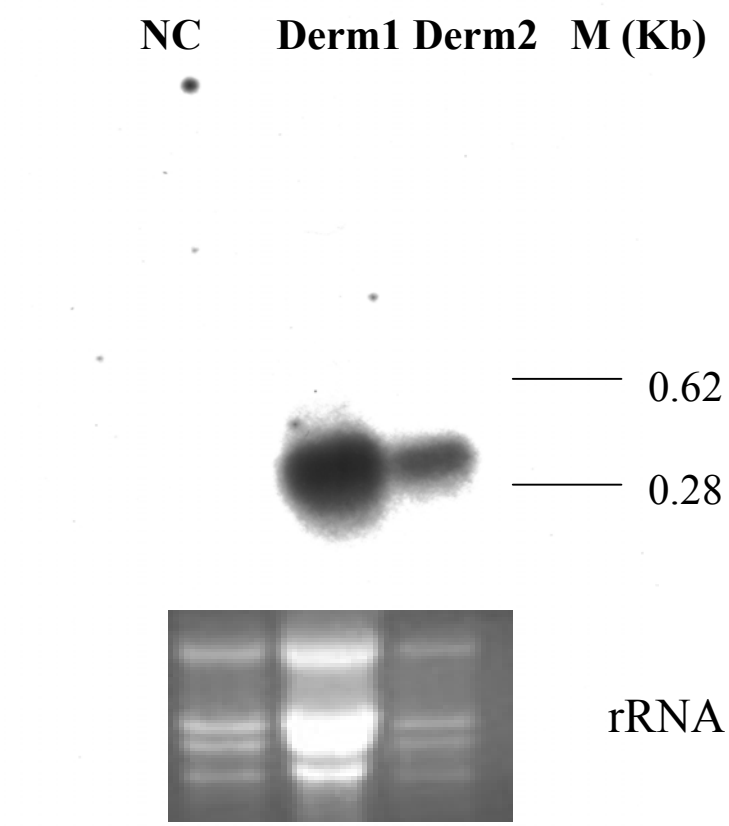
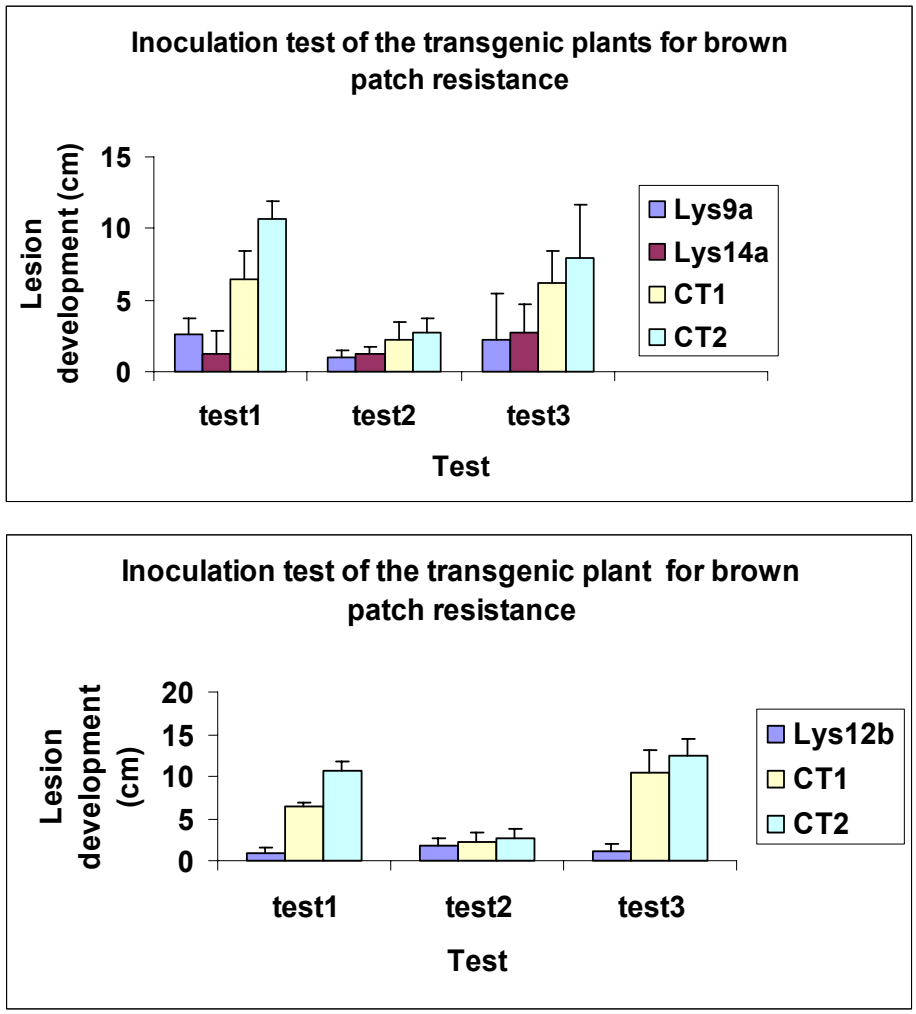


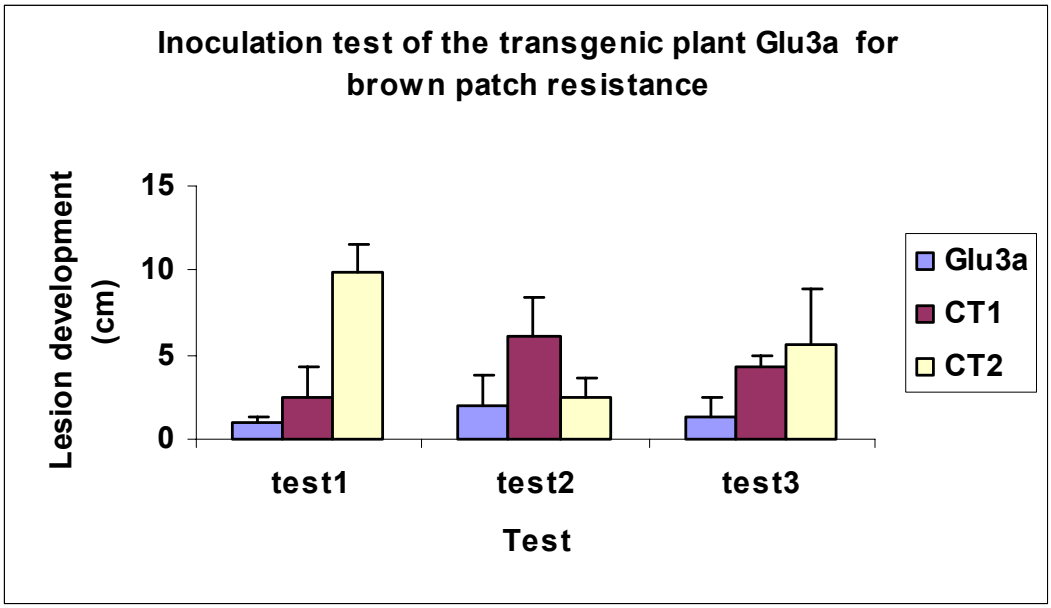
Fig II-8 Northern blot analysis of the Derm transgenic plants expressing the dermaseptin SI fusion gene, using the dermaseptin SI coding and *nos* terminator as the probe. NC, negative control, RNA from a non-transformed tall fescue plant; Derm refers to transgenic plants; M, molecular weight markers. Photo of rRNAs from EtBr stained gel is shown as a loading control.

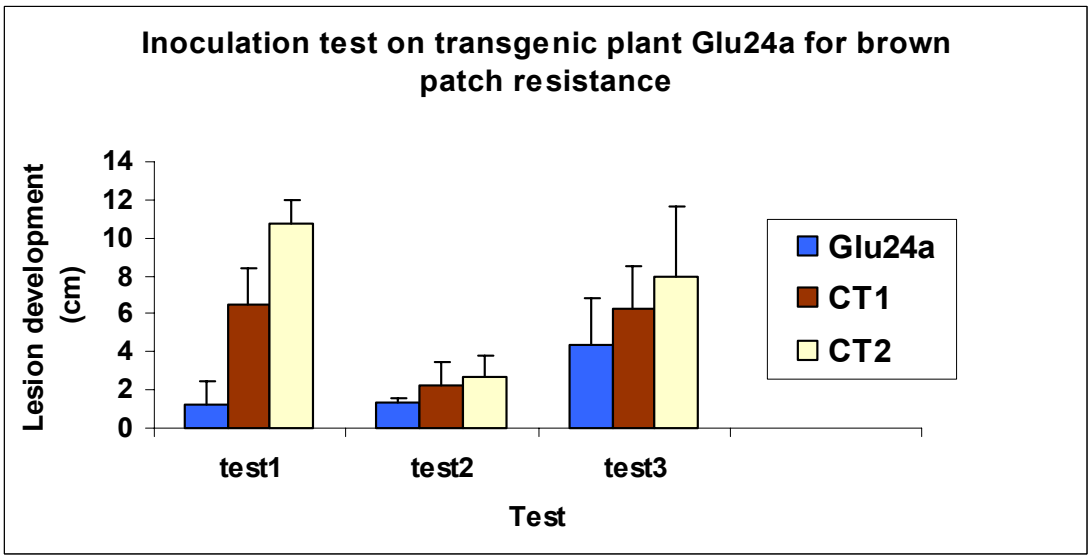


(A)

Fig. II-9. *R. solani* inoculation test of three transgenic plants containing T4 phage lysozyme gene. (A). Lesion development data of the Lys transgenic plants (Lys9a, 12b, and 14a) and two non-transformed plants (CT1 and 2). (cv. Coronado). Lesion development was determined 14 days after inoculation of leaves by measuring the total distance in cm from the point of inoculation where lesions developed. (B). Inoculated leaves of Lys9a and a control plant (Ck). Leaves were collected and photo taken 2 weeks after the inoculation.

Fig. II-9 (B)





(A)

Fig II-10. *R. solani* inoculation test of the Glu transgenic plants containing *AGLU1* gene. (A). Lesion development data of two Glu transgenic plants in comparison to non-transformed control plants (CT1 and CT2). Glu3a was from cv. Matador and Glu24a was from cv. Coronado. They were compared with control plants of their own cultivars. (B). Inoculated leaves of Glu24a and a control plant (Ck). Leaves were collected and photo taken 2 weeks after inoculation, cv. Coronado.

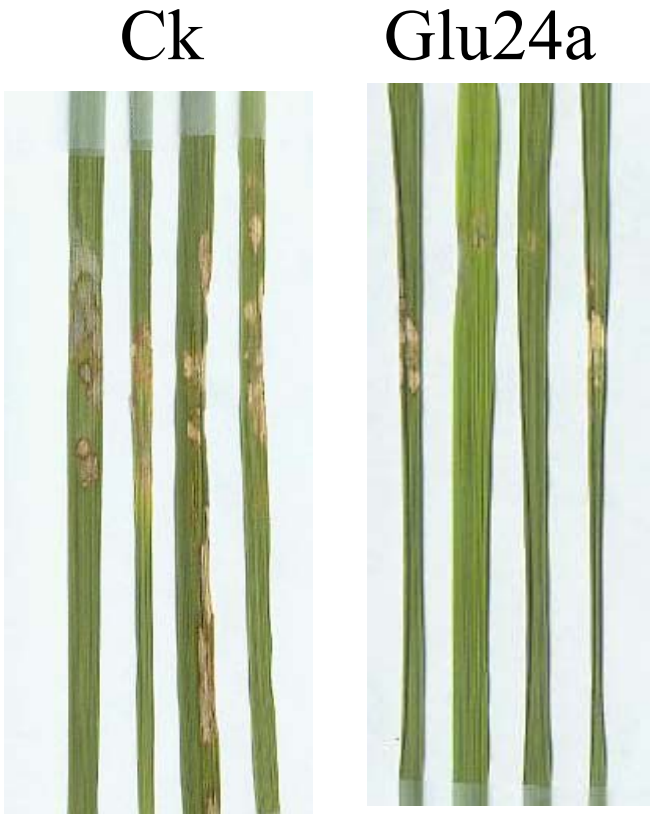
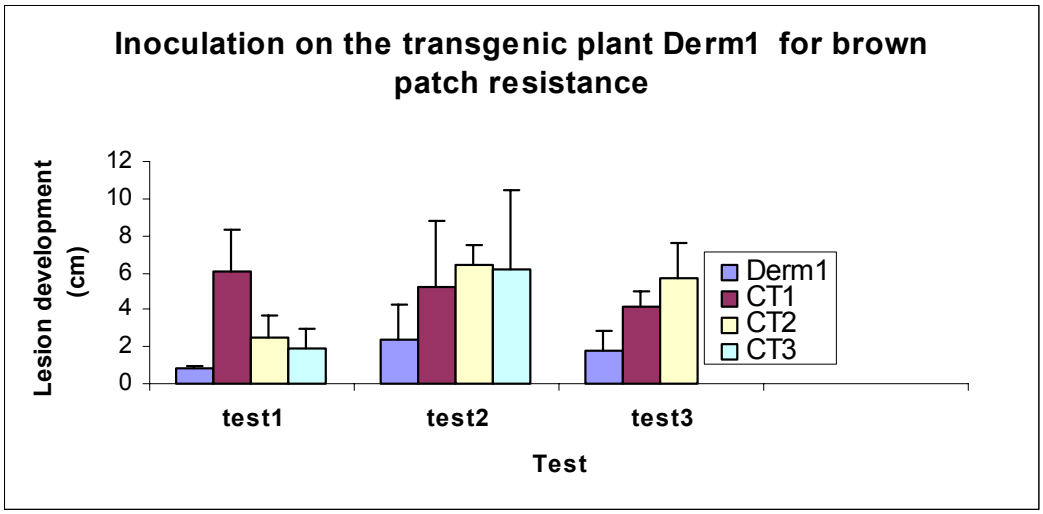


Fig. II-10(B)

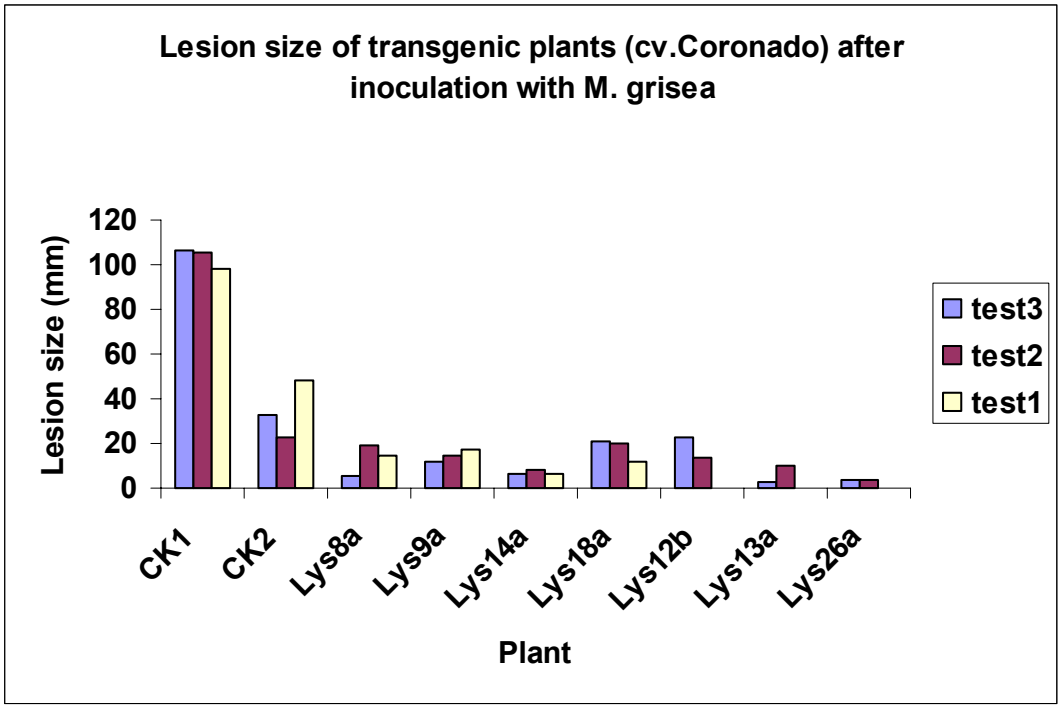
(A)



(B)

Fig. II-11. *R. solani* inoculation test of the transgenic plant Derm-1 containing the dermaseptin SI fusion gene. (A). Lesion development data of Derm-1 in comparison with three non-transformed control plants (CT1, 2, and 3). (B). Inoculated leaves of Derm-1 and a control plant (Ck).

(A)



(B)

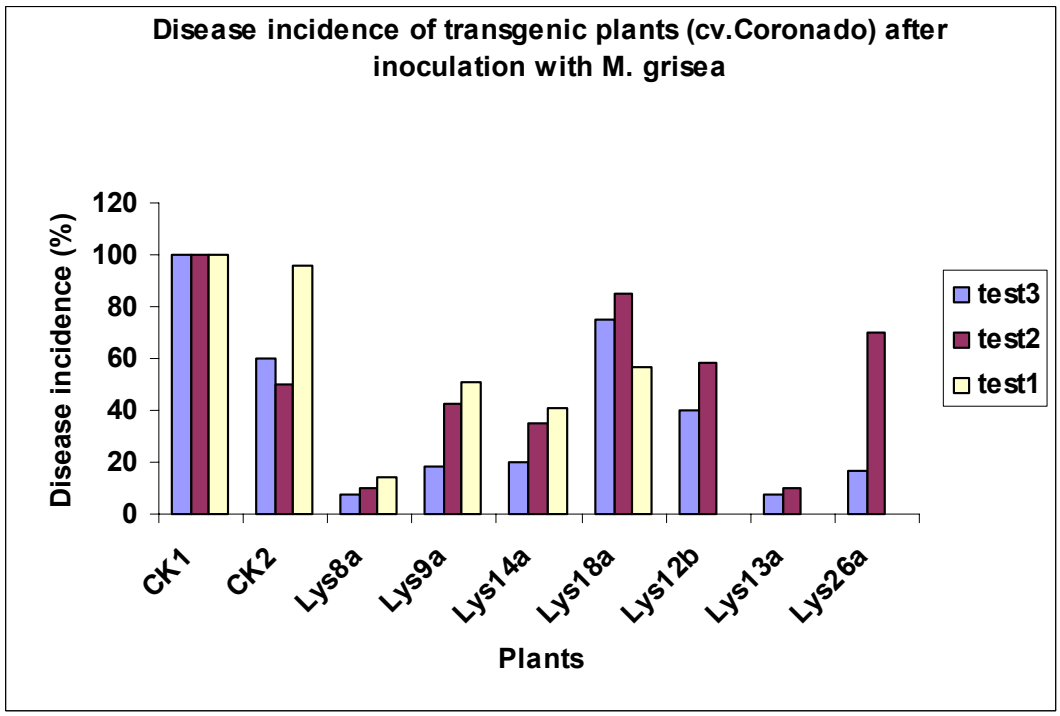
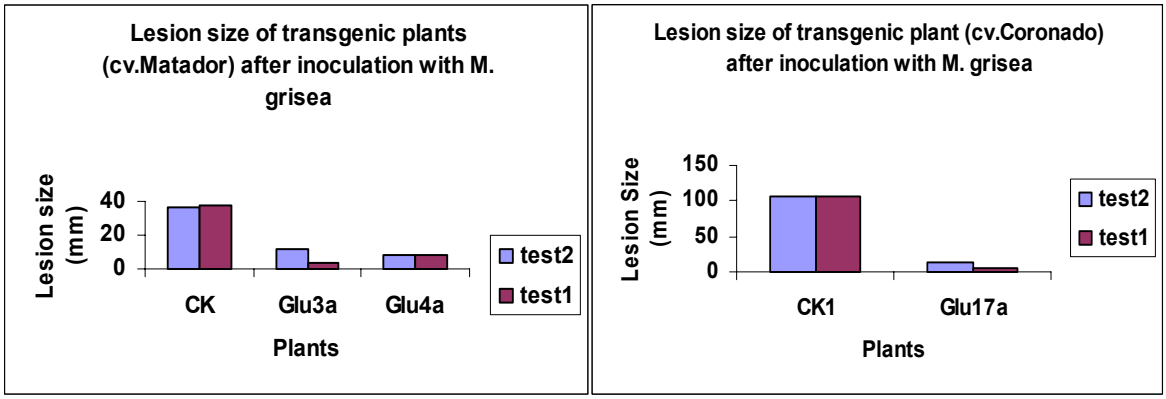


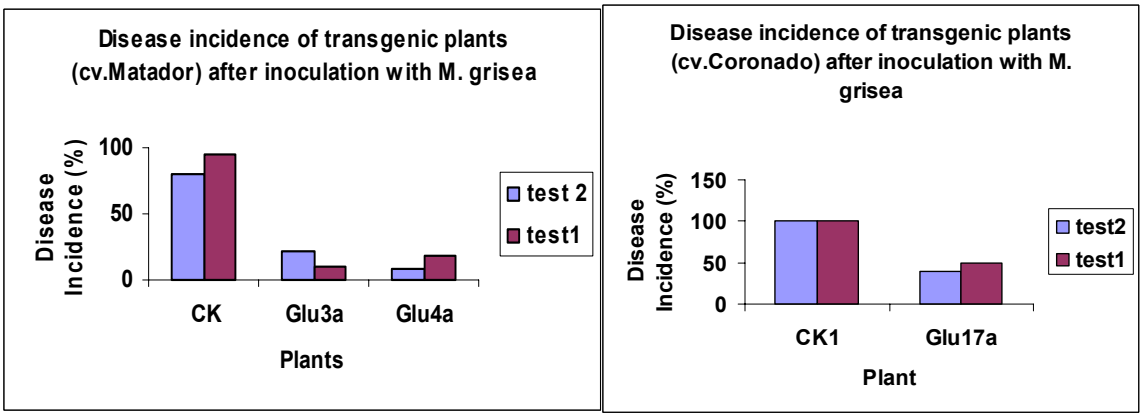
Fig. II-12. *M. grisea* inoculation test of the transgenic plants containing the T4 lysozyme gene. (A). Lesion size data of 7 transgenic plants resistant to *M. grisea* in comparison with two non-transformed control plants (CK1 and CK2, cv. Coronado). (B). Disease incidence data of the above plants. Lys refers to transgenic plants containing T4 lysozyme gene. (C). Inoculated leaves of two transgenic plants, Lys9a and Lys12b and a control plant.

CK1 Lys9a Lys12b

Fig. II-12 (C)



A



B

Fig. II-13. *M. grisea* inoculation test of three transgenic plants expressing *AGLU1* gene. (A). Lesion size data of the transgenic plants in comparison to non-transformed plants. (B). Disease incidence data of the above plants. Glu refers to transgenic plants containing alfalfa *AGLU1* gene. (C). Inoculated leaves of transgenic plant Glu3a and a control plant (Ck) collected and photographed 2 weeks after inoculation (cv. Matador).

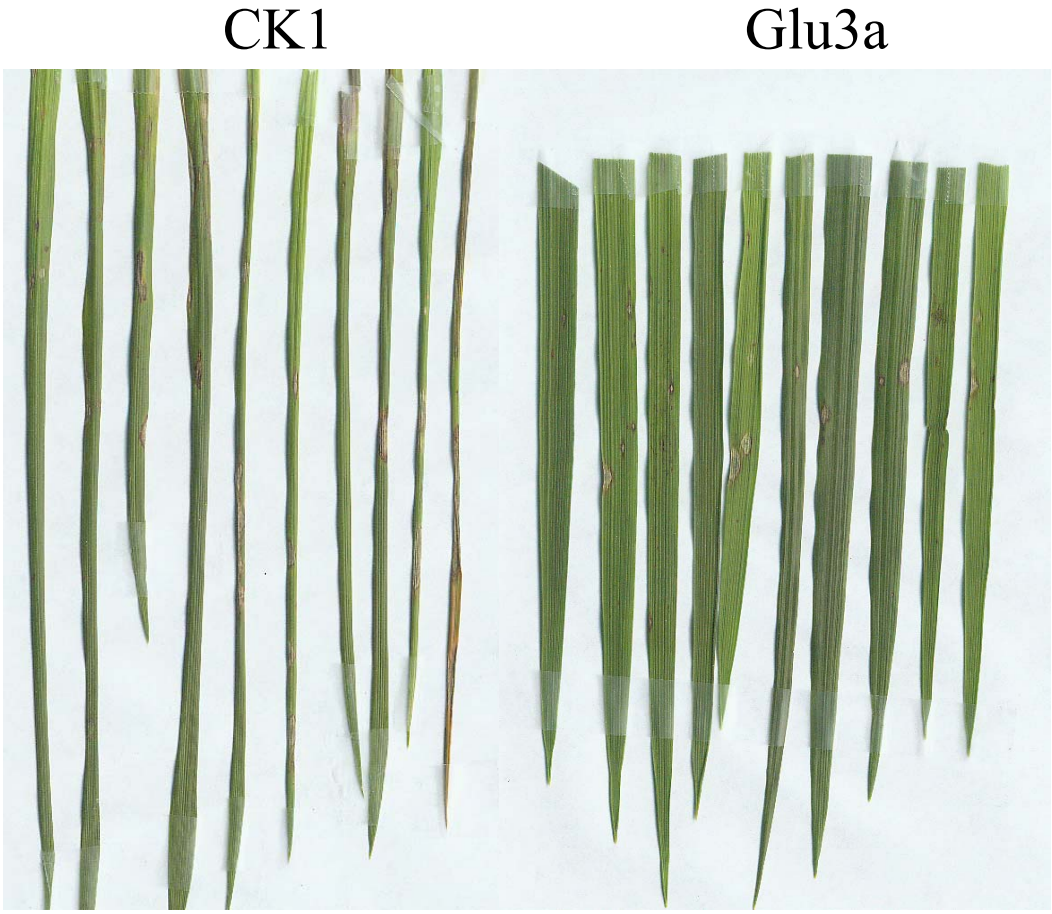
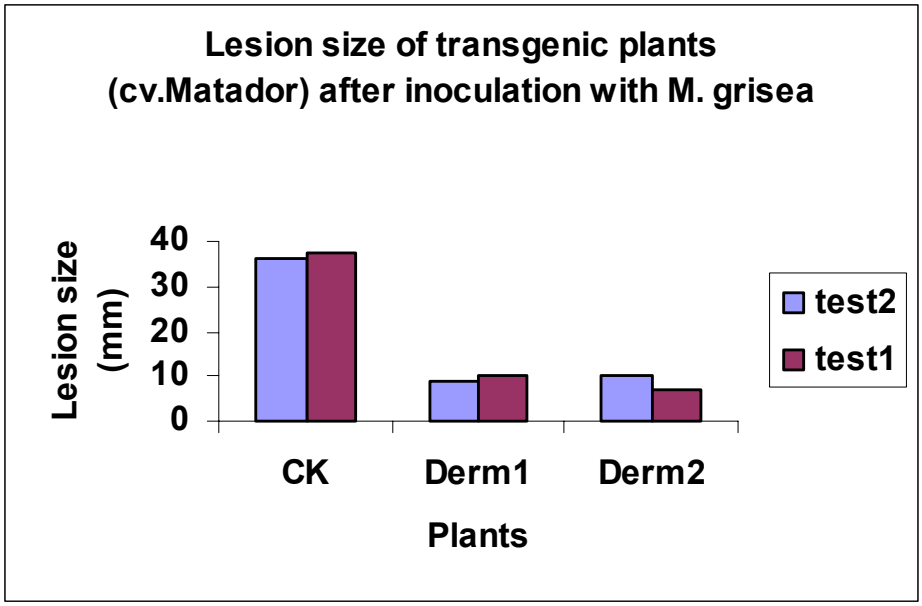


Fig. II-13 (C)

(A)



(B)

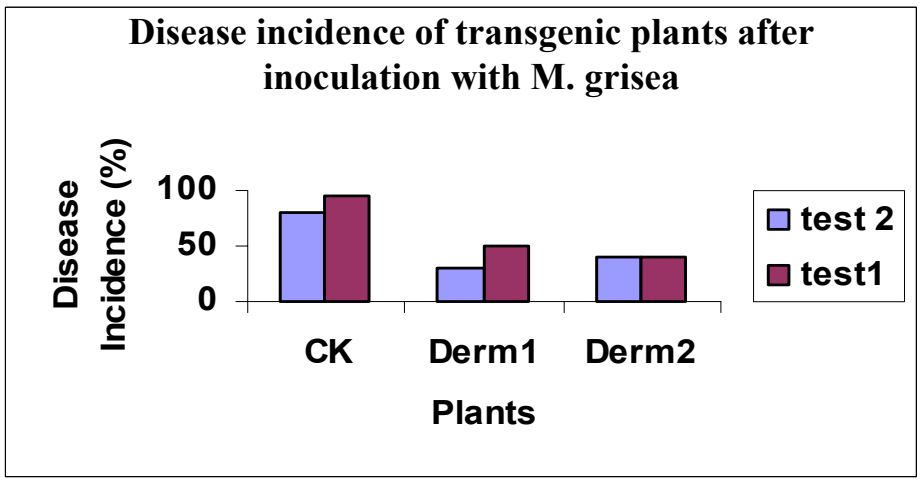
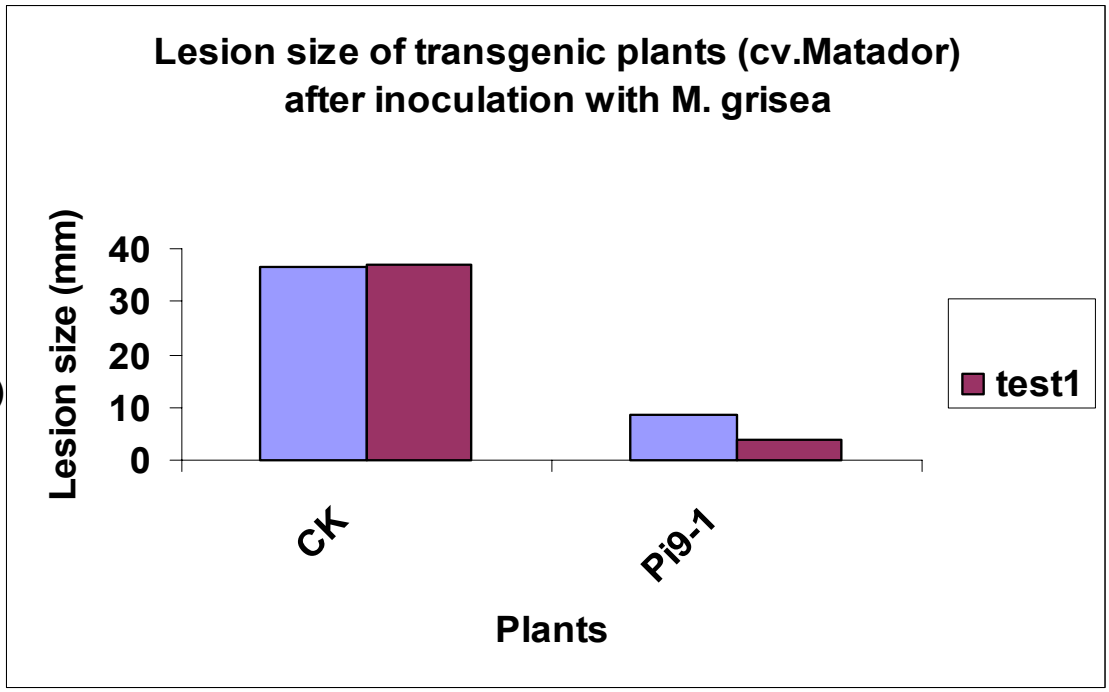


Fig II-14. *M. grisea* inoculation test of the Derm transgenic plants containing dermaseptin SI fusion gene. (A). Lesion size data of the transgenic plants in comparison to a non-transformed plant (CK). (B). Disease incidence data of the above plants. Derm refers to transgenic plants containing the dermaceptin SI fusion gene. (C). Inoculated leaves of transgenic plant Derm-1 and a control plant (Ck).

CK Derm-1

Fig II-14 (C)

(A)



(B)

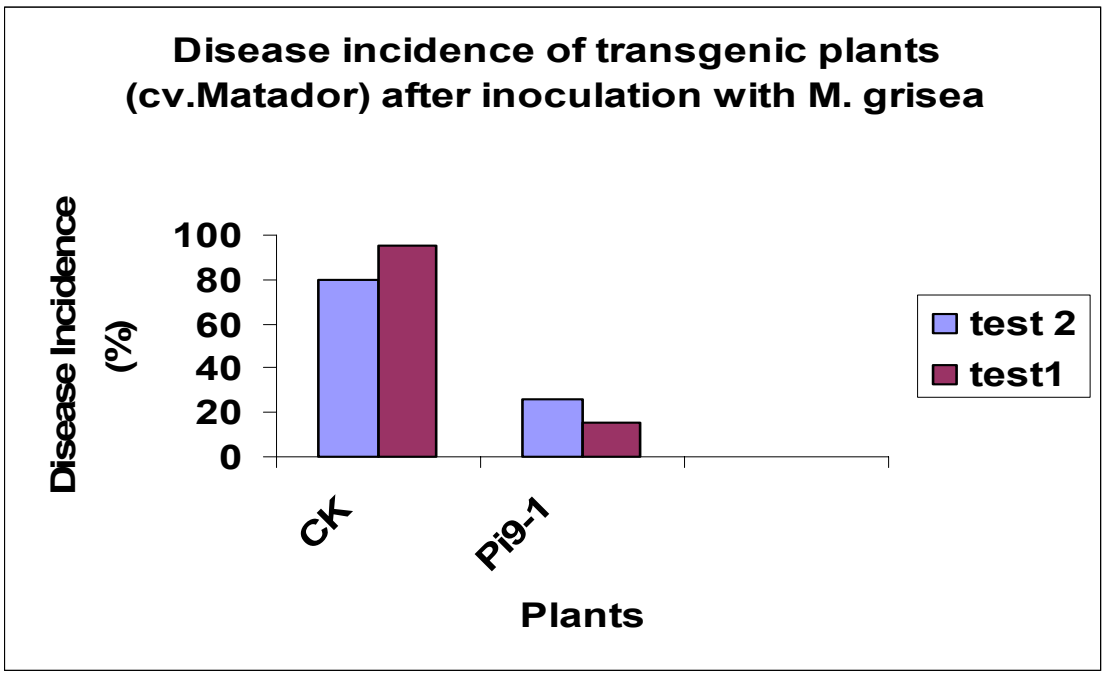
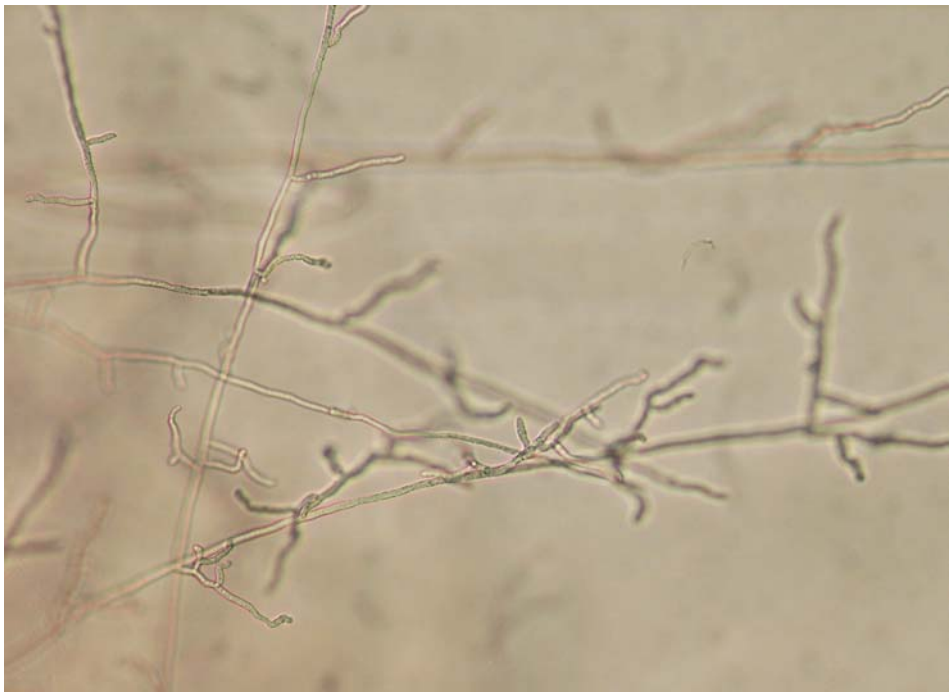


Fig II-15. *M. grisea* inoculation test of the Pi9-1 transgenic plant containing the rice *Pi9* gene. (A). Lesion size data of the Pi9-1 transgenic plant in comparison to a non-transformed plant (CK). (B). Disease incidence of the above plants. (C). Inoculated leaves of transgenic plant Pi9-1 and a control plant (Ck).

CK Pi9-1

Fig. II-15 (C)

CK



Lys9a

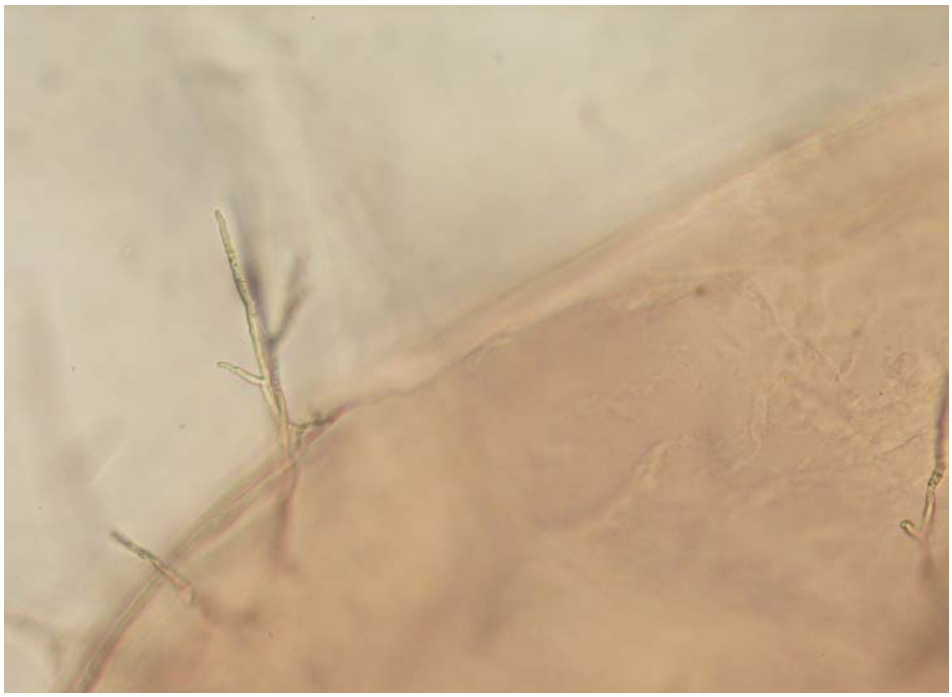


Fig. II-16. Comparison of the growth of hyphae between transgenic plants Lys9a and non-transformed plant (CK, cv. Coronado) by *in vitro* leaf sap assay of transgenic plants inoculated with *R. solani.* Photos were taken 24 hrs after inoculation.

Table II-1a. Statistical analysis of lesion size on transgenic plants (cv. Coronado) two weeks after inoculation with *R. solani*

		Lesion size	
Plant	Gene	Mean (cm)	Resistance significant P value
Lys9a	T4 phage lysozyme	1.96±0.90	0.0063
Lys12b	T4 phage lysozyme	1.20±0.41	0.0227
Lys14a	T4 phage lysozyme	1.76±0.90	0.0047
Glu24a	AGLU1	2.30±1.81	0.0177

Table II-1b. Statistical analysis of lesion size on transgenic plants (cv. Matador) two weeks after inoculation with *R. solani*

		Lesion size	
Plant	Gene	Mean (cm)	Resistance significant P value
Glu3a	AGLU1	1.43±0.48	0.0555
Derm1	Dermaseptin SI	1.66±0.92	0.0098

$P < 0.01$ indicates highly significant difference between transgenic plants and non-transformed plants, $P < 0.05$ indicates significant difference between transgenic plants and non-transformed plants, and $P < 0.1$ indicates slight difference between transgenic plants and non-transformed plants.
The inoculation tests were not performed in the same experiments and thus the values of the control plants varied and are not listed here. The lesion size of the transgenic plants is not directly comparable.

Table II-2a. Statistical analysis of lesion size and disease incidence on transgenic plants (cv. Matador) 10 days after inoculation with *M. grisea*

		Leaf lesion		Disease incidence	
Plant	Gene	mean (mm)	P value	mean (%)	P value
CK		36.85±0.49		87.5±10.61	
Glu3a	AGLU1	7.83±6.19	0.0001	15.95±8.41	0.0007
Glu4a	AGLU1	8.33±0.24	0.0001	12.85±7.28	0.0006
Derm 1	Dermaseptin SI	9.25±0.92	0.0002	39.90±14.28	0.0042
Derm 2	Dermaseptin SI	8.63±1.94	0.0002	40.00±0	0.0043
Pi9-1	Rice Pi9	6.33±3.37	0.0001	20.55±7.85	0.0009

$P < 0.01$ indicates highly significant difference between transgenic plants and non-transformed plants, $P < 0.05$ indicates significant difference between transgenic plants and non-transformed plants, and $P < 0.1$ indicates slight difference between transgenic plants and non-transformed plants.

Table II-2b. Statistical analysis of lesion size and disease incidence on transgenic plants (cv. Coronado) 10 days after inoculation with *M. grisea*

		Leaf lesion		Disease incidence	
Plant	Gene	mean (mm)	P value	mean (%)	P value
CK1		103.3±12.8		100±0	
CK2		34.63±7.02		68.61±24.1	
Lys8a	T4 phage lysozyme	12.78±7.01	<0.0001	10.68±3.57	<0.0001
Lys9a	T4 phage lysozyme	14.37±2.81	<0.0001	37.36±17.3	0.0003
Lys14a	T4 phage lysozyme	6.97±0.96	<0.0001	31.81±10.69	0.0001
Lys18a	T4 phage lysozyme	17.43±5.16	<0.0001	72.22±14.37	0.2185
CK1		105.9±0.49		100±0	
CK2		27.85±7.28		55.00±7.07	
Lys12b	T4 phage lysozyme	18.20±5.94	0.0021	49±12.73	0.0897
Lys13a	T4 phage lysozyme	6.22±4.93	0.0003	8.55±2.05	0.0038
Lys26a	T4 phage lysozyme	3.64±0.19	0.0002	43.35±37.7	0.0534
Glu17a	AGLU1	10.31±5.23	0.0006	45.00±7.07	0.062

Table II-3. Summary of tall fescue transformation for resistance to *R. solani* and *M. grisea*.

Transgenes	Total number of transgenic plants	Number of resistant plants to Brown patch	Gray leaf spot	Both diseases
T4 Lysozyme	14	3	7	3
AGLU1	12	2	3	1
Dermaseptin SI	2	1*	2	1
Pi9	1	N/A	1	N/A
Total	29	6	13	5

* Only one plant was tested.