

ABSTRACT

ZHOU, BINBIN. Novel Approaches to Genetically Engineer Tall Fescue (*Festuca arundinacea* Schreb.) for Brown Patch Resistance. (Under the direction of Dr. Rongda Qu).

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season turf and forage grass widely used for home lawns in North Carolina and other transition zone states in the US. Tall fescue has higher heat- and drought-tolerance compared to other cool season turfgrass, and with proper irrigation, it can sustain a whole year green lawn, which increases its popularity. The most serious and frequently occurring disease of tall fescue is brown patch which is caused by a basidiomycete fungus – *Rhizoctonia solani*. The objective of this research was to genetically engineer tall fescue to improve its resistance to brown patch disease.

Foreign genes were introduced into two cultivars, ‘Coronado’ or ‘Kentucky31’, of tall fescue through *Agrobacterium*-mediated transformation. The transgenes we used in this project belong to two categories: 1. A gene encoding antimicrobial peptide; 2. Gene segments from *Rhizoctonia solani*. In the first category, we used *Pen4-1* gene from shrimp, which encodes an antimicrobial peptide – penaeidin. Among the six transgenic plants containing *Pen4-1* gene, five of them displayed significantly higher level of brown patch resistance (averagely 42.7% improvement in resistance compared to wild type tall fescue). For the second category, we introduced gene segments from *Rhizoctonia solani* essential to the pathogen and utilized RNA interference technology to inhibit fungus gene expression, thus inhibiting the fungal infection. Based on this hypothesis, the transgenic tall fescue plants should be able to produce small interference RNAs (siRNAs) that target the fungus genes and

inhibit the expression of these target genes. Four gene constructs were used to transform tall fescue, two constructs with the natural gene segment sequences and the other two with modified sequences (nucleotides) of the gene segments. In total, 19 transgenic plants were confirmed with Southern blots and 12 of them showed the accumulation of siRNAs. Among these plants, six displayed improved resistance against *Rhizoctonia solani*, demonstrating that tall fescue's resistance against the fungal pathogen *Rhizoctonia solani* can be improved by introducing selected gene segments of *Rhizoctonia solani*. These segments are from the genes encoding the following proteins: RNA polymerase, importin beta-1 subunit, cohesin complex subunit Psm1, and ubiquitin ligase E3.

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Novel Approaches to Genetically Engineer Tall Fescue (*Festuca arundinacea* Schreb.) for
Brown Patch Resistance

by
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DEDICATION

I dedicate my dissertation to my parents Mr. Zhefan Zhou and Mrs. Jujiao Li, for their full support to my education and decision to study abroad in the United States.

BIOGRAPHY

Binbin Zhou was born in Xinchang, a small and beautiful town in southeast of China. As a little boy, he was fascinated by the mystery of nature, which reflected later in his interest in science, particularly biology.

He obtained his Bachelor degree with a major in Applied Biological Science at Zhejiang University in 2009. In 2008 he did a summer internship at Dr. Rongda Qu's laboratory and enjoyed his time very much by helping and learning from the researchers in Qu's lab. When he went back to China after that summer, he applied for graduate study and was admitted as a Master student in Dr. Qu's laboratory at North Carolina State University in 2009, and started his research on turfgrass biotechnology.

In order to study further in the research, he applied to switch from the Master program to the Ph.D. program in the fall of 2011 and continued his research in Dr. Qu's lab.

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PART I: INTRODUCTION AND LITERATURE REVIEW

Engineered disease resistance in plants

The battle between plants and pathogens has been around for hundreds of millions of years since their first origin, and humans are involved in this war and stand on plant's side as we live on the plants. For dozens of centuries, we have been cultivating plants for food and other resources just by simple selection of suitable plants for certain area, or some simple manipulation like rotations. The fast development of modern biology due to a series of breakthroughs including the invention of the microscope, the findings of Mendel's laws and the discovery of the double helix structure of DNA, enables us to look deeper into the battle between plants and pathogens, and achieve better understanding of the biological activities involved in the war, including what chemical and physical weapons the pathogens use to invade the plants, and what biological shields the plants use for defense. With the tool of genetic and genomic analysis, we now have made significant progress on understanding the interactions between plants and their pathogens, which enables us to develop more strategies to help plants fighting against the pathogens.

Although there is a large variety of bacterial and fungal pathogens in nature, normally they do not win the battle because of the genetic diversity. Plants evolved to have efficient, induced responses to help them win the battle during this plant pathogen interaction. These systems include pre-formed structural and chemical barriers, and complex mechanisms of molecular recognition and signal transduction which can activate subsequent defense responses (*Plant Biotechnology and Agriculture: Prospects for the 21st Century*, 2012). An elegant 'zigzag' model has been proposed by Jones and Dangl in 2006 to better explain the

innate immunity in plant pathogen interactions (Jones & Dangl, 2006), in which the war between plant and pathogen was separated into four phases (Figure I-1). On the front line of the war stands the plant defense system formed by pattern recognition receptors (PRRs), which are cell surface receptors that can recognize pathogen-associated molecular patterns (PAMPs). After the detection of PAMPs, PRRs activate an innate immune response in the plant, which is termed as PAMP-triggered immunity (PTI). Thus, the plant has the upper hand with the help of PTI during the first phase of plant-pathogen interaction. During the second phase, some pathogens successfully break into the plant defense by secreting effectors into the plant cell which can suppress PTI responses, resulting in effector-triggered susceptibility (ETS). In phase III, plants evolve to develop certain cytoplasmic resistant (R) proteins that can directly or indirectly recognize those effectors secreted by pathogens, and activate plant immunity again which is called effector-triggered immunity (ETI). ETI is a quicker and stronger immune response compared to PTI and typically leads to systemic acquired response (SAR) and programmed cell death, which is also referred as the hypersensitive response (HR). Under such high selection pressure, pathogens fight back by modifying the current effectors or develop new effectors to evade the recognition of R proteins, suppressing the ETI response and resulting in ETS again. In turn, plant receptors are improved during the selection and regain the ability to recognize the effectors and induce ETI again. As nature has no prejudice to both plants and pathogens, neither of them can always win the battle, thus victory and defeat alternate between plants and pathogens as evolution proceeds.

However, the modern agriculture practices which cultivate large areas of genetically identical crops decrease the power of the plants shield, allowing pathogens to quickly adapt and defeat plant immunity and result in huge losses in yield (*Plant Biotechnology and Agriculture: Prospects for the 21st Century*, 2012). Thus, an important goal for plant breeding is to produce plants with broad-spectrum and durable resistance. There are two types of disease resistance for plants, which are qualitative resistance and quantitative resistance. Qualitative resistance is a complete resistance, which is normally specific to certain pathogens and is controlled by a single R gene. It is relatively easy for the pathogens to evolve to overcome such resistance due to the high selection pressure, and pathogens can afford the loss of avirulence genes without loss of fitness. On the other hand, quantitative resistance of the plant is always partially effective, and normally is controlled by several resistance genes, thus much harder for the pathogen to overcome the resistance.

Currently, classic or marker-assisted breeding and genetic engineering are two strategies to produce plants with durable disease resistance (Michelmore, 2003). While compared to the time-consuming classic breeding strategy, genetic engineering technology enables us to incorporate favorable traits into plants in a relatively short time period (technically), and most of which solve problems by functional gene transfer from a different species, even from a different kingdom. To date, the genes of interest transformed into plants have various sources, for instance, plant defense-related genes from plants, pathogen-derived genes from the pathogens, genes coding for antimicrobial protein/peptides from animal or insect, and the dsRNA for essential pathogen genes adopted by the recently developed RNA interference (RNAi) technology.

Strategies for engineered disease resistance

As mentioned in the introduction, during the evolution, plants have developed strategies to fight against pathogen infections. Sometimes the pathogen is successful and can cause disease, and sometimes the host plant displays successful resistance. By understanding and interpretation of the strategy used by plants to protect themselves from disease infection, several genetic engineering strategies have been applied to disease resistance. Collinge et al. (2008) reviewed these strategies and divided them into three categories. As shown in Figure I-2, there are 3 types of successfully applied transgenic strategies: 1, direct interference or inhibition of pathogenicity, by constitutive (1a) or pathogen induced (1b) expression of genes that encode antimicrobial proteins/peptides. The direct inhibition of pathogen could also result from antimicrobial secondary metabolites, detoxification, or quenching pathogen signals, and the transferred gene could be from plants or other organisms, alone or in combination; 2, modify the plant pathogen detection recognition (2a) or the downstream defense regulatory pathways (2b); and 3, the mimicry of pathogen attack to prepare the plant for prime recognition of a specific pathogen, which is normally against viruses pathogen (Collinge et al., 2008). Here I will present some cases for each strategy, and make a comparison to generalize the current status of genetic engineering for disease resistance in plants.

Strategy 1: Direct inhibition of pathogen disease

During the co-evolution of plant and pathogen interactions, plants evolved to form certain defense mechanisms, and the study of these mechanisms leads to the discovery of various defense proteins (e.g., pathogenesis-related proteins, R proteins), small peptides, and

secondary metabolites possessing direct antimicrobial activities (Field, Jordan, & Osbourn, 2006; Hammerschmidt, 1999; Loon et al., 2006). Antimicrobial proteins (AMP) might interrupt directly the pathogen life cycle or interfere with the pathogen infection process. For instance, chitinase is one of the early studied plant defense enzymes, as shown by *in vitro* data it can degrade a major component of the fungal cell walls - chitin (Collinge et al., 1993). Due to its theoretical efficacy against a broad spectrum of fungus, genes encoding chitinases have been transferred into plants such as melon, peanut, and banana, and showed resistance to certain fungal diseases (Bezirganoglu et al., 2013; Iqbal et al., 2012; Kovacs et al., 2013).

Besides plants, there are many other sources of genes encoding the AMPs. For instance, penaeidins are a family of AMPs originally identified in the haemocytes of penaeid shrimp, which have been confirmed with broad-spectrum of antimicrobial activity (Cuthbertson et al., 2006). The introduction of a penaeidin gene, *Pen4-1*, into creeping bentgrass, significantly improved its resistance against the fungal pathogen *Rhizoctonia solani* (Zhou et al., 2011). Besides the animals, AMPs could also derive from the virus which is capable of infecting fungus. KP4 is such a protein encoded by a symbiotic virus of certain strains of smut fungus *Ustilago maydis*. To help the host outcompete other corn smut strains, the fungus infected with such a virus would secrete KP4 to kill other smut fungi which are not infected by the virus. The gene encoding KP4 has been transformed into wheat and corn and showed good inhibition on the growth of the smut fungi (Allen et al., 2011; Clausen et al., 2000; Schlaich et al., 2007). Indeed, the antimicrobial protein even doesn't have to be natural. A synthetic peptide, D4E1, derived from an insect antimicrobial peptide, was proved to be effective in transgenic cotton against fungi (*Fusarium verticillioides*, *Aspergillus flavus*,

Thielaviopsis basicola), and in transgenic poplar against bacterial pathogens (*Agrobacterium tumefaciens*, *Xanthomonas populi* pv. *populi*) (Mentag et al., 2003; Montesinos, 2007; Rajasekaran et al., 2005).

Quorum-sensing is the communication system used by bacteria for numerous activities including the virulence gene expression (Cui & Harling, 2005). AiiA is an enzyme identified from a bacterial strain which interferes with the signaling process of the soft rot pathogen, *Erwinia carotovora*. Transgenic expression of AiiA in potato and *Amorphophallus konjac* improved their resistance against this bacterial pathogen (Ban et al., 2009; Dong et al., 2001).

Phytoalexins are antimicrobial secondary metabolites produced in plants. Pathogens must be able to detoxify these phytoalexins in order to infect the host. Thus transferring phytoalexin synthesis genes from one species to another species of plants could introduce pathogen resistance. However, the manipulation of phytoalexin levels usually requires the knowledge about the complete set of involved mechanisms, for instance, the way to connect the primary and secondary metabolic pathways, the competition between enzymatic branches using the same precursors, and the enzymes involved in transcriptional and post-transcriptional regulation (Groffsky et al., 2012). The successful cases of using phytoalexins have been reported by transgenic expression of a grapevine stilbene synthase gene in tobacco, barley, wheat and rice (Hain et al., 1993; Leckband & Lorz, 1998; Tian et al., 1998). Other than phytoalexins, defensins have also been applied to plant disease resistance improvement. Plant defensins are small, cysteine-rich peptides that have been demonstrated to inhibit fungal and bacterial pathogens and even insects. One of the plant

defensins isolated from the seeds of alfalfa *Medicago sativa*, alfAFP, has been expressed in potato and conferred good resistance against the fungal pathogen *Verticillium dahlia*, and more importantly, the resistance was maintained in the field test (Gao et al., 2000).

Another strategy that falls into this category is the transgenic plants targeting toxins, which is normally used by necrotrophic pathogens to kill their host cells. For instance, trichothecene mycotoxin deoxynivalenol (DON) is a toxin produced by Fusarium head blight (FHB) on wheat and barley. DON can inhibit protein synthesis by interacting with the ribosomal protein L3 at the peptidyltransferase center. It has been shown that transgenic tobacco and wheat expressing the N-terminal fragment of the yeast L3 can decrease the toxicity of DON, reducing the ability of DON for protein synthesis inhibition because of its intimate interaction with L3 protein (Di et al., 2010).

Strategy 2: Utilization of plant defense related genes

It is known that plants have their own strategies to fight against pathogen infections. As a result, plants developed complex mechanisms for pathogen recognition and defense responses, during which a number of genes are involved. By modifying gene expression in these processes, disease resistance can be improved.

One of the most commonly used gene source is the plant specific resistance (R) gene, which was first proposed by Flor as a gene-for-gene hypothesis (Flor, 1955, 1971). This theory points out that for each gene conferring resistance in the host, there is a corresponding avirulence gene (Avr) in the pathogen with which it interacts. R-genes encoded proteins often contain nucleotide binding sites and leucine-rich repeats (NBS-LRR), which normally can recognize the avirulence gene product, and then activate down-stream defense gene

expression to combat the pathogen (Shao et al., 2003). The expression of resistance genes often triggers a rapid death of the infected cells, called the hypersensitive response or programmed cell death (PCD) (Dangl, Dietrich, & Richberg, 1996; Pennell & Lamb, 1997), which can slow down or stops the development of the disease. This finding is invaluable to our understanding of the interaction between the pathogens and plants, providing a great perspective for disease control through genetic engineering. It was shown that R-genes can be functional for pathogen resistance when transformed into another species or even a different genus. For instance, the recently reported transgenic tomato expressing the R-gene *Bs2* from pepper displayed extremely low levels of bacterial spot disease on the highly susceptible VF 36 background (Horvath et al., 2012). Expression of R-genes in other plant species including rice, maize, and *Brassica napus* also showed positive results for disease control (Hennin et al., 2001; Wang et al., 1996; Zhao et al., 2005).

Genetic engineering with R-gene falls under the concept of Strategy 2a (Figure I-2), which recognizes the infection of pathogen and then induces the subsequent defense process. The strategy 2b uses the downstream genes that relate to defense signaling pathways, for instance, the *NPR1* gene. *NPR1* was first identified from *Arabidopsis* through genetic screening for systemic acquired resistance (SAR)-compromised mutants (Durrant & Dong, 2004). Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens has been achieved by expressing the *Arabidopsis NPR1* gene in transgenic carrots (*Daucus carota L.*) (Wally et al., 2009).

However, the resistance obtained by transgenic expression of these types of genes generally has one drawback – the resistance is non-durable. Several cases have indicated that

pathogen populations eventually evolve to adapt to the R gene and regain pathogenicity (Hovmøller et al., 1997; McDonald & Linde, 2002). In the gene for gene relationship, when the Avr gene in the pathogen is inactivated by mutation, the R gene in the host will lose its effectiveness as the recognition process between these two gene products is interrupted. Gradually this mutated pathogen race outcompetes other lines and the host loses its resistance. In order to gain durable resistance, different types of R genes can be stacked in one host, or this strategy can be combined with other strategies discussed in this review.

Strategy 3: Pathogen derived resistance

The third strategy is to prime the plant for disease resistance, by another mechanism called pathogen derived resistance (PDR). PDR originated from the insertion of genes derived from viral pathogens into the host plant to obtain resistance. With its mechanism now understood as RNAi, its application has been extended to control insects, nematodes, and fungal and bacterial diseases.

The concept of PDR was first demonstrated by Beachy lab in 1986 through their pioneer work on coat protein (CP)-mediated resistance to tobacco mosaic virus (TMV) (Beachy et al., 1990). In 1997, Beachy suggested two mechanisms regarding the application of PDR in transgenic plants against virus infection: 1, Expression of genes that encode certain viral proteins in the host plant can protect these plants against certain viruses; 2, The second strategy is through the nucleic acid mediated resistance (Beachy, 1997). The mechanism of protein-mediated defense is still not clearly elaborated. On the other hand, three types of RNA-mediated virus resistance are shown in Collinge's review: (A) Sense or antisense viral sequences, (B) Inverted repeats/hairpin RNA of viral sequence, (C) Sequences

of engineered microRNAs (miRNA) targeted against viruses (Collinge et al., 2008).

Successful applications of all the three approaches have been reported and it seems that the third method performs the best in terms of improving plants' disease resistance (Qu et al., 2007; Smith et al., 2000).

With the significant findings of Fire and Mello in 1998 which showed that double-stranded RNA has much more powerful silencing ability than either strand individually, the study of RNA-mediated gene silencing advanced rapidly in the following years, and our understanding of the molecular mechanism of RNAi has gradually taken shape (Fire et al., 1998). During the gene silencing process via RNAi, two kinds of small RNA molecules play important roles: small interfering RNAs (siRNA) and miRNA. Both small RNAs are 21 to 25 nucleotides in length and both can induce sequence-specific mRNA molecular degradation in the cytoplasm. The introduction of foreign genes into the host cell leads to the formation of dsRNA, for instances, via antisense transcription, viral infection, or artificial expression. The dsRNA is processed by Dicer in the cytoplasm, resulting in ~ 21-bp siRNA with 2-nt 3' overhangs. The siRNAs are then incorporated with an RNA-induced silencing complex (RISC), which mediates the cleavage of the passenger-strand of siRNA, leading to the binding of its complementary mRNA and the subsequent degradation. On the other hand, miRNAs have their own genes in the genome and often functions in development. The primary miRNA transcript is formed in the nucleus and transported into the cytoplasm via Exportin-5, although the exact process of this exporting remains unclear (Park et al., 2002). After the processing of pre-miRNA in the cytoplasm with a different Dicer, the mature single-stranded miRNA is loaded onto the catalytic center of RISC – AGRONAUTE1

(AGO1), which utilizes its Dicer activity to cleave and repress the related mRNA expression (Duan et al., 2012).

The understanding of RNA-mediated gene silencing promotes the application of RNAi technology on plant improvement. As viruses propagate inside plant cells, silencing their gene in the plant cells via RNAi should be an effective way to gain the resistance. A recently published paper on cassava brown streak disease (CBSD) is a good case to illustrate it (Ogwook et al., 2012). Truncated full-length (894-bp) and N-terminal (402-bp) portions of the CBSV coat protein (Δ CP) sequence was introduced into cassava plants. Seven siRNA-producing lines from each gene construct were tested with wild type cassava for virus resistance. The transgenic cassava showed significant difference from the wild type on both viral disease development and root infection after harvest. The success of this case provides proof of principle for the control of CBSD by RNAi-mediated technology. Other successful cases include the application of hpRNA sequences against geminivirus *Vigna mungo* yellow mosaic virus (VMYMV) and bean golden mosaic virus (BGMV), suggesting that this RNA silencing strategy may be applicable to engineered resistance against geminiviruses (Pooggin et al., 2003; Rodriguez-Negrete et al., 2009).

Besides the virus resistance, RNAi is also a powerful tool for plant insect resistance. One of the successful examples in using the RNA silencing-mediated transgenic method lies on the control of cotton bollworm in the field via targeting its gene, CYP6AE14. The expression of dsRNA of this gene in cotton caused the retarded development of bollworm larva, and the larva fed with CYP6AE14-derived hpRNA transgenic cotton showed reduced expression of CYP6AE14 (Mao et al., 2007). To conclude the previous studies on application

of RNAi for insect control in plants, there are three major findings: (1) Oral intake of dsRNA is effective not only for genes expressed in midgut but also for those expressed in other tissues/organs (Turner et al., 2006); (2) Feeding insects with bacteria that express dsRNA is also effective to induce the RNAi for the gene of interest (Arakane et al., 2004); (3) Genetic engineered plants that express dsRNA can be used to feed the insect and induce the corresponding RNAi inside insects (Mao et al., 2007). It seems that in spite of the hostile environment of the insect gut due to numerous RNases, the RNAi signal can still enter the midgut cells remaining intact and initiate the gene silencing (Turner et al., 2006).

In addition to the successful application of RNA-mediated gene silencing to creating virus or insect-resistant plants, similar strategies have been developed for fungal diseases. However, unlike plant viral pathogens, which replicate and propagate inside the infected plant cells, there is no direct and obvious means for the transfer of dsRNA or siRNA from plant to fungus. The closest interaction between fungus and plants is the haustorium formed during the infection which is the interface responsible for the nutrient uptake by fungus and signal exchange between fungus and host (Panstruga, 2003). Is it possible that gene silencing signals can be transferred from plants to fungus through this interaction? That is, can the transgenic plant expressing dsRNA of essential fungal genes induce gene silencing in the fungal pathogen? This approach was described in 2004 in a patent application by Niblett (Niblett, 2004), and proof of concept was shown in tobacco, potato and soybean by Niblett and Bailey (Niblett & Bailey, 2012). Work by Tinoco and colleagues in 2010 also verified that a plant expressing dsRNA could cause gene silencing of corresponding target genes in fungal cells infecting the plant tissues. In this study, they engineered the tobacco plants to

express a *GUS* gene-interfering cassette [hairpin (hp) *GUS*]. When the transgenic and non-transgenic plants were infected by *Fusarium verticillioides* which was transformed to express *GUS*, far less *GUS* gene expression was detected from the fungus infecting transgenic plants compared to those on control plants (Tinoco et al., 2010). This experiment demonstrated that the expression of dsRNA inside the plants can lead to target gene silencing inside the fungus infecting the plant tissue, which was termed “host-induced gene silencing” (HIGS).

Following this report, several HIGS related papers were published on barley and wheat with the powdery mildew fungus (Nowara et al., 2010), and rust fungus (Yin et al., 2011). The expression of double-stranded or antisense RNA targeting fungal transcripts in barley and wheat induced the silencing of an effector gene of *Blumeria graminis* (Avra10), which resulted in reduced fungal development (Nowara et al., 2010). In another case, gene fragments of rust fungus *Puccinia striiformis* f. sp. tritici were delivered into plant via the *Barley stripe mosaic virus* system and the expression of corresponding genes in rust fungus were reduced (Yin et al., 2011). Both cases indicated that *in planta* RNAi approach might be a promising strategy to engineer fungal pathogen resistance crops.

PDR achieved through HIGS is still at an infant stage, and its application to disease control requires further development of RNAi technology with several aspects. For instance, how to achieve high gene silencing efficiency? How to achieve multi-target gene silencing against diverse pathogen sources in the environment? What is the best way to deliver the silencing inducer? And most importantly, how to make this strategy feasible to fulfill both environmental and economic requirements?

Future perspectives

Genetic engineering for disease resistance has been studied for 30 years. However, there are still very few GM disease resistant cultivars commercially available in the market. The commercialization of GM disease resistant plants needs to deal with several obstacles: the efficacy and specificity for disease control, the competitive strength of GM crops compared to substitution strategy, and the economic incentive for the market, and the public acceptance. As molecular biologists aiming to improve plants disease resistance, we should endeavor to develop the technology for higher efficacy products and durable resistance and at the same time, minimize the environmental impact and reduce the cost. From my point of view, mixing the strategies listed above for disease resistance, instead of focusing on only one strategy or one gene, might be helpful to gain durable resistance and increase the efficacy.

Tall fescue genetics, morphology, and breeding

Turfgrass is one of the gifts from nature, harnessed by human beings to fulfill our aesthetic and other realistic requirements. During the long process of cultivation, various species have been chosen for their favorable characteristics for use as a turf and their ability to survive under stresses (biotic and abiotic). Nowadays, turfgrass has been widely used in home lawns, parks, golf courses, roadside, and sports fields. With over 2 million acres of turfgrass, North Carolina ranks eighth in the U. S. in total turfgrass acreage. As in the transition zone, 37% (742,600 acres) of the turf area in North Carolina is covered by tall fescue, partially due to its drought tolerance, to make it one of the most important turfgrass species in the state (TurfFiles:Crop Profile for Turfgrass in North Carolina (2004)).

Tall fescue (*Festuca arundinacea*) was introduced into North and South America by European settlers in the late 19th century. While not until the release of cultivars Alta and Kentucky-31 in 1940s, had it become a prominent turfgrass species in the United States (*Turfgrass Biology, Genetics, and Breeding*, 2003). Kentucky-31 stands out from all the cultivars due to its wide adaptation to variant environment, including soil condition, rainfall and sunlight (Murray & Powell, 1979). In the past few decades, new cultivars with finer and greener leaves have been bred and commercialized (*Turfgrass Biology, Genetics, and Breeding*, 2003).

Tall fescue is classified as a cool-season grass, belonging to a group of grasses utilizing C₃ carbon fixation for photosynthesis and normally thriving in spring and fall. The other group is warm season grasses, which use C₄ carbon fixation for photosynthesis and grow vigorously during late spring and summer. Tall fescue has bunch-type growth habit and rarely has rhizomes. It has erect, stout, smooth culms up to 2 m tall, a medium-fine leaf texture, and 3-12 mm wide leaf blade that lacks a mid-rib (*Turfgrass Biology, Genetics, and Breeding*, 2003). In the United States, tall fescue has been widely adapted to the eastern part of the country, and parts of the Pacific Northwest, and California.

Genetic composition and its growth environment are the two factors that impact the phenotype of a plant. Plant breeders endeavor to identify and select those favorable phenotypes conferred from genotypes, other than those desirable phenotypes caused by the environment factors. And also, traits with higher heritability will be more interesting to the breeders as it will be easier to be preserved by selection during the long process of breeding. Traits can be classified as two types, one is qualitative trait, and the other is quantitative trait.

Qualitative trait stands for the phenotype that falls into different classes, and is typically inherited by one or a few genes, while quantitative trait has continuous variation and is normally controlled by several genes, which means that quantitative trait is the accumulative effect of multiple genes. Regions of DNA contributing to a specific trait are called quantitative trait loci or QTLs. Identifying QTLs and figuring out their correlation to the favored traits has been a great strategy to improve plant quantitative traits. In tall fescue, a good example of a qualitative trait analysis is the resistance to stem rust (Barker et al., 2003). Other quantitative traits include forage yield, seed yield, digestibility, and drought stress tolerance.

Tall fescue has a huge genome around 5.27 to 5.83×10^6 kb (Seal, 1983), which is more than 12 times larger than the genome of rice (*Oryza sativa* L.). Most of commercial tall fescue cultivars have allohexaploid genome with 42 chromosomes ($2n=6x=42$) and is designated as PPG₁G₁G₂G₂. The P and G genomes are claimed to be from two different sources respectively, diploid meadow fescue (*Festuca pratensis*), and the tetraploid *Festuca arundinacea* var. *glaucescens* Boiss (Eizenga, Schardl et al. 1998). Tall fescue is primarily an outcrossing species which has a high level of self-incompatibility. The heterozygous state endows tall fescue with a high level of genetic variation in the populations. Thus, every plant in a tall fescue cultivar is a unique genotype, and cross-pollination has been the most applicable procedure for tall fescue cultivar breeding.

Tall fescue breeding can be divided into two categories, classical approach and molecular approach. With classical approach, cross-pollination and self-incompatibility are the two main traits exploited by the breeders for cultivar development. Mainly, the purpose

of tall fescue breeding is to get the cultivars that obtain the favored traits and at the same time has certain level of uniformity on agronomic and morphological characteristics. Purposeful selection after open pollination of several parent plants is applied for increasing the frequency of favorable alleles in a population. Based on the different cycles of selection, there are two types of selection, ecotype selection and recurrent selection. Ecotype selection involves testing the tall fescue germplasm collected from a source and directly increasing seed of the germplasm for cultivar release, while recurrent selection goes through several cycles of trait selection from multiple generations. Ecotype selection can generate new populations in shorter periods of time and played an important role in tall fescue breeding, for instance, KY-31 was developed by this manner (Vaylay & van Santen, 1999). In recurrent selection, selected plants are intermated in isolated environment (no pollen from other genotypes) and the subsequent seeds are harvested for the next generation's trait evaluation. This manner can accumulate favorable alleles in the population in a more powerful way and thus is a better choice for improving traits with lower heritability although it takes a longer time period.

Compared to the time-consuming classical breeding, molecular breeding provides an alternative way to create new gene combinations in tall fescue germplasm in a shorter period. With the development of gene technology and the transformation technology, molecular approach has a great potential in tall fescue breeding. For instance, new gene combinations can be achieved by somaclonal variation (Roycastle et al., 1994), doubled haploids (Jones et al., 1997), and genetic transformation (direct gene transfer and *Agrobacterium tumefaciens*-mediated gene transfer). The transgenic approach has been

applied on forage-type tall fescue for several traits improvement, like forage quality, resistance to disease and abiotic stresses, and the manipulation of grass development. Reports have been published regarding the forage lignin biosynthesis and improved digestibility (Casler & Kaeppler, 2001) and disease resistance (Dong et al., 2007; Zhou et al., 2011).

Other than transgenic technique, a method that has great potentials is marker-assisted selection. However, this requires an extensive molecular marker map of tall fescue genome, and knowledge of genes identified with agronomic importance. To make the molecular technology a more powerful tool for turfgrass breeding, we need to fill a lot of gaps in our understanding about the genetics, biochemistry and physiology that function in many complex processes.

For the application of transgenic technique onto turf grass breeding, there is still a long way to go after the achievement of genetic transformation. Field evaluation is the most important and the very necessary step to confirm the favorable traits brought by the transgene. Thus, there are mainly two questions that need to be answered for the test carried out in the wild environment other than in the controlled greenhouse: how stable is the expression of the transgene? And what's the actual phenotype of the transgenic plant in the environment? In addition, another concern about growing the transgenic grass in the field is biosafety. An illustration can be found in papers regarding alfalfa mosaic virus (AMV) immune transgenic plants (Kalla et al., 2001; Spangenberg et al., 2001), in which several layers of non-transgenic grass were planted surrounding the central transgenic grass and the transgene flow was assessed to confirm the suitability of the field test design. Another simpler and faster method to control the gene flow in the field test is just to prevent the plant from flowering by

frequent mowing and monitoring, though it's not the most ideal way as no offspring can be obtained for further analysis.

After evaluation of the phenotype of transgenic tall fescue in the wild environment, the next step is to integrate the transgenic plant into breeding program in order to develop novel elite transgenic cultivars. A good way to achieve this is to introgress the transgene into elite parents and to get stable and uniform transgene expression in the later generations. There are several steps involved in the strategy for producing transgenic elite plants which have homozygous transgenes (Spangenberg, Kalla et al. 2001). First of all, cross the elite non-transgenic grass with the transgenic grass that stands out in the field evaluation; then pick out the progeny (F1) pertaining the transgene with antibiotic selection from the offspring of the former crossing; do a diallel crossing between the F1 plants and select plants in F2 for those having homozygous transgenes (with quantitative PCR or inheritance test). Once the elite grass plants with homozygous transgenes were obtained, we can compare it with elite non-transgenic grass, send it to other locations for further evaluation, and finally produce seeds that can be used by other growers.

Tall fescue pathology and its disease resistance

With 2.1 million acres of lawn, North Carolina ranks eighth in the U. S. in total turfgrass acreage (35 million acres). The annual cost for pesticide on turfgrass management in North Carolina is around \$46 million. Tall fescue is the most important turf species in North Carolina, with 37% of the total turf acreage. Fungicides are substantial costs to maintain turfgrass lawns. In 1999, \$2.5 million was spent on fungicides for disease control

and 90% of that was applied for brown patch and large patch (Crop Profile for Turfgrass in North Carolina, 2004). Farmers and turf breeders have endeavored to develop disease resistant cultivars for decades. Many diseases can impair tall fescue health, such as brown patch, *pythium* blight, grey leaf spot, rust and net blotch. One of the major diseases is brown patch caused by *Rhizoctonia solani*. The knowledge about the biology of this pathogen and the current situation regarding the resistance development are valuable for tall fescue breeding.

R. solani is a soil-born basidiomycete pathogen which mostly reproduces asexually in the form of vegetative mycelium. It has a wide host range and worldwide distribution, with herbaceous plants as their main targets. It can cause brown patch disease on many cool-season grasses, like fescues, ryegrasses, bentgrasses, and bluegrasses (Cho et al., 2011; Tredway & Burpee, 2001). Also, it is the pathogen that causes sheath blight in rice and aerial blight in soybean. Tall fescue is very sensitive to brown patch disease in summer, especially in hot and humid weather. As the name of the disease describes, the standard symptom is patches with brown or tan color on the leaves, which are actually leaf lesions with irregular shapes.

R. solani has many isolates which belong to different anastomosis groups (*AG*). An anastomosis group stands for a collection of genetically-related isolates which can anastomose hyphae between each other. This fungus can overwinter in the soil in the form of sclerotia which protects the fungus with thick outer layers. When the conditions are favorable to the pathogen (compatible host and suitable environment), *R. solani* can adhere to the external surface of the host by profuse hypha growth and form infection structure. The

structure used by the fungus for infection can be short swollen hyphae, appressorium or repetitive T-shaped hyphal branches based on different host specificity (reviewed by Garcia et al. 2006). The next step is the penetration of the host cells normally with the enzymes secreted from the pathogen. Several extracellular enzymes have been found involved in this process, like cutinase, cellulase, pectinase and pectin lyase (Sherwood, 1966; Lister et al., 1975). After penetration, more hyphae grow into the plant cells by secreting cell wall degrading enzymes. The colonization of the fungus causes collapse of the entire cytoplasm and kills the plant cells. Then necrosis and sclerotia will form in the infected tissue after invasion, resulting in the symptom of brown patch on tall fescue. *R. solani* is necrotrophic fungus, thus it can grow on, and extract nutrition from, the dead tissue. When there is another plant available, a new cycle begins.

Normally there are several ways to control the brown patch. First of all, selecting cultivars and the seeding density less susceptible to brown patch is critical for the management. It was concluded that the wider leaves of Kentucky-31 may contribute to its tolerance to brown patch than another cultivar ‘Mojave’ (Green II et al., 1999). In another study, it was claimed that low density canopy played an important role for Kentucky-31 and Fawn being less susceptible than the other four cultivars tested (Giesler et al., 1996). Recently, new tall fescue cultivars such as ‘TarHeel’ and ‘Wolfpack’ were bred for brown patch resistance (Fraser et al., 1998, 1999). Besides the cultivar and blade density factors, less nitrogen fertilization in late spring or summer and less frequent irrigation can reduce the possibility of infection. Another effective control is the fungicide application, on a preventative or curative way, based on different weather conditions and disease development.

Although these management practices can reduce brown patch severity in tall fescue, there is no complete genetic resistance to brown patch found in any tall fescue cultivars. Thus, developing transgenic tall fescue to improve the brown patch resistance opens another door for turfgrass breeders. In recent years, several transgenes have been introduced into turfgrasses and demonstrated certain level of brown patch resistance in the laboratory conditions. For instance, A pepper esterase (*PepEST*) gene has been introduced into creeping bentgrass (*Agrostis stolonifera*) and showed enhanced resistance to brown patch disease (AG-2-2 (IIIB)) (Cho et al., 2011); Hong Luo's group has transferred a shrimp antimicrobial peptide Penaeidin 4-1 into creeping bentgrass and gained resistance to dollar spot and brown patch (AG-2-2 (IIIB)) (Zhou et al., 2011); Besides, Dong et al. have transformed tall fescue with T4 lysozyme and dermaseptin gene, resulting in certain levels of brown patch (AG1-IA) and grey leaf spot disease resistance (Dong et al., 2007, 2008). With more resistance genes verified and tested in tall fescue, there will be more transgenic tall fescue lines that show resistance to *R. solani*. However, a thorough evaluation of disease resistance for these transgenic lines grown in the field other than controlled greenhouse is still needed.

Aims of the project

The purpose of this project is to transform foreign genes into tall fescue through *Agrobacterium*-mediated transformation, and test the resistance of the transgenic plants against *Rhizoctonia solani* (causing brown patch disease). The transgenes we used in this project can be sorted into two categories: the gene in the first category comes from other organisms which has verified antifungal activities, like the penaeidin gene from shrimp; The other category are gene segments that come from *Rhizoctonia solani* itself and utilize the

HIGS approach to inhibit the expression of essential fungal genes to interfere the fungal growth.

The first approach is to transform foreign genes into tall fescue, get these genes expressed, and utilize their corresponding proteins' antifungal activity, to confer the plant with pathogen resistance. Genes have been selected based on previous research from other groups and the gene we used in this project is a shrimp gene – Penaeidin4-1 (Pen4-1). As one of the many invertebrates, shrimp lacks the adaptive immune system and the innate immune system becomes their main defense against microorganism infection. During this process, the antimicrobial peptides play an important role. Penaeidins are proteins originally isolated from the haemocytes of penaeid shrimp, which has been proved to have antimicrobial activity against a broad spectrum of microbial targets (Cuthbertson et al., 2004). The penaeidin family is divided into four classes and Pen4-1 is an isoform within the class number 4 penaeidins isolated from Atlantic white shrimp (*Litopenaeus setiferus*) (Cuthbertson et al., 2004). It has both cysteine-rich domain (CRD) and proline-rich domain (PRD), during which the 6 cysteine residues form 3 disulfide bridges, and the proline-rich domain has potential resistance to proteases (Cuthbertson et al., 2006). Pen4-1 has inhibitory activity against several Gram-positive bacteria species and Gram-negative bacteria at relatively high concentration, and also against multiple plant pathogenic fungal species (Cuthbertson et al., 2004, 2006). Pen4-1 has been introduced into creeping bentgrass by Luo's group, resulting in improved resistance against two important fungal pathogens, *Sclerotinia homoeocarpa* and *Rhizoctonia solani* (Zhou et al., 2011). In this study, I introduced the Pen4-1 gene into tall

fescue to see whether the transgene also improves resistance to the *R. solani* strain that infects tall fescue.

The second approach is to utilize the RNAi technology to introduce *R. solani* dsRNA gene constructs into tall fescue to silence certain essential genes of fungus during their infection, and the fungus will reduce or lose their pathogenicity when expression of these genes are suppressed. In the first step, the essential genes of the pathogenic fungus (*R. solani*) are identified. In vitro assays are performed to evaluate the inhibitory effects of the dsRNAs on fungal pathogen growth. The most effective dsRNAs will then be used to make constructs and transform tall fescue. It is hypothesized that during the process of fungal infection, the siRNAs formed from the dsRNA in transgenic tall fescue cells will get into the fungus and silence the corresponding gene expression inside the fungus. With expression suppression of these essential genes, the fungal growth could be inhibited and the plants would show less symptoms to the *R. solani* infection.

References

- Allen, A., Islamovic, E., Kaur, J., Gold, S., Shah, D., & Smith, T. J. (2011). Transgenic maize plants expressing the Totivirus antifungal protein, KP4, are highly resistant to corn smut. *Plant Biotechnology Journal*, 9(8), 857–864.
- Barker, R. E., Pfender, W. F., & Welty, R. E. (2003). Selection for Stem Rust Resistance in Tall Fescue and Its Correlated Response with Seed Yield. *Crop Science*, 43(1), 75. doi:10.2135/cropsci2003.7500
- Casler, M. D., & Kaepler, H. F. (2001). Molecular Breeding for Forage Quality in Forage Crops. In G. Spangenberg (Ed.), *Molecular Breeding of Forage Crops* (Vol. 10, pp. 175–188). Springer Netherlands. doi:10.1007/978-94-015-9700-5_10
- Charles Niblett. (2004). US patent US20060095987.

- Cho, K. C., Han, Y. J., Kim, S. J., Lee, S. S., Hwang, O. J., Song, P. S., ... Kim, J. I. (2011). Resistance to *Rhizoctonia solani* AG-2-2 (IIIB) in creeping bentgrass plants transformed with pepper esterase gene PepEST. *Plant Pathology*, 60(4), 631–639. doi:10.1111/j.1365-3059.2011.02433.x
- Clausen, M., Krauter, R., Schachermayr, G., Potrykus, I., & Sautter, C. (2000). Antifungal activity of a virally encoded gene in transgenic wheat. *Nature Biotechnology*, 18(4), 446–449.
- Collinge, D., Lund, O., & Thordal-Christensen, H. (2008). What are the prospects for genetically engineered, disease resistant plants? In D. Collinge, L. Munk, & B. M. Cooke (Eds.), *Sustainable disease management in a European context* (pp. 217–231). Springer Netherlands. doi:10.1007/978-1-4020-8780-6_2
- Cuthbertson, B. J., Büllsbach, E. E., Fievet, J., Bachère, E., & Gross, P. S. (2004). A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. *The Biochemical Journal*, 381(Pt 1), 79–86. doi:10.1042/BJ20040330
- Cuthbertson, B. J., Büllsbach, E. E., & Gross, P. S. (2006). Discovery of synthetic penaeidin activity against antibiotic-resistant fungi. *Chemical Biology & Drug Design*, 68(2), 120–127. doi:10.1111/j.1747-0285.2006.00417.x
- Di, R., Blechl, A., Dill-Macky, R., Tortora, A., & Tumer, N. E. (2010). Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves resistance to *Fusarium* head blight. *Plant Science*, 178(4), 374–380. doi:10.1016/j.plantsci.2010.02.003
- Dong, S., Tredway, L. P., Shew, H. D., Wang, G. L., Sivamani, E., & Qu, R. (2007). Resistance of transgenic tall fescue to two major fungal diseases. *Plant Science*, 173(5), 501–509. doi:10.1016/j.plantsci.2007.08.002
- Dong, S., Shew, H. D., Tredway, L., Lu, J., Sivamani, E., Miller, E., & Qu, R. (2008). Expression of the bacteriophage T4 lysozyme gene in tall fescue confers resistance to gray leaf spot and brown patch diseases. *Transgenic Research*, 17(1), 47–57. doi:10.1007/s11248-007-9073-3
- Duan, C. G., Wang, C. H., & Guo, H. S. (2012). Application of RNA silencing to plant disease resistance. *Silence*, 3(1), 5. doi:10.1186/1758-907X-3-5
- Field, B., Jordan, F., & Osbourn, A. (2006). First encounters - deployment of defence-related natural products by plants. *New Phytologist*, 172(2), 193–207. doi:10.1111/j.1469-8137.2006.01863.x

- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, *391*(6669), 806–811. doi:10.1038/35888
- Fraser, M.L., Rose-Fricker, C.A., Meyer, W.A., & Funk, C.R. (1998). Registration of ‘TarHeel’ tall fescue, *Crop Science*, *38*, 539.
- Fraser, M.L., Rose-Fricker, C.A., Meyer, W.A., & Funk, C.R. (1999). Registration of ‘Wolfpack’ tall fescue, *Crop Science*, *39*, 872.
- Gao, A. G., Hakimi, S. M., Mittanck, C. A., Wu, Y., Woerner, B. M., Stark, D. M., ... Rommens, C. M. (2000). Fungal pathogen protection in potato by expression of a plant defensin peptide. *Nature Biotechnology*, *18*(12), 1307–10. doi:10.1038/82436
- Gonzalez Garcia, V., Portal Onco, M. A., & Rubio Susan, V. (2006). Review. Biology and systematics of the form genus *Rhizoctonia*. *Spanish Journal of Agricultural Research*, *4*(1), 55. doi:10.5424/sjar/2006041-178
- Großkinsky, D. K., van der Graaff, E., & Roitsch, T. (2012). Phytoalexin transgenics in crop protection—Fairy tale with a happy end? *Plant Science*, *195*, 54–70.
- Hain, R., Reif, H. J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., ... Stenzel, K. (1993). DISEASE RESISTANCE RESULTS FROM FOREIGN PHYTOALEXIN EXPRESSION IN A NOVEL PLANT. *Nature*, *361*(6408), 153–156. doi:10.1038/361153a0
- Hammerschmidt, R. (1999). Phytoalexins: What have we learned after 60 years? *Annual Review of Phytopathology*, *37*, 285–306. doi:10.1146/annurev.phyto.37.1.285
- Hovmøller, M. S., Østergård, H., Munk, L., Crute, I. R., Holub, E. B., & Burdon, J. J. (1997). Modelling virulence dynamics of airborne plant pathogens in relation to selection by host resistance in agricultural crops., 173–190.
- Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–9. doi:10.1038/nature05286
- Jones, L. K. (1997). Breeding Field Crops (Fourth Edition.). *Experimental Agriculture*, *33*(01), 113–119.
- Kalla, R., Chu, P., & Spangenberg, G. (2001). Molecular Breeding of Forage Legumes for Virus Resistance. In G. Spangenberg (Ed.), *Molecular Breeding of Forage Crops* (Vol. 10, pp. 219–237). Springer Netherlands. doi:10.1007/978-94-015-9700-5_13

- Leckband, G., & Lorz, H. (1998). Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance. *Theoretical and Applied Genetics*, *96*(8), 1004–1012. doi:10.1007/s001220050832
- Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J., Tao, X. Y., Wang, L. J., ... Chen, X. Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotechnol*, *25*(11), 1307–1313. doi:10.1038/nbt1352
- McDonald, B. A., & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annu Rev Phytopathol*, *40*, 349–379. doi:10.1146/annurev.phyto.40.120501.101443
- Michelmore, R. W. (2003). The impact zone: genomics and breeding for durable disease resistance. *Current Opinion in Plant Biology*, *6*(4), 397–404.
- Murray, J. J., & Powell Robert C. E1 - Bush, Lowell P., J. B. E.-B. (1979). Turf (pp. 293–306).
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., ... Schweizer, P. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell*, *22*, 3130–3141.
- Ogwok, E., Odipio, J., Halsey, M., Gaitan-Solis, E., Bua, A., Taylor, N. J., ... Alicai, T. (2012). Transgenic RNA interference (RNAi)-derived field resistance to cassava brown streak disease. *Mol Plant Pathol*, *13*(9), 1019–1031. doi:10.1111/j.1364-3703.2012.00812.x
- Plant Biotechnology and Agriculture: Prospects for the 21st Century*. (2012). Altman, A. & Hasegawa, P.M. (Eds.) Academic Press. Amsterdam.
- Pooggin, M., Shivaprasad, P. V, Veluthambi, K., & Hohn, T. (2003). RNAi targeting of DNA virus in plants. *Nat Biotechnol*, *21*, 131–132.
- Qu, J., Ye, J., & Fang, R. (2007). Artificial MicroRNA-Mediated Virus Resistance in Plants. *J Virol*, *81*, 6690–6699.
- Rodriguez-Negrete, E. A., Carrillo-Tripp, J., & Rivera-Bustamante, R. F. (2009). RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J Virol*, *83*, 1332–1340.

- Roylance, J. T., Hill, N. S., & Parrott, W. A. (1994). Detection of Somaclonal Variation in Tissue Culture Regenerants of Tall Fescue. *Crop Sci.*, 34(5), 1369–1372. doi:10.2135/cropsci1994.0011183X003400050041x
- Schlaich, T., Urbaniak, B., Plissonnier, M.-L., Malgras, N., & Sautter, C. (2007). Exploration and swiss field-testing of a viral gene for specific quantitative resistance against smuts and bunts in wheat. In A. Fiechter & C. Sautter (Eds.), *Green Gene Technology: Research in an Area of Social Conflict* (Vol. 107, pp. 97–112). doi:10.1007/10_2007_046
- Seal, A. G. (1983). DNA variation in Festuca. *Heredity*, 50(3), 225–236. doi:10.1038/hdy.1983.26
- Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G., & Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature*, 407(6802), 319–320. doi:10.1038/35030305
- Spangenberg, G., Kalla, R., Lidgett, A., Sawbridge, T., Ong, E. K., & John, U. (2001). Breeding Forage Plants in the Genome Era. In G. Spangenberg (Ed.), *Molecular Breeding of Forage Crops* (Vol. 10, pp. 1–39). Springer Netherlands. doi:10.1007/978-94-015-9700-5_1
- Tian, W. Z., Ding, L., Cao, S. Y., Dai, S. H., Ye, S. Q., & Li, L. C. (1998). Rice transformation with a phytoalexin gene and bioassay of the transgenic plants. *Acta Botanica Sinica*, 40(9), 803–808.
- Tinoco, M. L. P., Dias, B. B. A., Dall’Astta, R. C., Pamphile, J. A., & Aragão, F. J. L. (2010). In vivo trans-specific gene silencing in fungal cells by in planta expression of a double-stranded RNA. *BMC Biology*, 8(1), 27. doi:10.1186/1741-7007-8-27
- Tredway, L. P., & Burpee, L. L. (2001). Rhizoctonia Diseases of Turfgrass. *The Plant Health Instructor*.
- Tumer, N. E., & Di, R. (n.d.). New transgenic plant that exhibits increased resistance to toxins targeting eukaryotic ribosomal L3 protein compared to a non-transgenic control plant, useful for providing resistance against trichothecene fungal toxins. Univ Rutgers State New Jersey (Rutf).
- TurfFiles:Crop Profile for Turfgrass in North Carolina (2004). (n.d.).
- Turfgrass Biology, Genetics, and Breeding*. (2003). Casler, M.D. & Duncan, R.R. (Eds.) John Wiley & Sons.

- Turner, C. T., Davy, M. W., MacDiarmid, R. M., Plummer, K. M., Birch, N. P., & Newcomb, R. D. (2006). RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Mol Biol*, *15*(3), 383–391. doi:10.1111/j.1365-2583.2006.00656.x
- Van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. In *Annual Review of Phytopathology* (Vol. 44, pp. 135–162). doi:10.1146/annurev.phyto.44.070505.143425
- Vaylay, R., & van Santen, E. (1999). Grazing Induces a Patterned Selection Response in Tall Fescue. *Crop Science*, *39*(1), 44. doi:10.2135/cropsci1999.0011183X003900010007x
- Yin, C., Jurgenson, J. E., & Hulbert, S. H. (2011). Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. *Mol Plant Microbe Interact*, *24*(5), 554–561. doi:10.1094/MPMI-10-10-0229
- Zhou, M., Hu, Q., Li, Z., Li, D., Chen, C. F., & Luo, H. (2011). Expression of a novel antimicrobial peptide Penaeidin4-1 in creeping bentgrass (*Agrostis stolonifera* L.) enhances plant fungal disease resistance. *PLoS One*, *6*(9), e24677. doi:10.1371/journal.pone.0024677

PART II: MATERIALS AND METHODS

I. Plasmid construction

Plasmid construction for Penaeidin 4-1 gene

Penaeidin class 4 isoform 1 (*Pen4-1*) gene was obtained from Luo's lab in Clemson University, who synthesized the modified full sequence of *Pen4-1* gene (Zhou et al., 2011). DNA construct pHL016 containing the coding sequence of *Pen4-1* was used as a template and PCR reaction was carried out using the primers: Pen4F1: 5'-AAATACCCGGGATGCACTCCTCCGGCTA-3' and Pen4R1 5'-ACTATGAGCTCTAGAGGTGGCAGCAGT-3'. The PCR product was introduced into pCAMBIA1300, resulting in a new plasmid pBZ7 (Figure II-2). *Pen4-1* gene was under the control of the rice *rubi3* promoter and *nos* gene terminator (Sivamani & Qu, 2006). Then pBZ7 was transformed into *Agrobacterium tumefaciens* strain EHA105 (pTOK47), resulting in a new *Agrobacterium* strain, ETBZ7.

BLAST *N. crassa* gene with *R. solani* database for target gene selection

A list of 32 essential genes of *Neurospora. crassa* was provided by Dr. Ana Bailey of Venganza Inc., based on their unpublished data showing that dsRNA transcribed from segments of these genes leads to the inhibition of spore germination of several fungal pathogens. The amino acid (AA) sequences of these essential genes were used as query sequences to perform 'tblast' in the *R. solani* database. The *R. solani* database we used in this project was constructed by Dr. Marc Cubeta in North Carolina State University (<http://rsolani.org/index.html>), which includes high quality of genome sequence of *R. solani*

anastomosis group 3 (AG-3). Based on the corresponding sequence ID in the *R. solani* database, homologous cDNA sequences of *R. solani* (mRNA sequences) were identified.

The cDNA sequences found by this approach were analyzed on the NCBI (National Center for Biotechnology Information) website for their conserved domains. The annotated protein function of the conserved domains was ensured to be the same as the corresponding query protein functions in *N. crassa*. Then ~400 bp cDNA sequences containing the conserved domains were chosen.

These gene segments were then analyzed by a siRNA Selection Program from MIT (<http://sirna.wi.mit.edu/home.php>). This program can scan the sequence of the whole gene segment and calculate the binding energies for both sense and antisense siRNAs based on the sequence patterns. The higher negative scores in the output indicate potentially lower binding energy and thus higher silencing activity. Only those gene segments with high potential siRNA activity were selected for further analysis.

PCR amplification for target gene and dsRNA synthesis

The gene segments identified by the previous step were amplified by PCR. Genomic DNA of *R. solani* (AG1-IA) was extracted and purified for PCR amplification. PCR pre-mix from Life Technologies was used to amplify segments around 400 bps. The molecular size of the PCR products was assessed from gel electrophoresis, and the sequences of the PCR products were determined. The sequences of the PCR products were compared to the original sequences from the *R. solani* database, and only the segments with identical or highly homologous sequences were selected for the next step of screening. For double strand RNA (dsRNA) synthesis *in vitro* the PCR primers were designed based on the target gene

sequences, with the addition of the promoter sequence for the T7 RNA polymerase. Since the MEGAscript® RNAi Kit (Life Technologies, Carlsbad, CA) was used later for dsRNA synthesis (utilizing the T7 promoter sequence), the T7 promoter sequence (5'-GCGTAATACGACTCACTATAGGGAGA-3') was added to the 5' end of both the forward and reverse primers.

In vitro test of dsRNA on growth of *R. solani*

The test of *in vitro* antifungal activities for a set of synthetic dsRNA molecules have been developed by Venganza Inc. and reported by Mumbanza et al. in 2013 (Mumbanza et al., 2013). Briefly, dsRNA molecules were incubated with a small portion of fungus, and after incubation, fungus spore number or mycelium growth speed in the following days was used as a measurement to indicate the inhibition effect of the dsRNA molecules.

With the amplified PCR products containing T7 promoter sequence, the corresponding dsRNA for each segment was synthesized *in vitro* using the MEGAscript® RNAi Kit. After overnight synthesis at 37 °C and purification, dsRNA concentration was determined and 7 µg dsRNA was dissolved into 50 µl distilled water. *R. solani* was cultured on ¼ Potato Dextrose Agar (PDA) (Sigma-Aldrich, St. Louis, MO) with 100 µg/ml ampicillin for 3 days. Under sterile conditions a small portion of *R. solani* mycelium was picked by forceps and incubated in 50 µl dsRNA solution at room temperature overnight. The mycelium was picked out the next day and cultured in the center of fresh plates of ¼ PDA medium. The growth of *R. solani* mycelium was examined every 12 hours, and the diameter of the colony spreading on the medium was measured. Ribosomal dsRNA solution and pure water without dsRNA were used as controls. Diameter of mycelium growth on the

PDA plate was measured 2 days after they were moved onto the fresh plate. The growth areas of the mycelium incubated with these dsRNA were calculated, and their area divided by the growth area of fungus mycelium incubated with water was used as the indicator for the percentage of inhibition. The slower growth of the *R. solani* indicated the greater inhibition effect of the corresponding dsRNA. This assay was repeated for 3 times, and those segments causing greater inhibition in this group were selected for further modification.

In addition to assessing the inhibitory activity of a single dsRNA segment, we also performed assays using a mixture of dsRNA segments from two genes that identified from the former step, using 3.5 μg of dsRNA for each gene (7 μg total in 50 μl solution) and their inhibition of mycelial growth was evaluated similarly. The combinations of dsRNA showing the greatest inhibitory effects were selected to be incorporated into plant transformation constructs.

Gene editing to avoid off-target effect

There were two principles for the gene modification: 1, the gene sequence after modification should keep its silencing effect for the target gene; 2, the sequences after modification should have minimized silencing effects on non-target genes.

In the absence of tall fescue genome sequence, to follow the first principle, the gene segment was analyzed on the MIT website (<http://sirna.wi.mit.edu/home.php>). It is a website that can mimic the dicer activity to cleave the dsRNA into 21 bp small interference RNA (siRNA) and arrange these siRNA from the most negative score to the positive score, to rank the silencing effect of each siRNA from strong to weak. The strongest 10 siRNA segments of each gene were marked in the whole gene sequence so not to be altered during the gene

editing process to preserve the silencing effect of the target gene after the editing.

For the second principle, the gene segment was used as query to perform nucleotide BLAST on NCBI website, against all of the sequences available on NCBI except the fungus database (Table II-2). Nucleotide modification or editing would be carried out when 18 or more identical base pairs were found in the BLAST. Once the blast result showed that the homologous sequence has 18 or more nucleotides, one of the nucleotides in this fragment was changed. And the sequence after such modification was re-blasted until all the homologous sequence has less than 18 nucleotides. The number 18 was chosen because the upper limit of contiguous base pairs that are generally able to avoid off-target effects is 18 (Xu, Zhang, Kang, Roossinck, & Mysore, 2006).

In addition to the edited version of these gene segments, we also include their original version of gene segments without such modification for transgenic construct making. Thus, there were 2 versions for each gene segments.

Moreover, to avoid accidental expression of any unwanted proteins, stop codons were introduced at the 5' termini of the 6 possible reading frames for each gene segment.

Gateway cloning and transformation

After gene editing, a total of four gene segments each containing fused segments of two genes, were introduced into an *Agrobacterium* binary vector, respectively. For convenience, Gateway® cloning technology (Life Technologies) was used and pANIC8A, designed to make RNAi constructs for monocot plants (Figure II-1, Mann et al., 2012), was chosen as the destination vector. By following the manufacturer's instruction, AttL1(5'-CAAATAATGATTTTATTTTGAC

TGATAGTGACCTGTTTCGTTGCAACAAATTGATAAGCAATGCTTTTTTATAATGCC
AACTTTGTACAAAAAAGCAGGCT-3') and attL2 (5'-ACCCAGCTTTCTT
GTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAG
GTCACTATCAGTCAAAAATAAAATCATTATTTG-3') were placed on the two termini of
the fused gene sequences, and the whole RNAi segments were synthesized and cloned in the
pUC57 vector by GenScript (Piscataway, NJ), which was used directly as a pENTR vector.
The RNAi constructs were verified with PCR, restriction digestion, and nucleotide
sequencing. Verified vectors were then transformed into *Agrobacterium* EHA105
individually (by freeze thaw method (Holsters et al., 1978)) for tall fescue transformation. A
total of four such fusion constructs were made.

II. *Agrobacterium* mediated transformation

Callus induction and preparation

Tall fescue cultivar “Coronado” (Rose-Fricker et al. 1999) was used for RNAi and
Pen4-1 construct transformation. Callus was induced from mature seeds of tall fescue. The
mature seeds were dehusked and sterilized as described (Dong & Qu, 2005). The embryo of
the seeds was sliced longitudinally to wound it, and callus was initiated from these wounded
seeds by growing them one month on callus induction medium – MS10 (Table II-1). Then
good quality callus (yellowish and compact) was picked and subcultured two to three times
on MS5 medium (Table II-1), with 3 weeks per subculture. Callus around 3 months old was
ready for *Agrobacterium tumefaciens*-mediated transformation.

***Agrobacterium tumefaciens*-mediated transformation**

Embryogenic callus with good quality (light yellowish and compact) (Dong & Qu, 2005) were picked and put onto a petri dish with two layers of filter papers, which were wet with 2 ml of infection medium (Table II-1). Transformation was carried out three days after the callus was desiccated on the filter paper. *Agrobacterium* was grown overnight in 10 ml YEP medium (10 g Yeast Extract, 10 g Bacto peptone, 5 g NaCl per liter), in an incubator shaker at 230 rpm, 28 °C. After approximately 16 hours, the OD₆₀₀ of the bacterium culture would reach 0.8 to 1.0. *Agrobacterium* cells were collected by centrifugation at 4000 g for 10 min, and then resuspended in 25 ml of the infection medium (Table II-1). *Agrobacterium* suspension was incubated again in the same shaker for 2 more hours prior to use. Then the prepared calli were mixed with *Agrobacterium* in 50 ml centrifuge tubes, and placed in 42 °C for 3 minutes as a heat-shock treatment (Patel, Dewey, & Qu, 2013), followed by slight agitation at room temperature for another 10 minutes. The calli were then blotted with 3 layers of filter paper to remove excessive *Agrobacterium*, and again were transferred to a petri dish with two layers of filter papers wet with 2 ml of infection medium and kept for three days of co-cultivation, in a 25 °C growth chamber. After co-cultivation, the calli were moved onto the resting medium for one week before transferring to Selection 1 medium for two weeks, followed by two rounds of Selection 2 medium, two weeks each (Table II-1). Callus with active growth after the hygromycin B selection were moved to regeneration medium and kept in a lighted growth chamber at 25 °C under a 16/8 h (day/night) photoperiod (140 mmol m⁻² s⁻¹ of cool white fluorescent light). The regenerated shoots were then transferred to rooting medium and kept in the same environment (Table II-1). In one to

two months, the new plants were ready to be grown in potting soil (Metro-Mix 200, Scotts, Marysville, OH) in the greenhouse.

III. Characterization of transgenic plants

Southern blot analysis

Southern blot was carried out for all of the putative transgenic events. Genomic DNA of tall fescue plants was isolated from the leaf using CTAB method (M. G. Murray & Thompson, 1980) and 20 µg of the DNA of each sample was digested overnight in 150 µl buffer for complete digestion. For all transgenic plant DNA, the restriction enzyme *HindIII* (NEB, Ipswich, MA) was used to digest genomic DNA of transgenic plants. The next day, the digested DNA was precipitated and separated by electrophoresis in a 1% agarose gel and blotted onto Hybond-N+ membranes (Amersham, Little Chalfont, Bucks, UK) according to the manufacturer's instructions. For each gel, around 100 pg corresponding plasmid DNA was used as a positive control. A 710 bp segment of *hph* coding sequence was synthesized by PCR with primers (5'- GGATATGTCCTGCGGGTAAA-3'; 5'- CCGTCAACCAAGCTCTGATA-3') using plasmid pBZ7 as a template, and a 510 bp segment of *OsAct1* promoter sequence was synthesized with primers (5'- CACGTCTCGCAGCCAAA-3'; 5'-CTCGCCCACCCAAACTAC-3') and pANIC8A plasmid as the template. The probes labeled with [α -³²P]-dCTP (Amersham) were synthesized using the Primer-It[®] II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX), and hybridized with the blot membrane in MiracleHyb[™] hybridization solution (Stratagene) overnight at 65 °C. The membrane after hybridization was washed once with 2× SSC (saline-sodium citrate), 0.1% SDS (Sodium dodecyl sulfate) for 5 minutes at room

temperature, and twice with $0.5 \times \text{SSC}$, 0.1% SDS for 15 minutes at 68 °C. Radioactive signal on the membrane was detected by 3 days exposure with Kodak BioMaxMS film (Kodak Eastman, Rochester, NY) and developed in a film processor.

Detection of siRNAs by northern blot

Total RNA was isolated from tall fescue leaf with TRIzol Reagent (Invitrogen) according to its protocol. Northern blot was carried out according to Lu et al. with modifications (Lu et al., 2004). Twenty μg total RNA was incubated at 65 °C for 10 min and then separated by gel electrophoresis using 15% Mini-PROTEAN® TBE-Urea Gel (Bio-Rad), and then transferred to Hybond-N⁺ membrane (Amersham) with Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). UV crosslinking was carried out to fix the RNA on the Hybond-N⁺ membrane (Amersham). After air drying, the membrane was pre-hybridized in 5X SSC, 5X Denhardt's solution and 0.5% SDS at 50 °C for 2–3 h. For all of the gel blots, the whole segment of the transgene sequences in a construct was labeled with ³²P as described above and used to detect siRNA from their corresponding transgenic plants. The segment was synthesized by PCR using primers pANICFP: 5'-TACTTCTGCAGGTCGACTCTA -3' and GUSR: 5'- AACGTATCCACGCCGTATTC-3'. A 21nt segment and a 25 nt segment complimentary to the probe were used as positive control and molecular marker. Membranes with RNA was hybridized with the probe at 50 °C overnight, and washed with 2X SSC for 5 min at 50 °C once, followed by 2-3 times of washing with 2X SSC, 1% SDS at 50 °C for 20 min. Signals were detected by autoradiography on BioMaxMS films (Kodak) at -80 °C and developed with a film processor.

qRT-PCR

Tall fescue plants transformed with Pen4-1 were analyzed by qRT-PCR. Total RNA was extracted from plant leaves using RNeasy_Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. During the process, RNase-free DNase RQ1 (Promega, Madison, WI) was added to eliminate genomic DNA contamination. Eight hundred nanograms of total RNA was used to synthesize cDNA with iScript™ cDNA synthesis Kit (Bio-Rad, Hercules, CA) and the reaction condition was set according to the kit protocol. One µl out of the total 10 µl cDNA synthesized was used for each real time PCR reaction. The iTaq™ Universal SYBR Green Supermix (Bio-Rad) was used for the real time PCR and it was carried out in the Mx3005P QPCR System (Agilent, Santa Clara, CA). Primers Pen4qF: 5'-CTCCAGGCCCATCTTCATC-3' and Pen4qR: 5'-CGTACCTGAAGCAGCAGAG-3' were used for Pen4-1 gene qRT-PCR. As an internal control, RT-PCR of actin gene was performed by using primers TFAcF: 5'-TCTTACCGAGAGAGGTTACTCC-3' and TFAcR: 5'-CCAGCTCCTGTTCATAGTCAAG-3'. The reaction was performed following the manufacturer's protocol and was repeated three times.

IV. Inoculation tests of transgenic plants

***Rhizoctonia solani* for brown patch disease**

The brown patch inoculation test was carried out in a plastic box which has two layers of cheese cloth on a plastic grid. The cheese cloth was soaked with distilled water to maintain high humidity inside the box. Four fully extended leaves with comparable ages were cut from each transgenic event of tall fescue, and laid on the cheese cloth in the box. *R.*

solani subgroup AG1-IA isolated from turfgrass was obtained from Dr. Lane Tredway's lab and was used for inoculation. Firstly, a piece of filter paper pertaining the fungus stored in - 80 °C was put on a petri dish with half strength of Potato Dextrose Agar (PDA) medium (Sigma-Aldrich). Four days later, a piece of agar from the medium of freshly growing fungus was sliced and placed onto new PDA medium. The *R. solani* on the new plate after three days of growth was ready to be used for inoculation. A small piece (2 mm x 2 mm) of agar covered with *R. solani* mycelium was sliced from the petri dish and placed onto the center of the detached leaf surface. The inoculated leaves were kept in the sealed plastic box with high degree of humidity. The box was kept in a growth chamber that has a cycle of 16 hour of light, 28 °C and 8 hour of dark, 24 °C. Distilled water was sprayed into the box once a day to keep the high moisture in the box. Four days after the inoculation, the photo of leaf lesion was taken and analyzed with the software "SigmaScan", using the Macro analysis from University of Arkansas (Karcher & Richardson, 2005) (<http://turf.uark.edu/turfmacro/index.htm>). The lesion area and the healthy area were measured and the calculated disease severity was used for statistical analysis. The inoculation experiment was replicated at least three times.

Statistical analysis

Disease severity was used for statistical analysis. The data were analyzed with SAS. V. 9.3 (SAS Institute, Cary, NC) using "Generalized linear mixed model" procedure (PROC GLMMIX) with LSMEANS statement. Dunnett's method was applied to compare each transgenic line performance with non-transformed control plants.

References

- Dong, S. J., & Qu, R. D. (2005). High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. *Plant Science*, *168*(6), 1453–1458. doi:10.1016/j.plantsci.2005.01.008
- Fricker, C. A., Fraser, M. L., Meyer, W. A., & Funk, C. R. Registration of “Coronado” tall fescue. (1999). *Crop Science*, *39*, 288
- Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M., & Schell, J. (1978). Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular & General Genetics : MGG*, *163*(2), 181–7.
- Karcher, D. E., & Richardson, M. D. (2005). Batch Analysis of Digital Images to Evaluate Turfgrass Characteristics. *Crop Science*, *45*(4), 1536. doi:10.2135/cropsci2004.0562
- Lu, S., Shi, R., Tsao, C.-C., Yi, X., Li, L., & Chiang, V. L. (2004). RNA silencing in plants by the expression of siRNA duplexes. *Nucleic Acids Research*, *32*(21), e171. doi:10.1093/nar/gnh170
- Mumbanza, F. M., Kiggundu, A., Tusiime, G., Tushemereirwe, W. K., Niblett, C., & Bailey, A. (2013). In vitro antifungal activity of synthetic dsRNA molecules against two pathogens of banana, *Fusarium oxysporum* f. sp. *cubense* and *Mycosphaerella fijiensis*. *Pest Management Science*, *69*(10), 1155–62. doi:10.1002/ps.3480
- Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, *8*(19), 4321–4326. doi:10.1093/nar/8.19.4321
- Patel, M., Dewey, R., & Qu, R. (2013). Enhancing *Agrobacterium tumefaciens*-mediated transformation efficiency of perennial ryegrass and rice using heat and high maltose treatments during bacterial infection. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 1–11. doi:10.1007/s11240-013-0301-7
- Sivamani, E., & Qu, R. (2006). Expression enhancement of a rice polyubiquitin gene promoter. *Plant Molecular Biology*, *60*(2), 225–39. doi:10.1007/s11103-005-3853-z
- Xu, P., Zhang, Y., Kang, L., Roossinck, M. J., & Mysore, K. S. (2006). Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiol*, *142*(2), 429–440. doi:10.1104/pp.106.083295

Zhou, M., Hu, Q., Li, Z., Li, D., Chen, C.-F., & Luo, H. (2011). Expression of a novel antimicrobial peptide Penaeidin4-1 in creeping bentgrass (*Agrostis stolonifera* L.) enhances plant fungal disease resistance. *PloS One*, 6(9), e24677.
doi:10.1371/journal.pone.0024677

PART III. RESULTS AND DISCUSSIONS

I: Penaeidin 4-1

Results

Production of transgenic tall fescue containing Pen4-1 gene

The embryogenic calli derived from mature seeds were transformed with *Agrobacterium* ETBZ7 which is EHA105 (pTOK47, pBZ7) with the binary vector pBZ7 containing the *Pen4-1* gene construct within its T-DNA region. Finally, a total of 6 T₀ transgenic plants were obtained through *Agrobacterium*-mediated transformation (Figure III-I-1). For the gene construct, a rice strong, constitutive promoter (*rubi3*) was used to drive the expression of the gene of interest, and *CaMV 35S* promoter was used to drive the expression of an antibiotic (*hygromycin B*) resistance *hph* gene (Figure II-2).

Southern blot analysis of transgenic tall fescue plants

To verify the integration of the foreign DNA into the plant genome, Southern blot hybridization was carried out with the *hph*-specific probe (Figure II-2). Southern blot analysis of the hygromycin B resistant plants confirmed the transgenic nature of these six plants (Figure III-I-2A). Different hybridization patterns among the analyzed plants revealed that they were from independent transformation events. Line P9-1 and P9-2 might develop from the same transgenic event as judged by their identical hybridization pattern on the membrane. No appreciable difference in plant morphology was observed between transgenic and wild type plants.

Pen4-1 expression in transgenic tall fescue plants

Quantitative RT-PCR (qRT-PCR) was carried out to assess the relative expression level of the *Pen4-1* gene in the leaves of transgenic tall fescue plants. The qRT-PCR result revealed variable expression levels of the transcript among different transgenic lines (Figure III-I-2B). No expression of *Pen4-1* gene was detected in the wild type tall fescue plants. Among the Pen4-1 plants, lines 7, 9-1, 9-2 and 11 had higher expression of *Pen4-1* than lines 5 and 8-1 (Figure III-I-2). No obvious correlations were found between gene transcription level and gene copy number in the transgenic plants.

Inoculation with *Rhizoctonia solani*

Detached leaves of the transgenic plants were inoculated with the *R. solani* isolate AG1-IA, which infects tall fescue. Digital photos were taken four days after inoculation and analyzed with software SigmaScan. Statistical analysis of the replicated data using Dunnett's method demonstrated that for all the six plants verified by Southern blot, only one line (No. 11) of the transgenic tall fescue plants didn't show improved resistance, the other 5 transgenic lines showed highly improved resistance against *R. solani* infection, among which four lines (No. 5, 8-1, 9-1, 9-2) showed highly significant difference ($P < 0.01$) and one line (No. 7) showed significant difference ($P < 0.05$) compared to the wild type plant (Figure III-I-4). Averagely, these five plants showed 42.7% improvement in brown patch resistance compared to the wild type tall fescue. However, no obvious correlation was found between the gene transcript level and the degree of disease resistance.

Discussion

The result of inoculation test in this study indicated that the expression of foreign gene *Pen4-1*, which encodes one of the penaeidin proteins isolated from Atlantic white shrimp, conferred the transgenic tall fescue plants significantly higher resistance against the *R. solani* infection. This is the second report of genetically engineering turfgrass with this gene for enhanced fungal pathogen resistance. Zhou et al. firstly incorporated this gene into the genome of creeping bentgrass (*Agrostis stolonifera* L.) and achieved higher resistance against the infection of *R. solani* and *sclerotinia homoeocarpa* which causes dollar spot disease (Zhou et al., 2011). The group of *R. solani* they tested on creeping bentgrass is AG2-2 (IIIB), which is a different group from AG1-IA and does not infect tall fescue. Our study demonstrated that it is also feasible to incorporate this gene into tall fescue for improved resistance against another group of *R. solani*, AG1-IA. Thus the *Pen4-1* gene is effective to confer high resistance against different groups of *R. solani* and a different fungus species, indicating that this gene could be a promising one for broad-spectrum fungal disease resistance.

Since brown patch disease in tall fescue and creeping bentgrass is caused by different groups of *R. solani*, it's not always the case that the resistance gene against one group would have similar effects on another group. For instance, *PepEST* is a resistance gene against AG2-2 (IIIB) discovered in ripe fruit of pepper (*Capsicum annuum*), which codes for an esterase (Kim et al., 2001). The incorporation of this gene into creeping bentgrass improved its resistance against the fungal pathogens *R. solani* AG2-2 (IIIB) and *Sclerotinia homoeocarpa* (dollar spot) (Cho et al., 2011). Transgenic plants pertaining this gene also

indicated higher resistance against rice blast fungus (*Magnaporthe grisea*) (Kim et al., 2001), and the anthracnose fungus *Colletotrichum gloeosporioides* (Ko et al., 2005). However, when we introduced this gene into tall fescue plant, no resistance against *R. solani* group AG1-IA was observed (data not shown), indicating that this gene can confer plants the resistance to *R. solani* AG2-2 (IIIB), but not to the AG1-IA at all, which causes brown patch in tall fescue.

The broad spectrum resistance against the fungal pathogens of *Pen4-1* was revealed by Cuthbertson and colleagues around a decade ago (Cuthbertson et al., 2004, 2006). It turns out that Pen4-1 is able to inhibit the infection of multiple fungal species, including *B. cinera*, *P. crustosum* and *F. oxysporum* (Cuthbertson et al., 2004), and a serial multidrug-resistant fungal species including *Cryptococcus neoformans* and *Candida spp.* (Cuthbertson et al., 2006). Such a broad spectrum of antimicrobial activity is rare and it prompts us to look into its intriguingly antimicrobial mechanism. The penaeidins are originally identified in the haemocytes of penaeid shrimp, during which the peptides play an important role for the shrimp's innate immune system (Cuthbertson et al., 2004). The unconstrained proline-rich N-terminal domain (PRD) and the cysteine-rich domain (CRD) with a stable α -helical structure (six cysteines engaged in three intramolecular disulfide bridges) is a unique two-domain structure for penaeidins, and it is believed that the complexity of this structure might be the reason for such a broad range antimicrobial activity (Cuthbertson et al., 2005, 2004; Destoumieux et al., 2000; Yang et al., 2003). Generally, antimicrobial peptides are divided into four classes based on their sequences or three-dimensional structures: the proline-rich, the glycine-rich, pertaining α -helical structure, and those have disulfide bonds (Yang et al.,

2003). It is claimed that the multifunctional properties and the broad spectrum of activity of the penaeidins might result from the presence of all these features in the same molecule (Yang et al., 2003). The recombinant expression of penaeidins in *Saccharomyces cerevisiae* indicated that PRD might not be directly related to the penaeidin antimicrobial properties, thus it is hypothesized that this domain might be involved in the membrane targeting process, working complementary with the CRD which likely plays an important role in the antimicrobial activity (Destoumieux et al., 1999; Cunthbertson et al., 2006).

As an AMP derived from shrimp rather than plant, Pen4-1's efficacy for antifungal activity in transgenic plants mainly depends on its translation efficiency and post-translational modification. It has been demonstrated in Zhou's work that transgenic creeping bentgrass pertaining the monocot plant preferred codons of *Pen4-1* sequence showed improved resistance against fungal pathogens (Zhou et al., 2011), and in this project, we used the transgene with monocot-preferred codons in tall fescue transformation. Actually, regarding the incorporation of non-plant-derived AMP genes into plant, both native AMP genes and codon modified AMP genes have been tested in previous studies. For instances, the native and codon-optimized *Aspergillus giganteus* antifungal protein AFP has been transformed into rice plants, and no different translational efficiency or different resistance level against *Magnaporthe grisea* was observed between these transgenic plants (Coca et al., 2004). In another case, the native sequence of two AMP genes from Chinese shrimp, *np3* and *np5*, have been transformed into rice and resulted in improved resistance against bacterial blight (Wang et al., 2011).

In conclusion, our study showed that transgenic tall fescue plants expressing the codon modified shrimp *Pen4-1* gene enhanced the resistance against the brown patch fungal pathogen *R. solani*, group AG1-IA, while the plants grew normally. The enhanced resistance is worth to be evaluated in field tests. It's another case to demonstrate the broad-spectrum antimicrobial activity of Pen4-1 protein, which could have a great potential to improve microbial disease resistance in other crops.

Reference

- Cho, K.-C., Han, Y.-J., Kim, S.-J., Lee, S.-S., Hwang, O.-J., Song, P.-S., ... Kim, J.-I. (2011). Resistance to *Rhizoctonia solani* AG-2-2 (IIIB) in creeping bentgrass plants transformed with pepper esterase gene PepEST. *Plant Pathology*, 60(4), 631–639. doi:10.1111/j.1365-3059.2011.02433.x
- Coca, M., Bortolotti, C., Rufat, M., Peñas, G., Eritja, R., Tharreau, D., ... San Segundo, B. (2004). Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology*, 54(2), 245–59. doi:10.1023/B:PLAN.0000028791.34706.80
- Cuthbertson, B. J., Büllsbach, E. E., Fievet, J., Bachère, E., & Gross, P. S. (2004). A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. *The Biochemical Journal*, 381(Pt 1), 79–86. doi:10.1042/BJ20040330
- Cuthbertson, B. J., Büllsbach, E. E., & Gross, P. S. (2006). Discovery of synthetic penaeidin activity against antibiotic-resistant fungi. *Chemical Biology & Drug Design*, 68(2), 120–7. doi:10.1111/j.1747-0285.2006.00417.x
- Cuthbertson, B. J., Yang, Y., Bachère, E., Büllsbach, E. E., Gross, P. S., & Aumelas, A. (2005). Solution structure of synthetic penaeidin-4 with structural and functional comparisons with penaeidin-3. *The Journal of Biological Chemistry*, 280(16), 16009–18. doi:10.1074/jbc.M412420200

- Destoumieux, D., Bulet, P., Strub, J. M., Van Dorsselaer, A., & Bachère, E. (1999). Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. *European Journal of Biochemistry / FEBS*, 266(2), 335–46.
- Destoumieux, D., Munoz, M., Bulet, P., & Bachère, E. (2000). Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). *Cellular and Molecular Life Sciences : CMLS*, 57(8-9), 1260–71.
- Kim, Y. S., Lee, H. H., Ko, M. K., Song, C. E., Bae, C. Y., Lee, Y. H., & Oh, B. J. (2001). Inhibition of fungal appressorium formation by pepper (*Capsicum annuum*) esterase. *Mol Plant Microbe Interact*, 14(1), 80–85. doi:10.1094/MPMI.2001.14.1.80
- Ko, M. K., Jeon, W. B., Kim, K. S., Lee, H. H., Seo, H. H., Kim, Y. S., & Oh, B.-J. (2005). A *Colletotrichum gloeosporioides*-induced esterase gene of nonclimacteric pepper (*Capsicum annuum*) fruit during ripening plays a role in resistance against fungal infection. *Plant Molecular Biology*, 58(4), 529–41. doi:10.1007/s11103-005-7088-9
- Wang, W., Wu, C., Liu, M., Liu, X., Hu, G., Si, H., ... Fu, Y. (2011). Resistance of Antimicrobial Peptide Gene Transgenic Rice to Bacterial Blight. *Rice Science*, 18(1), 10–16. doi:10.1016/S1672-6308(11)60002-4
- Yang, Y., Poncet, J., Garnier, J., Zatylny, C., Bachère, E., & Aumelas, A. (2003). Solution structure of the recombinant penaeidin-3, a shrimp antimicrobial peptide. *The Journal of Biological Chemistry*, 278(38), 36859–67. doi:10.1074/jbc.M305450200
- Zhou, M., Hu, Q., Li, Z., Li, D., Chen, C.-F., & Luo, H. (2011). Expression of a novel antimicrobial peptide Penaeidin4-1 in creeping bentgrass (*Agrostis stolonifera* L.) enhances plant fungal disease resistance. *PLoS One*, 6(9), e24677. doi:10.1371/journal.pone.0024677

II: Host Induced Gene Silencing

Results

Target gene selection from the *R. solani* database

Genomic DNA sequence of *Rhizoctonia solani* was searched to identify “essential genes” for the fungus to survive as the target genes for HIGS based on the knowledge and genomic sequence of a closely-related species, *Neurospora crassa*. From the BLAST (Basic Local Alignment Search Tool) results using AA sequences of 32 essential *N. crassa* proteins (Bailey, personal communication), 20 homologous *R. solani* proteins were identified, leading to the corresponding cDNA sequences.

Those sequences were further reviewed and screened using NCBI database and MIT website. Finally, 15 gene segments were selected to proceed to the next step of PCR amplification (Table III-1).

PCR amplification and dsRNA synthesis

For the gene sequences identified by the previous steps, the actual segments were obtained by PCR using *R. solani* genomic DNA as templates, with corresponding primers designed from the previously obtained sequences. The T7 RNA polymerase promoter sequence was also incorporated into the PCR products for subsequent use for dsRNA synthesis. After verification of PCR product sizes and sequences, twelve gene segments were obtained, and their dsRNAs were synthesized *in vitro* using the T7 RNA polymerase. During this process, *R. solani* 5S ribosomal RNA (rRNA) gene segment was also prepared as a control for the subsequent screening process. About 50 µg of dsRNA was synthesized from each gene segment for further analysis.

In vitro* dsRNA bioassays to determine inhibition activity against *R. solani

growth

The inhibitory activity of the synthesized dsRNAs was assessed *in vitro* by soaking the mycelium in the dsRNAs and measuring the inhibition of radial growth of the fungus.

During overnight incubation the dsRNA was believed to be absorbed by the fungus and the corresponding gene expression would be suppressed as measured by inhibition of radial growth of the fungal colony. This would indicate the importance of the gene to fungal growth as well as the potential efficacy of the dsRNA in a plant transformation construct. It was observed that dsRNA of the rRNA gene segment inhibited fungal growth by 50% at the concentration tested (7 μ g dsRNA/50 μ l dH₂O). Thus the dsRNA of a gene that had greater inhibition effect than the ribosomal dsRNA would be recognized as a good candidate for the RNAi gene construct. After three repetitions, 6 genes with higher inhibition effect were selected. In order to achieve better silencing effect with a single transgene construct, we combined two gene segments in one construct, thus we can silence two genes at the same time. Based on three repetitive test with combined dsRNA segments, two groups of gene segments were found to have the highest inhibition effect on mycelium growth, which were: RNA polymerase (RNApoly) combined with Importin beta-1 subunit (Imbs), and Cohesin complex subunit (Coh) combined with Ubiquitin ligase E3 (UbiE3) (Table III-2 and Figure III-II-1). Thus gene segment RNApoly was fused to Imbs (RNApoly::Imbs), and gene segment Coh with UbiE3 (Coh::UbiE3), to form two transgene constructs for further modification and transgenic construct design.

Transgene modification or “editing” to avoid “off target” hits

As the dsRNA of these selected genes were going to be expressed in tall fescue, and tall fescue is also livestock forage, any “off target” effect to tall fescue or to livestock could be a potential “side effect”. To avoid this potential effect, the nucleotide sequences were modified in an attempt to minimize their impact on other genes of either tall fescue or other organisms. Thus there were two principles for the gene modification: 1, the gene sequence after modification should keep its silencing effect for the target gene; 2, the sequences after modification should have minimized silencing effects on non-target genes. Gene editing details were described in the **Materials and methods** with section **Gene editing to avoid off-target effect**.

As a result, four edited gene segments were obtained from the four native gene segments identified in the previous step. Considering that modified gene segments might lose their efficacy for their RNAi activity as the gene sequences were edited, we also used the original version of these gene segments (without modification) to make gene constructs. Thus we could compare the efficacy of the siRNA activity produced from both the native segments and the edited segments. In addition, to avoid expression of any potentially allergenic proteins, stop codons were introduced near the 5' termini of all six possible reading frames for each gene segment (Table III-4).

Making RNAi constructs using Gateway technology

Based on the screening result from *in vitro* assay, two gene segments were fused in each RNAi construct, RNA polymerase with Importin beta-1 subunit (RNAPoly::Imbs), and Cohesin complex subunit with Ubiquitin ligase E3 (Coh::UbiE3). Therefore, based on the

gene modification result, there were two sets of these fused gene segments: the native sequences of RNAPoly::Imbs, and Coh::UbiE3, and the edited versions of RNAPoly::Imbs, and Coh::UbiE3. These segments were named NR (natural version of RNAPoly::Imbs), NC (natural version of Coh::UbiE3), and MR (modified version of RNAPoly::Imbs), MC (modified version of Coh::UbiE3) (Table III-5). Thus, a total of four fused gene segments were synthesized for making transformation constructs. .

These four segments were introduced into a destination vector – pANIC8A using the Gateway® cloning technology. pANIC8A is a binary vector especially designed for making RNAi constructs for monocot plants using maize *ubi1* promoter to drive the transgenes (Figure II-1A, Mann et al., 2012). In this way four binary vectors were obtained and the sequence verified vectors were then transformed into *Agrobacterium* strain EHA105 for tall fescue transformation. Four *Agrobacterium* lines were produced, which contained the binary vector containing NR (RNAPoly::Imbs), NC (CohP::UbiE3), MR and MC, respectively (Figure II-1).

Production of transgenic tall fescue with the RNAi constructs

Since we have four fusion RNAi constructs, we named the transgenic tall fescue plants transformed with these constructs as NR plants, NC plants, MR plants and MC plants, respectively. Over all, we obtained 4 independent transformants of NC plants, 4 MC plants, 3 MR plants and 9 NR plants.

All of the RNAi constructs were based on the pANIC 8A vector (Mann et al, 2012), which contained the PvUbi1:pporRFP reporter gene cassette. Three weeks after transformation, callus cultures were viewed under microscope for the detection of red

fluorescence. As shown in Figure III-II-2, red fluorescence was observed on the hygromycin B resistant callus with the microscope filter set for RFP (535.3 nm excitation, 600.5 nm emission), which confirms the successful integration of transgenic cassettes and their stable expression.

All of the RNAi plants grew normally except 2 NC plants: NC2 and NC5, while the growth of the other two NC plants (NC 21 and NC22) was not affected (Figure III-II-3, 4). NC2 and NC5 grew normally at the first 3 months after they were transplanted from the rooting medium to soil. However, after 3 months, their growth slowed down, and they eventually died in the greenhouse. In contrast, the growth of MC plants containing the modified version of the dsRNA targeting the same fungus genes was not affected. We speculate that NC transgenic plants might contain some siRNAs that could interfere the expression of certain important genes of tall fescue.

Southern hybridization analysis of RNAi plants

Southern blot analysis was performed to verify the presence and integration of the foreign DNA into the plant genome. The blot hybridization was carried out with the *hph*-specific probe (Figure II-1). In total, 20 hygromycin B resistant plants were analyzed with Southern hybridization, and the transgenic events were verified for each transgenic construct: 8 NR plants (Figure III-II-5A), 3 MR plants (Figure III-II-5 B), 4 NC plants and 4 MC plants (Figure III-II-5C). Because the restriction enzyme *HindIII* used to cut the genomic DNA of tall fescue cannot always distinguish various transformation events in Southern blot (Figure II-2), it is difficult to judge if the plants were independent transgenic lines based on their hybridization patterns on the Southern blot for some of the plants. However, most of the

transgenic plants were from separate petri dishes during selection and thus were from independent events. Some plants have multiple and/or more intensive bands (such as NR1, 14, 16, MR1, 2, NC21, 22, and all the four MC plants), suggesting they likely have multiple copies of the transgene. Others appear to have single transgene copies. Although they were from different petri dishes based on my note record, we cannot exclude the possibility that NC21 and NC22 may be from the same transformation event as judged by the hybridization patterns.

Northern hybridization analysis for siRNAs

Northern blot analysis was carried out to detect accumulation of the siRNAs in the transgenic plants, and the results are shown in Figure III-II-6. A majority of the RNAi transgenic plants had detectable amounts of siRNAs in plant leaves, but different levels of siRNA accumulation were observed (Figure III-II-6). As shown in Figure III-II-6, the size of the siRNAs detected on the blots varied slightly but are in the range between 21nt and 25nt. The following plants have detectable siRNA shown in Figure III-II-6: NR plant (A): 1, 2, 3, 11, 12 and 16; from MR plant (B): 1-4; from NC plant (C): 2 and 5; from MC plant (D): 5, 12 and 15. A few other plants, i.e., NR4, MR 2 and 3, NC21 and 22, and MC1 did not contain detectable levels of siRNAs.

The NC2 and NC5 plants contained siRNAs accumulation, but they did not survive in the greenhouse. In the contrary, plants NC21 and NC22 did not contain siRNAs, and grew normally in the greenhouse. This further supports our speculation that the NC RNAi construct might produce certain siRNA that silence some tall fescue genes that are essential for plant growth or development.

Inoculation with *Rhizoctonia solani*

All of the T₀ transgenic tall fescue (7 NR plants, 4 MR plants, 2 NC plants (the other 2 died) and 4 MC plants) were inoculated with *R. solani* isolate AG1-IA. Leaf lesion percentages were analyzed using SAS software and the statistical results revealed that 3 NR transgenic plants (NR1, NR12, NR14) have highly significant resistance (P<0.01), which showed 46.02%, 41.79% and 39.42% for the improved resistance compared to wild type plants, respectively; 1 NR plants (NR3) has slightly higher resistance (P<0.1) which showed 21.52% for the improved resistance; and 2 MC plants (MC12, MC15) have significantly higher resistance (P<0.05), which showed 37.40% and 45.75% for the improved resistance, respectively (Table III-5, Figure III-II-8). No resistance was found in the MR and NC plants.

Several of the plants, NR4, MR2, MR3, NC21, NC22, and MC1 were shown to be transgenic by Southern blots. But no siRNAs were detected by northern blots, and none of them showed disease resistance in the inoculation test. This further demonstrates that the observed resistance was caused by inhibition of *R. solani* growth due to the siRNA formed by the transgene, and HIGS is an effective technology to control *R. solani* in tall fescue.

Discussion

Host Induced Gene Silencing (HIGS) for plant disease resistance

Based on our results, we postulate that the dsRNA or siRNA produced in the host plant can be transferred into the fungus *R. solani* during the infection process, and lead to the silencing of the genes that might be involved in host-pathogen interaction or those fungal genes playing an important role in fungal development or fungal pathogenicity. Initially

HIGS was applied to plant virus resistance (Bonfim et al., 2007), nematode resistance and insect resistance (Lilley et al., 2012; Gordon & Waterhouse, 2007; Mao et al., 2007; Zha et al., 2011). Recently, HIGS has also been applied for fungal resistance. For instance, the accumulation of siRNA targeting an effector gene *Avra10* in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) resulted in improved host resistance against powdery mildew *Blumeria graminis* (Nowara et al., 2010), and complete immunity to *Fusarium graminearum* (*Fg*) was achieved by expressing a double-stranded RNA targeting the fungal sterol 14 α -demethylase (CYP51) genes in Arabidopsis and barley (Koch et al., 2013). However, the previous findings about using HIGS for higher fungus resistance were all focused on biotrophic or hemibiotrophic fungi. Our results with *R. solani* revealed that utilizing HIGS for high resistance against necrotrophic fungi was also feasible, and this extends the application of HIGS for plant disease control.

Using native gene sequence vs. edited gene sequence for HIGS

Comparing the NR plants and MR plants, the NR transgenic plants, having the native gene sequences, showed much higher levels of resistance than the MR transgenic plants containing the edited versions of the gene fragments. This likely resulted from the lower silencing ability of the edited sequences in the dsRNA, compared to the native sequences produced in the NR plants. This finding confirms our original concern that excessive modification of the native gene sequences to avoid off-target effect could compromise the silencing effect on the target gene.

For the other two groups of transgenic plants, 2 NC plants with detectable siRNAs died before being inoculated with the fungus, and the other 2 NC plants without detectable

siRNAs survived but didn't show any resistance, whereas 2 out of 3 MC plants with detectable siRNAs showed good resistance. This finding showed that siRNA derived from native gene sequences could interfere with the host plant's growth, while it might be feasible to modify the gene sequence to avoid this host growth interference and still keep the silencing effect against their target gene.

Combining these two findings, we conclude that using the original sequence for HIGS could work better than the modified gene sequence. However, for certain genes, using their original sequence for HIGS may interfere with the host plant growth and development, and this "side effect" could be avoided by certain level of sequence modification and still maintain their silencing efficacy. Thus for HIGS, the results using the original sequence or the modified sequence could vary from gene to gene. However, considering the environmental effect of such transgenic plants, sequence modification would be advisable to avoid the off-target gene silencing in other organisms, and this could be compensated by testing more target genes.

Transfer of silencing signals from host to fungus

Since the concept of parasite-derived resistance (PDR) proposed around 30 years ago by Sanford and Johnston (1985), the macromolecule exchange between the host and parasite has been an intriguing question for scientists. During the past decade, the mechanism and fundamental principle of applying RNA silencing for plant resistance have been elucidated due to the rapid development of RNAi based on Fire and Mello's findings in 1998. However, exactly how the silencing signal is transferred from the host to fungus remains unclear.

The transfer of silencing signal from plant to fungal pathogens has its peculiarity compared to the signal transfer between plants and other pests. For instance, viral DNA/RNA are directly exposed in the host cell and replicate and propagate inside the plant, thus the virus gene might be silenced during their replication inside the plant cytoplasm; Nematodes and insects normally harm plants by eating plant tissue like root, leaf or suck the sap from plant phloem. The long distance transport and movement of viral RNA genomes, endogenous cellular mRNAs and small non-coding RNAs in plant phloem have been verified (Kehr & Buhtz, 2008). The silencing signal can be transported from plant to nematodes and insects through ingestion, and then spread systemically inside the pests from the gut (Price & Gatehouse, 2008). Compared to the interaction between plant and viral pathogen, nematodes and insects, the interaction between bacteria and host plant might be closer to the fungus and plant interaction. Bacteria can penetrate plant cell wall and membrane using certain structures like Type III secretion system (five types are known so far), through which bacteria can colonize their host and inject effector proteins into host cell cytosol (Büttner & He, 2009). The secretion systems might be a good pathway for silencing signal transfer from host to bacteria, however, it has not been demonstrated and no research using HIGS for plant bacterial resistance has been reported.

Unlike bacterial pathogens that penetrate plant cell walls with a series of secretion systems, fungi interact with plants through a highly specialized cell called haustorium, a structure between the host plant membrane and fungal pathogen used to maintain the parasitic relationship through signal exchange and nutrient uptake (Voegelé & Mendgen, 2003). It has been proposed that silencing signals might be transferred into the fungus

through the haustorium interface (Panwar et al., 2013). However, how exactly the silencing signal is transferred from plant cell cytosol through this interface to the fungal cell remains to be elucidated.

Exosomes as RNAi signal cargo for cross-species transport

Exosomes are endosome-derived small (30–100 nm) membrane microvesicles, that can fuse with plasma membranes and released the contents into the extracellular environment (Van et al., 2006). Therefore, exosomes play an important role for intercellular communication, involve in a series of biological functions including angiogenesis, cell proliferation, and tumor cell invasion and metastasis (Huang et al., 2013). More interestingly, exosomes are found to be able to transport mRNAs and non-coding RNAs to neighboring or distant cells (Pegtel et al., 2011). In 2013, Panwar *et al.*, who applied HIGS on wheat and improved its resistance against the rust fungus (*Puccinia triticina*), already postulated that host generated siRNA molecules might be transported into the fungus cell through the haustorium interface using exosomes as a carrier (Panwar et al., 2013). And recently, the findings about miRNA trafficking related to cancer cells make that postulation more plausible. Melo *et al.* showed that breast cancer cells can secrete exosomes which transfer miRNAs to normal cells and make them cancerous (Melo et al., 2014). While these exosomes derived from the cancer cell not only deliver miRNAs, but also key enzymes involved in miRNA biogenesis including Dicer, AGO2 and TAR (trans-activation response) RNA-binding protein (TRBP). Thus in terms of plant fungus interactions, it is also possible that exosomes transport silencing signals together with the siRNA biogenesis-related proteins from the host to fungal pathogen. However, what kind of form and size of the silencing

signal is carried in the exosome remains unknown. Are they siRNA, long single stranded RNA, or dsRNA? The research by Huang et al. on characterization of human plasma-derived exosomal RNAs by deep sequencing might shed some light on this problem (Huang et al., 2013). If it is feasible to isolate exosomes from transgenic plants with silencing signal, then carry out the deep sequencing of the RNA extracted from these exosomes might enable us to tell the size and the form of the silencing factor(s).

Necrotrophic fungus resistance obtained by HIGS

Compared to the previous studies on HIGS application for plant fungal disease resistance, the fungus we researched (*R. solani*) is necrotrophic, which destroys the host cells and extract nutrients from the dead host cells, while the fungi studied before on HIGS were biotrophic (wheat leaf rust) or hemibiotrophic (*Phytophthora infestans*) (Panwar et al., 2013; Eschen-Lippold et al., 2012), both of which have a stage to colonize and extract nutrients from living plant tissue. As discussed above, for biotrophic or hemibiotrophic fungus, the silencing signal could be transferred into the fungus by exosome biosynthesis pathway through the haustorium interface during the host-fungus parasitic interaction. In the contrary, for the necrotrophic fungus *R. solani*, the successful infection results in the disruption of the host cell. Thus, there is a question about the timing of the silencing signal transfer. Does it happen immediately after the penetration of fungal hypha, or after the host cell death when the cytosol is disrupted? If it is the latter, the next question is that if the silencing signal is still transferred through the exosomes, or the exosomes also get disrupted and everything inside is mixed with other cell debris? In this case, it will depend on whether the exosomes

can stay intact during the *R. solani* infection, and whether the silencing signal can survive in the disrupted environment.

Genetic information transfer across kingdom

Genetic material transfer between prokaryotes such as bacteria and other microorganisms has been well known for several decades, not to mention the gene transfer between viruses and their hosts. The research in the last 10 years revealed that the genetic material transfers were not limited between microorganisms which don't have a protective nucleus enveloping their DNA, but was also found between the eukaryotes like animals, fungi and plants. The gene transfer between different species or even different kingdoms is termed as horizontal gene transfer, compared to the traditional "vertical transfer" from parent to child (Skippington & Ragan, 2011). A recent Nature paper just indicated that the entire nuclear genomes can be transferred between plant cells just by grafting, a simple plant-plant interaction (Fuentes et al., 2014). Just as W. F. Doolittle said "the exchange of genetic information across species is far more pervasive and more radical in its consequences than we could have guessed just a decade ago" (Zhaxybayeva & Doolittle, 2011). More and more evidence is showing that not only DNA fragments, but also small RNAs (sRNA: 19–25 nt long) can be transferred between different kingdoms (Knip et al., 2014), which is happening in nature. For instance, the endogenous siRNA of a plant pathogen, *Botrytis cinerea*, can be transferred into *Arabidopsis* and tomato and silence the defense genes in the hosts, thereby enhancing its pathogenicity (Weiberg et al., 2013).

Applying HIGS to raise the plant resistance against fungal pathogens is a technique derived from the RNA silencing mechanism, whose effectiveness is established by the

siRNA transfer from the host plant to the pathogen. In nature DNA transfer between different organisms or kingdoms occurs and makes the genetically modified organisms (GMO) not so “unorthodox”; while on the other side, the possibility of transgene escape and outflow into other organisms brings more concern about the design of the transgene. Especially for HIGS, transfer of a gene fragment into other organisms might induce repression of non-target endogenous genes. Thus, sequence modification to avoid off-target effects in HIGS experiment design is very important if we want to apply this technique in the real life. Deeper knowledge of the RNA silencing mechanism especially sequence specificity, and more availability of the genetic information for all of the organisms related to the transgenic plant, will be helpful for us to achieve this goal.

References

- Bonfim, K., Faria, J. C., Nogueira, E. O. P. L., Mendes, E. A., & Aragão, F. J. L. (2007). RNAi-mediated resistance to Bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). *Molecular Plant-Microbe Interactions : MPMI*, 20(6), 717–26. doi:10.1094/MPMI-20-6-0717
- Büttner, D., & He, S. Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiology*, 150(4), 1656–64. doi:10.1104/pp.109.139089
- Eschen-Lippold, L., Landgraf, R., Smolka, U., Schulze, S., Heilmann, M., Heilmann, I., ... Rosahl, S. (2012). Activation of defense against *Phytophthora infestans* in potato by down-regulation of syntaxin gene expression. *The New Phytologist*, 193(4), 985–96. doi:10.1111/j.1469-8137.2011.04024.x
- Fuentes, I., Stegemann, S., Golczyk, H., Karcher, D., & Bock, R. (2014). Horizontal genome transfer as an asexual path to the formation of new species. *Nature*, 511(7508), 232–235. doi:10.1038/nature13291

- Gordon, K. H. J., & Waterhouse, P. M. (2007). RNAi for insect-proof plants. *Nature Biotechnology*, 25(11), 1231–2. doi:10.1038/nbt1107-1231
- Huang, X., Yuan, T., Tschannen, M., Sun, Z., Jacob, H., Du, M., ... Wang, L. (2013). Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics*, 14(1), 319. doi:10.1186/1471-2164-14-319
- Kehr, J., & Buhtz, A. (2008). Long distance transport and movement of RNA through the phloem. *Journal of Experimental Botany*, 59(1), 85–92. doi:10.1093/jxb/erm176
- Knip, M., Constantin, M. E., & Thordal-Christensen, H. (2014). Trans-kingdom Cross-Talk: Small RNAs on the Move. *PLoS Genetics*, 10(9), e1004602. doi:10.1371/journal.pgen.1004602
- Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., & Kogel, K.-H. (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14 α -demethylase-encoding genes confers strong resistance to Fusarium species. *Proceedings of the National Academy of Sciences of the United States of America*, 110(48), 19324–9. doi:10.1073/pnas.1306373110
- Lilley, C. J., Davies, L. J., & Urwin, P. E. (2012). RNA interference in plant parasitic nematodes: a summary of the current status. *Parasitology*, 139(5), 630–40. doi:10.1017/S0031182011002071
- Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J., Tao, X. Y., Wang, L. J., ... Chen, X. Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotechnol*, 25(11), 1307–1313. doi:10.1038/nbt1352
- Melo, S. A., Sugimoto, H., O'Connell, J. T., Kato, N., Villanueva, A., Vidal, A., ... Kalluri, R. (2014). Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis. *Cancer Cell*, 26(5), 707–721. doi:10.1016/j.ccell.2014.09.005
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., ... Schweizer, P. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *The Plant Cell*, 22(9), 3130–41. doi:10.1105/tpc.110.077040
- Panwar, V., McCallum, B., & Bakkeren, G. (2013). Endogenous silencing of *Puccinia triticina* pathogenicity genes through in planta-expressed sequences leads to the suppression of rust diseases on wheat. *The Plant Journal : For Cell and Molecular Biology*, 73(3), 521–32. doi:10.1111/tpj.12047

- Pegtel, D. M., van de Garde, M. D. B., & Middeldorp, J. M. Viral miRNAs exploiting the endosomal-exosomal pathway for intercellular cross-talk and immune evasion. *Biochimica et Biophysica Acta*, 1809(11-12), 715–21. doi:10.1016/j.bbagr.2011.08.002
- Price, D. R. G., & Gatehouse, J. A. (2008). RNAi-mediated crop protection against insects. *Trends in Biotechnology*, 26(7), 393–400. doi:10.1016/j.tibtech.2008.04.004
- Skippington, E., & Ragan, M. A. (2011). Lateral genetic transfer and the construction of genetic exchange communities. *FEMS Microbiology Reviews*, 35(5), 707–35. doi:10.1111/j.1574-6976.2010.00261.x
- Van Niel, G., Porto-Carreiro, I., Simoes, S., & Raposo, G. (2006). Exosomes: a common pathway for a specialized function. *Journal of Biochemistry*, 140(1), 13–21. doi:10.1093/jb/mvj128
- Voegelé, R. T., & Mendgen, K. (2003). Rust haustoria: nutrient uptake and beyond. *New Phytologist*, 159(1), 93–100. doi:10.1046/j.1469-8137.2003.00761.x
- Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., ... Jin, H. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science (New York, N.Y.)*, 342(6154), 118–23. doi:10.1126/science.1239705
- Wibberg, D., Jelonek, L., Rupp, O., Hennig, M., Eikmeyer, F., Goesmann, A., ... Schlüter, A. (2013). Establishment and interpretation of the genome sequence of the phytopathogenic fungus *Rhizoctonia solani* AG1-IB isolate 7/3/14. *Journal of Biotechnology*, 167(2), 142–55. doi:10.1016/j.jbiotec.2012.12.010
- Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., & He, G. (2011). Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. *PloS One*, 6(5), e20504. doi:10.1371/journal.pone.0020504
- Zhaxybayeva, O., & Doolittle, W. F. (2011). Lateral gene transfer. *Current Biology*: CB, 21(7), R242–6. doi:10.1016/j.cub.2011.01.045

Table II-1. Composition of media used in tall fescue transformation experiments

Medium components	Induction medium	Subculture medium	Infection medium	Resting medium	Selection1 medium	Selection2 medium	Regeneration medium	Rooting medium
	MS10	MS5	IFM	RSM	S1	S2	RG	RT
MS with Vitamins (g L ⁻¹)	4.4	4.4	4.4	4.4	4.4	4.4	4.4	2.2
Maltose (g L ⁻¹)	30	30	30	30	30	30	30	30
Phytigel (g L ⁻¹)	3	3	–	3	3	3	3	3
2,4-D (mg L ⁻¹)	10	5	5	5	5	3	–	–
BAP (mg L ⁻¹)	0.05	0.05	0.05	0.05	0.05	0.1	0.5	–
Acetosyringone (uM)	–	–	200	200	–	–	–	–
Timentin (mg L ⁻¹)	–	–	–	200	200	200	200	200
Hygromycin B (mg L ⁻¹)	–	–	–	–	150	250	–	50
pH	5.8	5.8	5.4	5.8	5.8	5.8	5.8	5.8
Callus growth time (weeks)	4	9	–	1	2	4	3~6	3~5

Table II-2: List of genomes on NCBI that were blasted with target gene sequences

Human
Mouse
Rat
<i>Arabidopsis thaliana</i>
<i>Oryza sativa</i>
<i>Bos Taurus</i>
<i>Danio rerio</i>
<i>Drosophila melanogaster</i>
<i>Gallus gallus</i>
<i>Pan troglodytes</i>
<i>Apis mellifera</i>

Table III-1. A list of 15 essential fungal genes of *Rhizoctonia solani*

No.	Protein name	Gene symbol
1	Coatomer alpha	NCU10066
2	RNA polymerase	NCU08616
3	ARP2/3	NCU01756
4	Cap methyltransferase	NCU03971
5	Proteosome Pre4	NCU07365
6	DNA polymerase Alpha	NCU07870
7	DNA polymerase Delta	NCU01192
8	Adenylate cyclase	NCU08377
9	Protein kinase C	NCU06544
10	RNA helicase	NCU03363
11	Phosphatidylinositol-4-phosphate 5-kinase	NCU02295
12	DNA repair helicase RAD25	NCU06438
13	Cohesin complex subunit Psm1	NCU01323
14	Importin beta-1 subunit	NCU02011
15	Ubiquitin ligase E3	NCU03947

Fifteen *R. solani* essential genes were identified by homologous protein sequence blast with *Neurospora crassa*. The middle column shows the annotation of the proteins for these corresponding genes in *N. crassa* and the right column stands for their corresponding gene symbols in NCBI.

Table III-2. Inhibition effect of mycelium growth of the dsRNA synthesized from selected gene segments

Inhibition% for selected gene segments				
Gene No.	test1	test2	test3	mean
2	50	37	86.4	57.80
13	50	22	45.6	39.20
14	58	54	45	52.33
15	100	54	81.8	78.60
2+14	97	88	95	93.33
14+15	83	77	85	81.67
2: RNA polymerase				
13: Cohesin complex subunit Psm1				
14: Importin beta-1 subunit				
15: Ubiquitin ligase E3				

Diameter of mycelium growth on the PDA plate was measured 2 days after they were moved onto the fresh plate. Mycelium growth area were calculated and all the numbers in the table were calculated by using growth area of the fungus mycelium incubated with certain dsRNA(s) divided by the growth area of fungus mycelium incubated with water, and multiplied with 100.

Table III-3 Original nucleotide sequences of the four gene segments of *R. solani* AG1-IA used for RNAi constructs

RNA poly
GACGAGCGCTTTGGAACGAGCATAACAGGGATCATATACTCTTGTTTCCTC CAGCTGAACCTCAACATTGCCAGTCCATTTCGAGAGGTAGTTTAGTGAATTT GTGACAGATGTTTGGTTCGGGTCTGACACAGCGTATGGCCACGCCATAGGG TGTGAATGAGGGACCTCGGTTCGTGAATGAGTTTCGGATGATCGAAATGA CATGGTTTCGTGAGGGAGGATGAGGTACCGGATTAATCGTTCGTTTCCGT TGACGATAAAGTACCCTCCAACTCTTCTGGTTCTTCGTGTCTTCGTACCA ATTGGGCAGAGCTAAGGCCTCGAAGATTGCATCGGTTTGACTTGAT
Importin beta-1 subunit
TCACGAGGGCAGTAACAAGGACTTGGAGTTGGCTGTCAATGTTTACGGAT CCAACGAGGAGTTCACAGATCCGTCCTAGGGTCCATGCAGTTGTATCTTTC ACAGCAGCATTGTGTGCGCCATCATGGCAATAAGTACTGGAAGTGCTTG GCTGACGAGTCCTGCAAGAACGTTTGGATCGGGCCCTTCAAGGATGGAAC CGAATACCATCACAGCAGCCTCACGCAAGTGCCAATCGTCGTGCTTGATGT TCGTCTCAATGAATGGAAGCACAACCTGGAACAATCGCGTCGTCGACTGCC TGGGCGAGGAGTGCAGAGACAGGTTCCAGCGGCCATGGAAATGTTCCATTC ATCTTCATCTGCATCTTCTTCCCTGCTGTGTGAGGAG
Cohesin complex subunit Psm1
CCTTACAATAGCGGTATCAAATACCACGCAATGCCTCCAATGAAGCGCTTC CGTGACATGGAGCAGTTGTCTGGTGGAGAGAAGACGGTCGCAGCTCTGGC TCTGCTATTCGCTATTCACAGGTATGTATTCTACCAGTGCCTCTGGGATGC ATTAACAATCTATTCTTCTCAGCTTCCAACCTTCACCTTTCTTCGTGCTCGA CGAAGTGGACGCGGCGTTGGATAACACAAACGTCGCTAAAGTTGCCAACT ATATTCGCCAACACTCGTCGGAGACCTTTCAGTTTATTGTCATCAGCTTGA AAGGATCGCTCTACGAAAAGGGAAATTCGTTGGTGGGAATCTATCGCGAC CAGGACGTGAACAGTTCGCGCACGCTGACACTAGACGTA
Ubiquitin ligase E3
GAGTAATCACTCTTCATCGTCGTACAGTATATCCAAGGATTACGTGTCATG ACTCCAAATGATTTGAAGAAGCGACTTATGATCAAATTCGACGGTGAAGA CGGTTTGGACTATGGCGGTCTCTCTCGTGAATTCTTCTTCCCTGCTCTCGCAC GAGATGTTCAACCCATTCTACTGTTTGTGTTGAGTACTCGGCACACGACAAC TATACCCTCCAGATTAACCCCGCAAGTGGTGTCAACCCAGAACAATTGAA CTACTTCAAATTCATTGGGCGCTGTGTGTCGGCTTGGGCATCTTCCACCGAAG ATTCCTGGACGCATACTTCATTGTCAGTTTCTACAAGATGATCTTGAAGAA AAAGATTACGCTTGCGGATTTGGAAAGTGTGGATGCGGAACCTTCATCGTG GAATGACTTGGATGCTGA

Table III-4. Modified nucleotide sequences of the four gene segments of *R. solani* AG1-IA used for RNAi constructs

NR sequence: GACGAGCGCTTTGGAACGAGCATAACAGGGATCATATACTCTTGTTTACTC CAGCTGAACCTCAACATTGCCAGTCCATTCGAGAGGTAGTTTAGAGAATTT GTGACAGATGTTAGGTCGGGTCTGACACAGCGTATGGCCACGCCATAGGGT GTGAATGAGGGACCTCGGTTTCGTGAATGAGTTTCGGATGATCGAAATGACA TGGTTTCGTTCGAGGGAGGATGAGGTACCGGATTAATCGTTCGTTTCCGTTG ACGATAAAGTACCCTCCAACTCTTCTGGTTATTCGTGTCTTCGTACCAATT GGGCAGAGCTAAGGCCTCGAAGATTGCATCAGTTTGACTTGATTCACGAGG GCAGTAACAAGGACTTGGAGTTGGCTGTCAATGTTTACGGATCCAACGAGG AGTTCACAGATCCGTCCTAGGGTCCATGCAGTTGTATCTTTCACAGCAGCA TTTGTGTTGCCCATCATGGCAATAAGTACTGGAAGTGCTTGGCTGACGAGT CCTGCAAGAACGTTTGGATCGGGCCCTTCAAGGATGGAACCGAATACCATC ACAGCAGCCTCACGCAAGTGCCAATCGTCGTGCTTGATGTTTCGTCTCAATG AATGGAAGCACAACCTGGAACAATCGCGTCGTCGACTGCCTGGGCGAGGAG TGCGAGACAGTTCCAGCGGCCATGGAAATGTTCCATTCAACTTCATCTGC ATCTTCTCCTGCTATGTCAGGAG
MR: sequence: GACGAGCGCTTTGGAACGAGCATAACAAGGATCATATACTCTTGTTTACTC CAGCTGAACCTCAACATTGCCAGTCCATTCGAGAGGTAGTTTATTTGAATTT GCGACAGATGTTTGGTCGAGTCTGACACAGCGTATGGCCACGCCATAGGGT GTGAATGAGGGACCTCGGTTTCGTGAATGAATTCGGATGATCGAAATGACA TGGTTTCGTTCGAGTGAGGATGAGGTACCGGATTAATCGTTCAATTCGTTG ACGATAAAGTACCCTCGAACTCTTTGGTTATTCGTGTCTTCGTACCAATT AGGCAGAGCTAAGGCCTCGAAGATTGCATCAGTTTGACTTGATTCACGAGG GCAGTAACAAGGACTTGGAGTTGGCTGTCAATGTTTACGGATCCAATCGAGG AGTTTACAGATCCGTCCTAGGCATCCATGCTGTTGTATCTTTCACAGCAGCAT TTGTGTTGCCCATCACGGCAATAAGTACTGGAAGTGCTTGGCTGACGAGTC CTGCAAGAACGTTTGGATCGGGCCCTTCAAGGATGGAACCGAATACCATCA CATCAGCCTCACGCAAGTGCCAATCGTCGTGCTTGATGTTTCGTCTCAATGA ATGGAAGCACAACCTGGAACAATCGCGTCGTCGACTGCCTGGGCAGGAGT GCGAGACAGTTCCAGCGGCCATGGTATTGTTCCATTCATCTTCTTCTCAT CTTCTCCTTCTATGTCAGGAG
NC sequence: CCTTAAATAGCGGTATCAAATACCACGCAATGCCTCCAATGAAGCGCTAC CGTGACATGGAGCAGTTGTCTGGTGGAGAGAAGACGGTCGCAGCTCTGGCT CTGCTATTCGCTATTCACAGGTATGTATTCTACCAGTGCCTCTGGGATGCAT

Table III-4 (continued)

<p>TAACAATCTATTCTTCTCA<u>T</u>CTTCCAAC<u>A</u>ATTCACCTTTCTTCGTGCTCGACG AAGTGGACGCGGGCGTTGGATAACACAAACGTCGCTAAAGTTGCCAACTAT ATTCGCCAACACTCGTCGGAGACCTTTCAGTTTATTGTCATCAGCTTGAAAG GATCGCTCTACGAAAAGGGAAATTCGTTGGTGGGAATCTATCGCGACCAGG ACGTGAACAGTTCGCGCACGCTGACACTAGACGTAGAGTAATCACTCTTCA TCGTTCGTACAGTATATCCAAGGATTACGTGTCATGACTCCAATGATTTGA AGAAGCGACTTATGATCAAATTCGACGGTGAAGACGGTTTGGACTATGGCG GTCTCTCTCGTGAATTCTTCTT<u>G</u>CTGCTCTCGCACGAGATGTTCAACCCATT CTACTGTTTGTGTTGAGTACTCGGCACACGACA ACTATAACCTCCAGATTAAC CCCGCAAGTGGTGTCAACCCAGAACACTTGA ACTACTTCAAATTCATTGGG CGCTGTGTCGGCTTGGGCATCTTCCACCGAAGATTCTGGACGCATACTTC ATTGTCAGTTTCTACT<u>T</u>AGATGATCTTGAAGAAAAGATTACGCTTGCGT<u>T</u>ATT TGGAAGTGTGGATGCGGAACTTCATCGTGGAATGACTTGGATGCT<u>AA</u></p>
<p>MC sequence:</p> <p>CCTTAA<u>A</u>AATAGCGGTATCAAATACCACGCAATGCCTCCAATGAAGCGCT<u>AC</u> CGTGACATGGAGCAGTTGTCTGGTGGAGAGAAGACGGTCGCAGCTCTGGCT CTGCTATTCGCTATTCACA<u>T</u>GTATG<u>A</u>AT<u>ACA</u>CCAGTGCC<u>A</u>CTGGGAT<u>ACC</u> TTAACAATCTAT<u>AC</u>GTCTCAT<u>T</u>CTTCCAT<u>CA</u>ATTCACCTTTCTTCGTGCTCT<u>TAC</u>G AAGTGGACGCGGGCGTTGGATAACACAAACGTCGCTAAAGTTGCCAACTAT ATTCGCCAACACTCGTCGGAGACCTT<u>A</u>CAGTTTATTG<u>A</u>CATCAGCTTG<u>TAA</u> GGATCGCTCTACG<u>TAA</u>AGGGAAATTCGTTG<u>T</u>TGGGAATCTATCGCGACCAG GACGTGAACAGTTCGCGCACGCTGACACTAGACGTAGAGTAATCACTCTTC ATCGTCGTACAGTATATCCAAGGATTACGTGTCATGACTCCA<u>T</u>ATGATTTG AAGAAGCGACTTATGATCAAATTCGACGGTGAAGACGGTTTGGACTATGGC GGTCTCTCTCGT<u>AA</u>ATTCTTCTT<u>G</u>CTGCTCTCGCA<u>A</u>GAGATGTTCAACCCAT TCTACTGTTTGTGTTGAGTACTCGGCACACGACA ACTAT<u>T</u>CCCTCCAT<u>TATTA</u> CCCCGCAAGTGGTGTCAACCCAGAACACTTGA ACTA<u>G</u>TTCAAATTCA<u>CTGG</u> GCGCTGTGTCGGCTTGG<u>T</u>CATCTTCCACCGAAGATT<u>A</u>CTGGACGCATACTTC A<u>C</u>TGTCAGTTTCTACT<u>T</u>AGATGATCTTGAAG<u>C</u>AAAAGATTACGCTTGCGT<u>T</u>ATT TGGAAGTGTGGATGCGGAACTTCATCGTGGAATGACTTGGATGCT<u>AA</u></p>

NR: natural version of RNA polymerase gene and Importin beta-1 subunit gene; MR: modified version of the same fragment as NR; NC: Cohesin complex subunit Psm1 gene and Ubiquitin ligase E3; MC: modified version of the same fragment as NC. The sequence with underscore was the second part of each fusion. The nucleotides highlighted with yellow color were those changed during gene modification.

Table III-5. Summarized results of *R. solani* inoculation with RNAi transgenic plants

Plant	Inoculated plants detected with siRNA	highly significant resistance (P<0.01)	significant resistance (P<0.05)	slightly resistance (P<0.1)
NR	6	3	0	1
MR	1	0	0	0
NC	0	0	0	0
MC	3	0	2	0

Among 6 NR plants with detectable siRNA, 3 of them showed highly significant resistance and 1 had slightly resistance; For 3 MC plants detected with siRNA, 2 of them showed significant resistance. None of the MR and NC plants tested in the inoculation showed any resistance. No NC plants with detectable siRNA were inoculated because they were dead at the time of inoculation, and the only 2 NC plants inoculated in the test didn't have detectable siRNA accumulation.

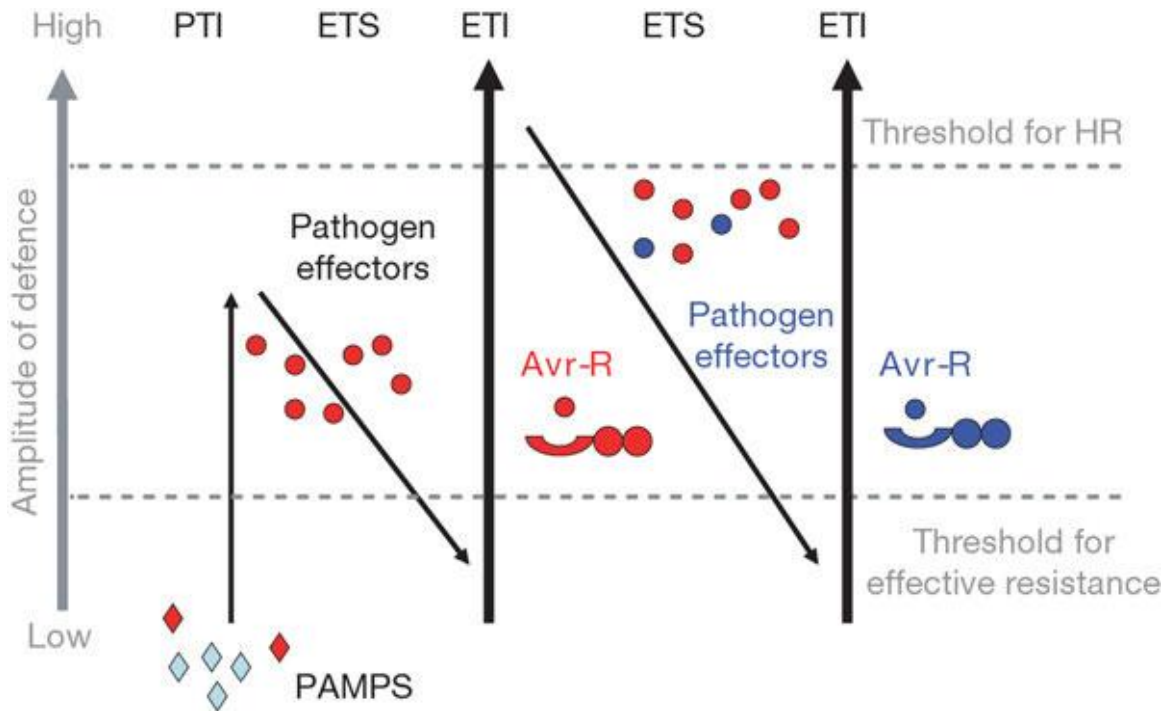


Figure I-1. The ‘zigzag’ model to show four phases of plant-pathogen interaction during their co-evolution

In phase I, plant cell surface-localized Pattern Recognition Receptors (PRRs) detect and recognize PAMPs from invading microbes to trigger immunity that prevents the potential pathogens from invasion; In phase II, the invading microbe delivers effectors into plant cells to suppress PTI, resulting in effector-triggered susceptibility (ETS) to establish the plant-pathogen relationship. In phase III, plant evolves to produce intracellular immune receptors (i.e. R proteins) that detect and recognize specific effectors resulting in effector triggered immunity (ETI); In phase IV, the effector recognized by R protein may undergo modification such that the pathogen evades ETI, or new effectors may evolve in the pathogen to suppress ETI, resulting in ETS again. Subsequently, ETI and ETS alternately occur as a consequence of the plant-pathogen co-evolution. Figure is adopted from Jones and Dangl’s review paper in 2006.

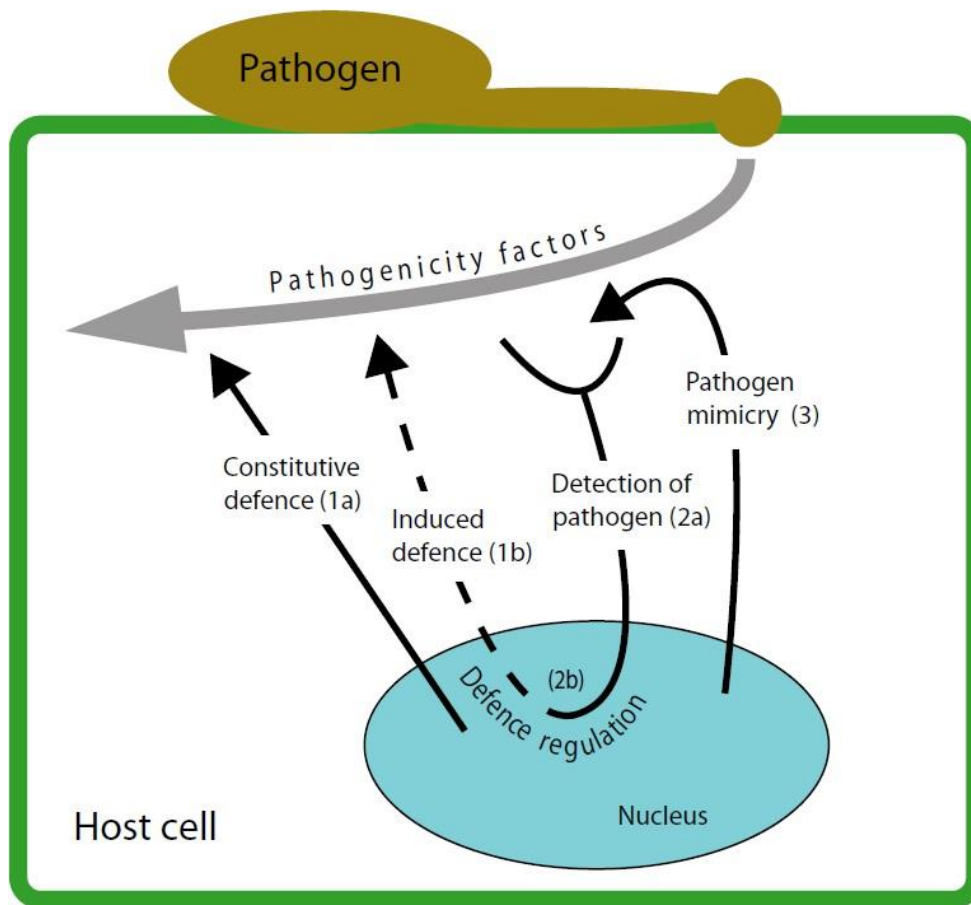


Figure I-2. A simplified model to illustrate three types of defense strategies successfully adopted for disease resistance in transgenic plants

Figure is adopted from Collinge's review paper in 2008.

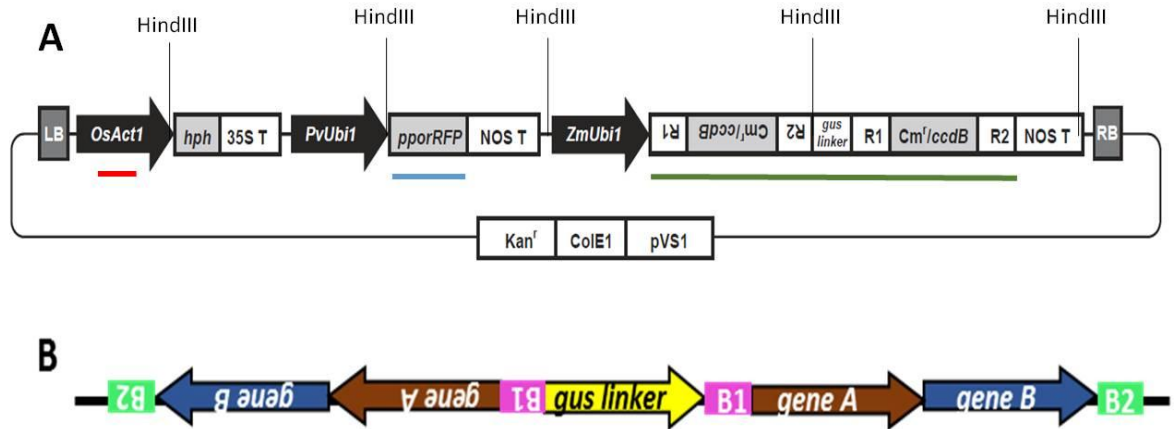


Figure II-1. Diagram of pANIC8A vector and binary construct

A: pANIC8A vector used for the gene construct (Mann et al., 2012). Red bar marks the *OsAct1* promoter sequence which was used as probe sequence for southern blots; Blue bar marks the *pporRFP* reporter gene whose expression product was a red fluorescent protein; Green bar marks the region replaced by the RNAi construct in B after the Gateway Cloning.

B: B1 and B2 were the products of recombination of attL1 with attR1 and attL2 with attR2 sequences after Gateway Cloning. Gene A and gene B are the targeted gene sequence we identified in the previous steps (RNAPoly::*Imbs*, or CohP::*UbiE3*). A total of four fusion constructs were made, which include two *R. solani* gene sequences modified slightly, NR (RNAPoly::*Imbs*) and NC (CohP::*UbiE3*), and another two sequences modified substantially, MR and MC.

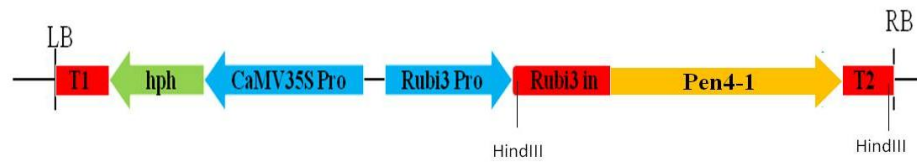


Figure II-2. Map of T-DNA region of binary vector pBZ7

Map of the T-DNA region containing *Penaeidin4-1* gene plant expression cassette in pBZ7. RB: right border, LB: left border, CaMV35S Pro: CaMV35S promoter, Rubi3 Pro: rice rubi3 promoter, Rubi3 in: Rubi3 intron, hph: hygromycin phosphotransferase gene coding region, Pen4-1: *Penaeidin4-1* gene, T1: CaMV35S terminator, T2: *Agrobacterium tumefaciens nos* gene terminator. Arrows indicate direction of transcription. The location of *HindIII* restriction sites used in Southern analysis is also indicated.

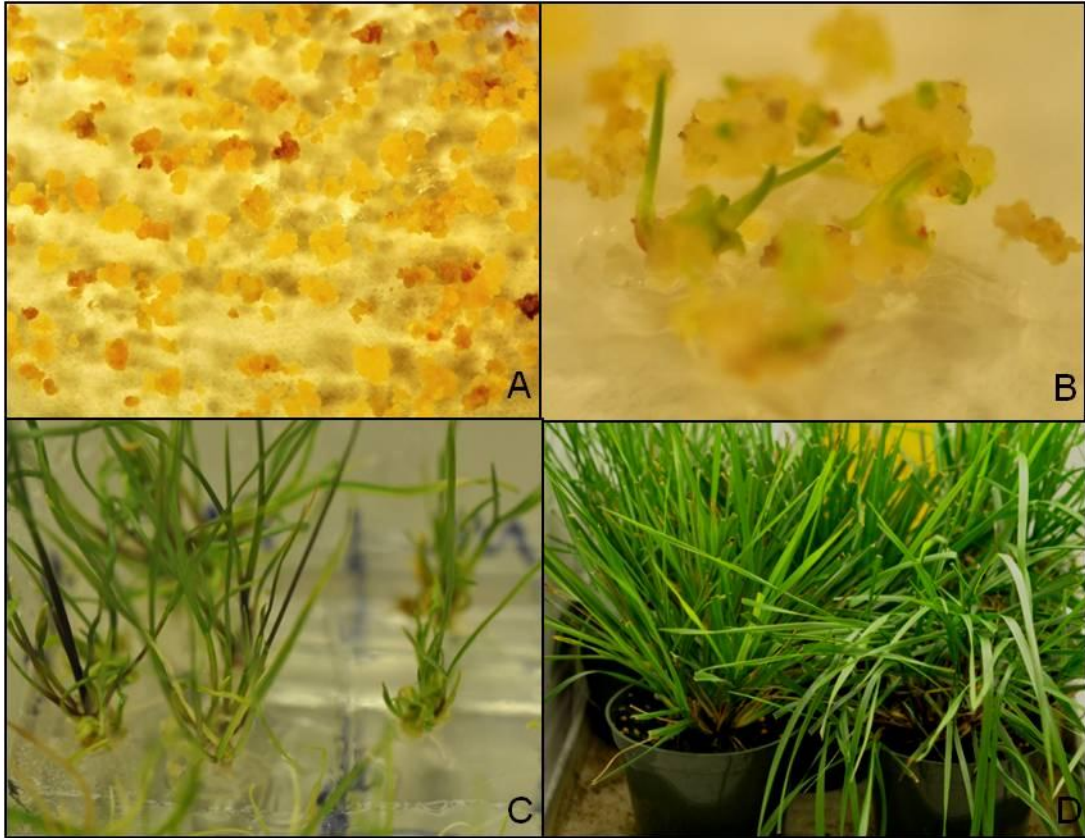


Figure III-I-1. *Agrobacterium tumefaciens*-mediated transformation of tall fescue with hyg B selection

A. Callus under hyg B selection after transformation; B. Hyg B resistant callus in regeneration medium for shoot formation; C. Root development of hyg B resistance plantlets; D. Transgenic plants growing in soil.

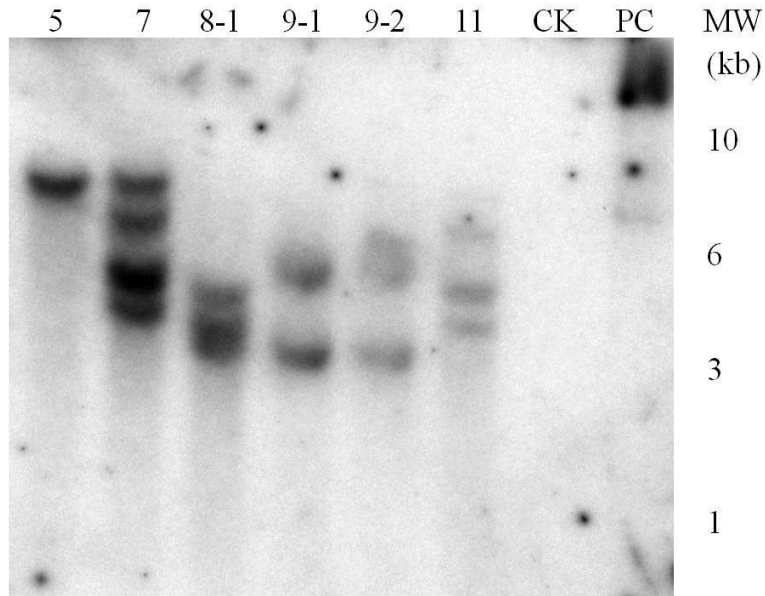


Figure III-I-2A. Southern blot analysis of Pen4-1 transgenic plants

Southern blot analysis of Pen4-1 transgenic plants using hph coding sequence as the probes. Genomic DNA was digested with *Hind*III. WT, negative control, DNA from a non-transformed tall fescue plant; 5-11, DNA from 6 putative transgenic plants, PC, positive control: 200 pg plasmid DNA, MW, molecular markers in kb.

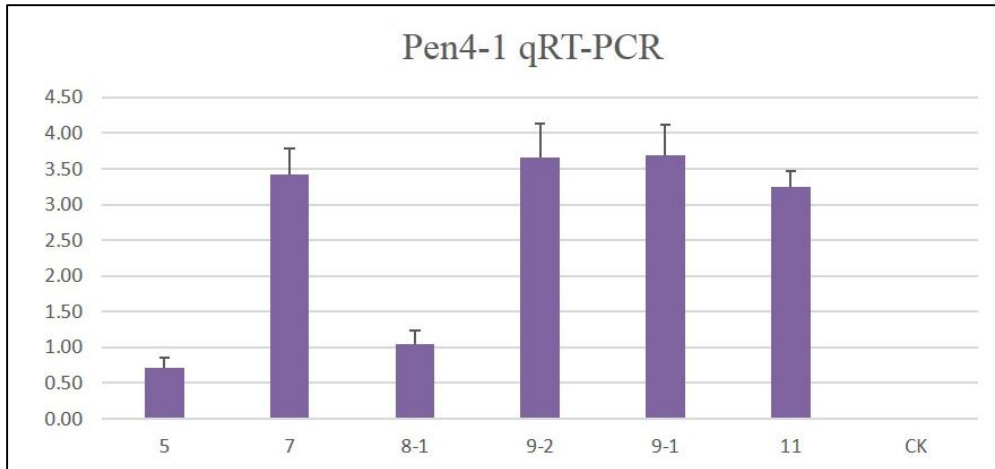


Figure III-I-2B. *Pen4-1* expression in transgenic tall fescue plants

The relative quantitative level of *Pen4-1* expression in transgenic lines was evaluated by real-time qRT-PCR. Ck stands for the wild type plants without transformation and it did not have detectable *Pen4-1* expression. The *Pen4-1* expression level of line 8-1 was used as a calibrator (expression level arbitrarily set at 1), and other lines' expression levels were expressed as the fold of the expression level in line 8-1.

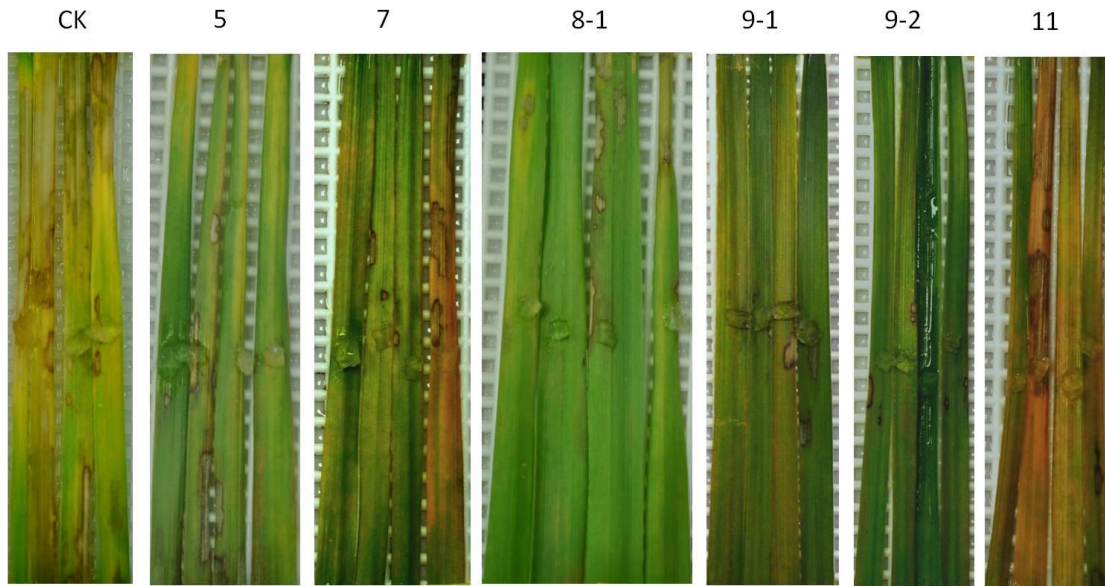


Figure III-I-3. Representative infection results of Pen4-1 plants inoculation tests
Detached leaves of tall fescue plants four days after inoculation with *Rhizoctonia solani*.

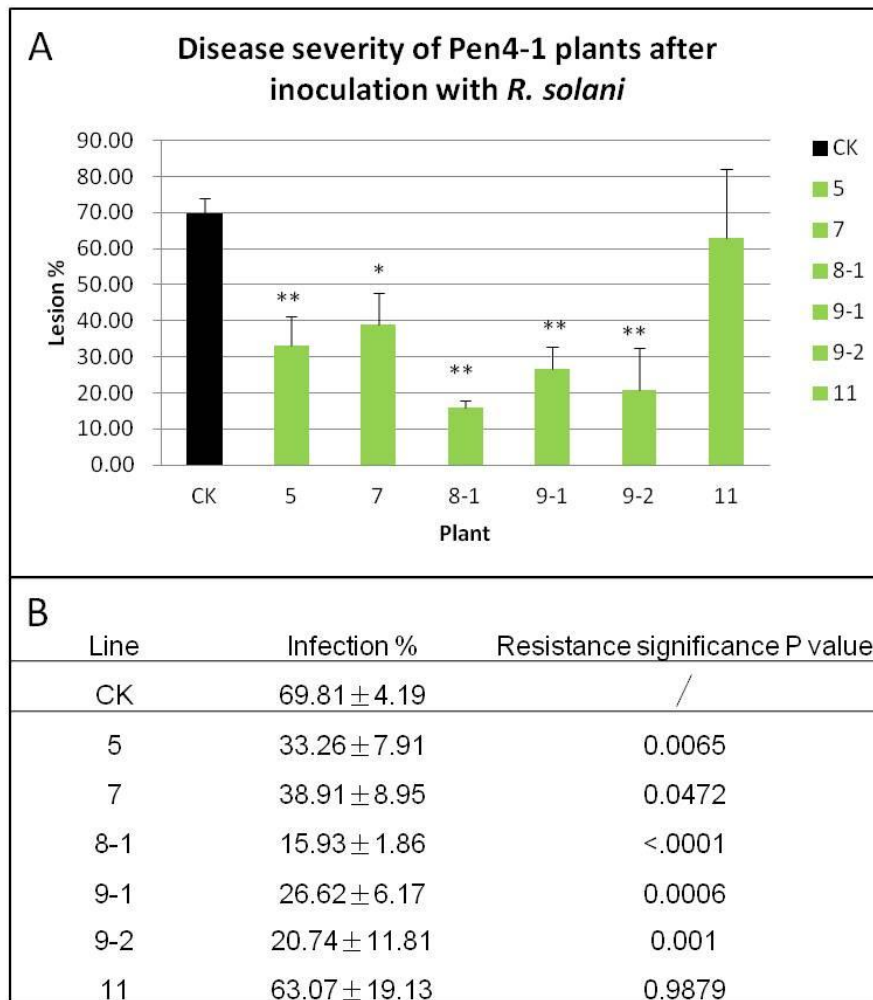


Figure III-I-4: Statistical analysis of *R. solani* resistance of Pen4-1 plants

A: Y-axis stands for the lesion area percentage after *R. solani* inoculation, X-axis represents the transgenic plants. * indicates significant improvement in resistance ($p < 0.05$) while ** labels plants with highly improved resistance ($p < 0.01$).

B: Output of the significance analysis using SAS software.

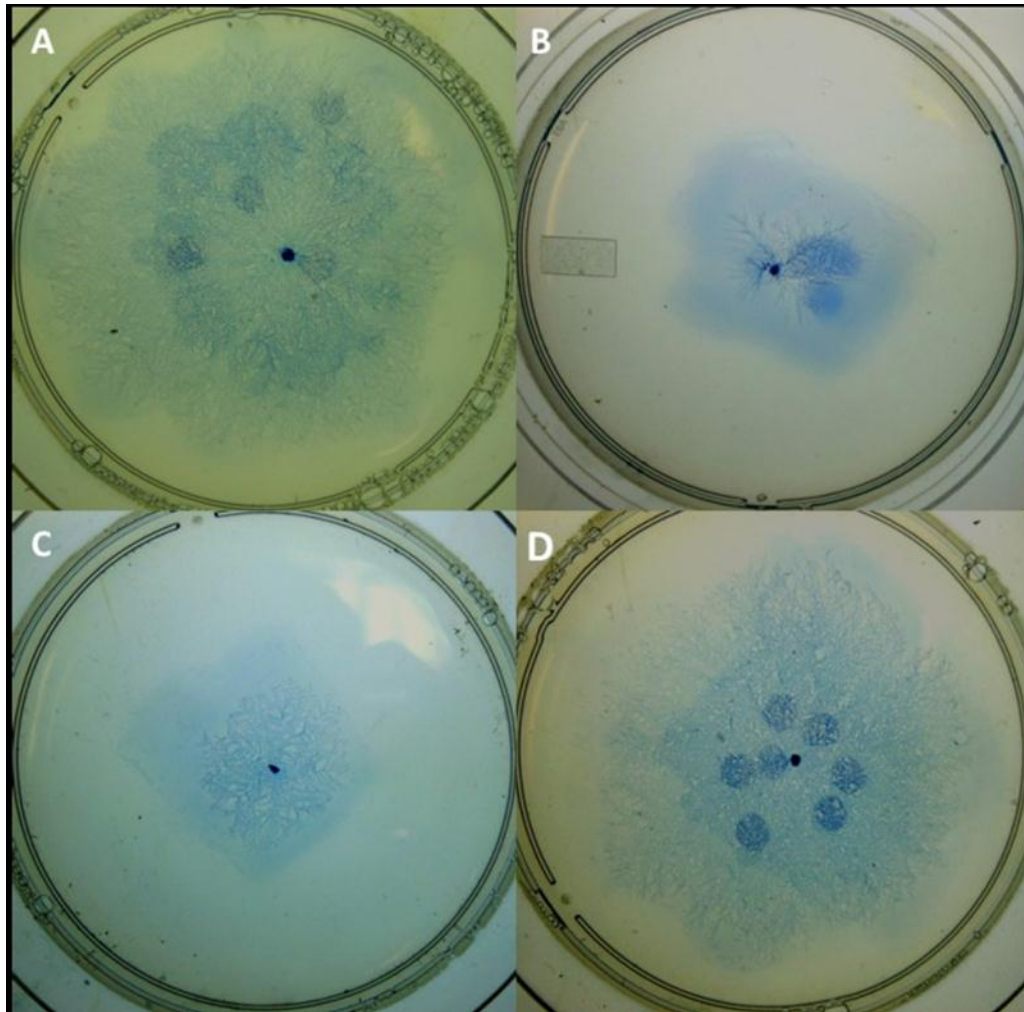


Figure III-II-1: Demonstration of the *in vitro* tests of the *Rhizoctonia solani* mycelium growth inhibition by selected dsRNAs

Mycelium of the *R. solani* was incubated with 50 μ l dsRNA solution overnight and then grown on PDA medium for two days. The blue color on the medium is the mycelia stained with lactophenol-trypan blue to make it visible to the camera. A shows the negative control where mycelium was treated in pure dH₂O and resulted in no inhibition effect. B and C showed good inhibition by the dsRNA combination for the mycelium growth. B is the dsRNA combination derived from RNApoly::*Imbs*, and C is the dsRNA combination derived from Coh::*UbiE3*. D shows the lower inhibition by another dsRNA combination.

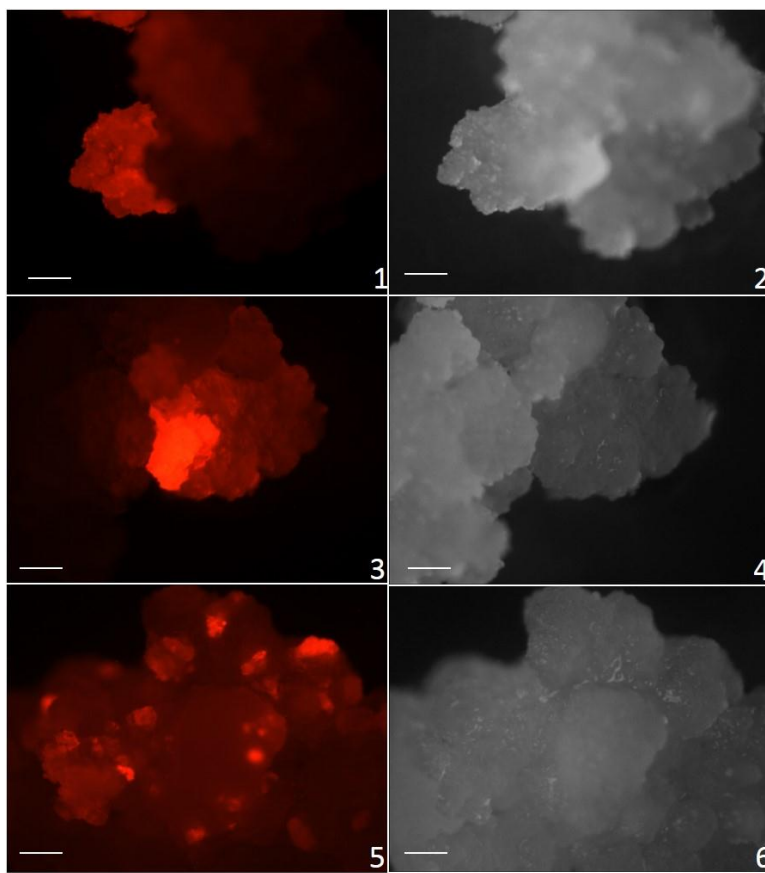


Figure III-II-2. Fluorescence microscopy showing *ppvRFP* expression in tall fescue callus after transformation

Figures 1, 3, 5 are photos of callus under RFP filter, and 2, 4, 6 are their photos under light field, respectively. The red color of callus under RFP filter demonstrated that the red fluorescence protein is expressed in the transgenic cell clusters. The scale bar represents 0.5 mm.

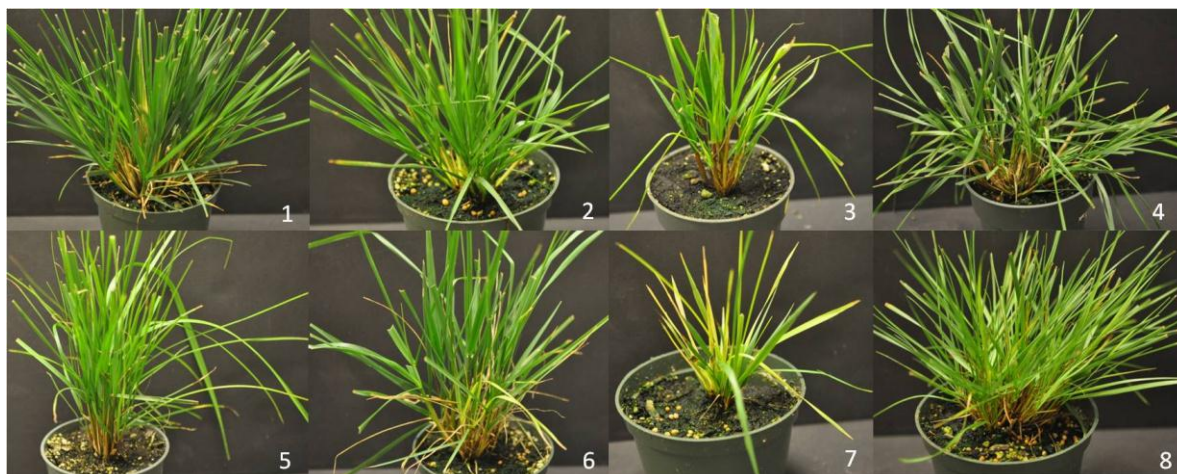


Figure III-II-3. Growth of NR and MR transgenic plants

Figures 1 to 4 are NR plants. 1, NR1; 2, NR3; 3, NR16; 4, NR12; 5, non-transgenic plant; 6 to 8 are MR plants. 6, MR1; 7, MR2; 8, MR3. All of the transgenic NR and MR plants grow normally.

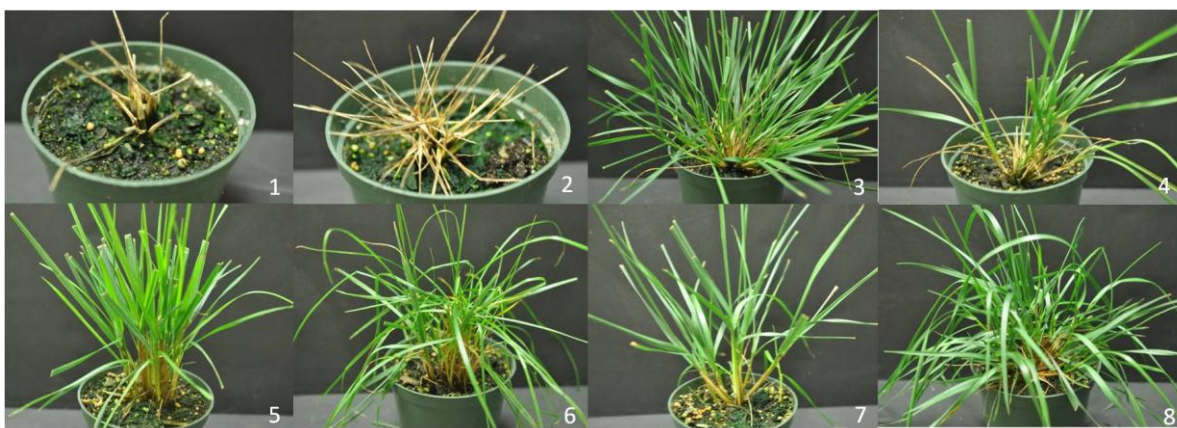


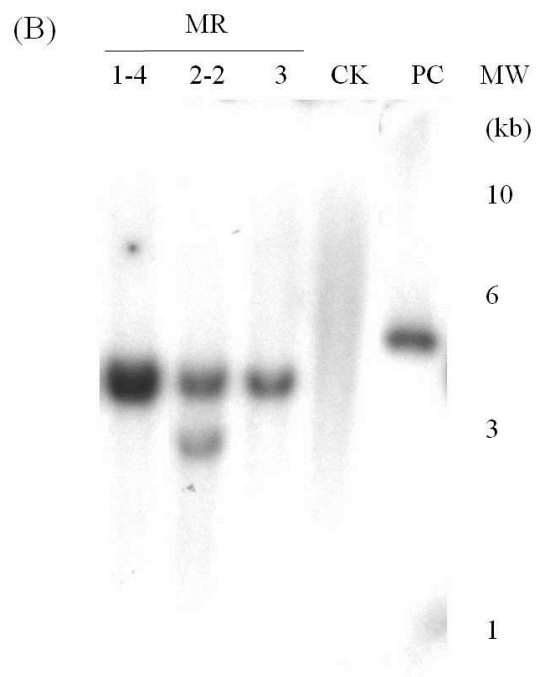
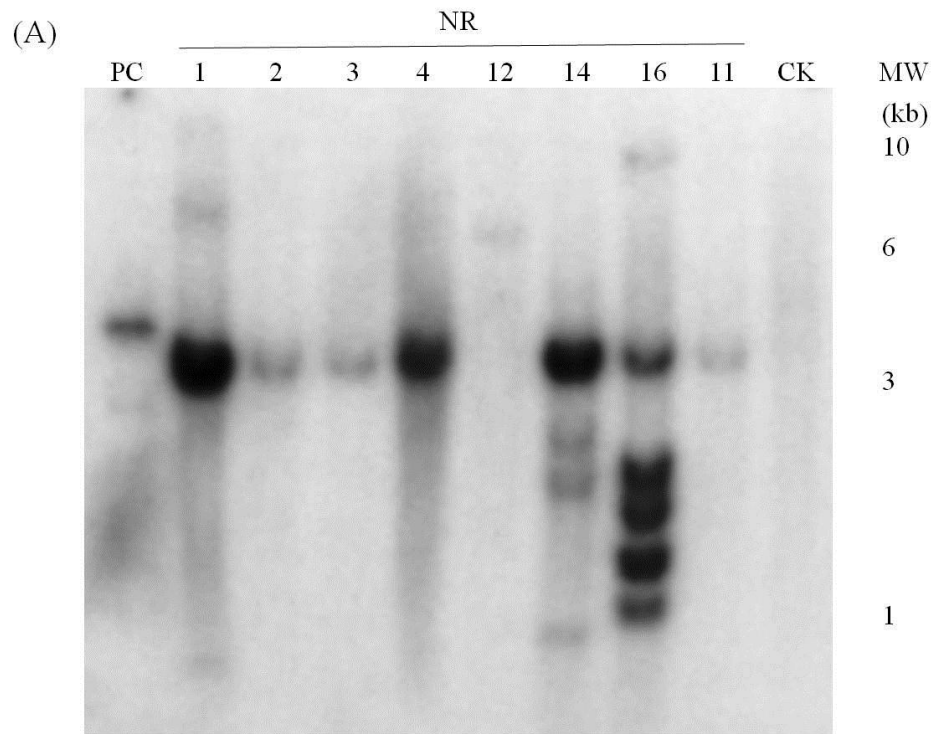
Figure III-II-4. Growth of NC and MC transgenic plants

Figures 1 to 4 are NC plants. 1, NC2; 2, NC5; 3, NC21; 4, NC22. 1 and 2 (NC2 and NC5) died whereas 3 and 4 (NC21 and NC22) grew normally. 5 to 8 are MC plants, all growing normally. 5, MC1; 6, MC5; 7, MC12; 8, MC15.

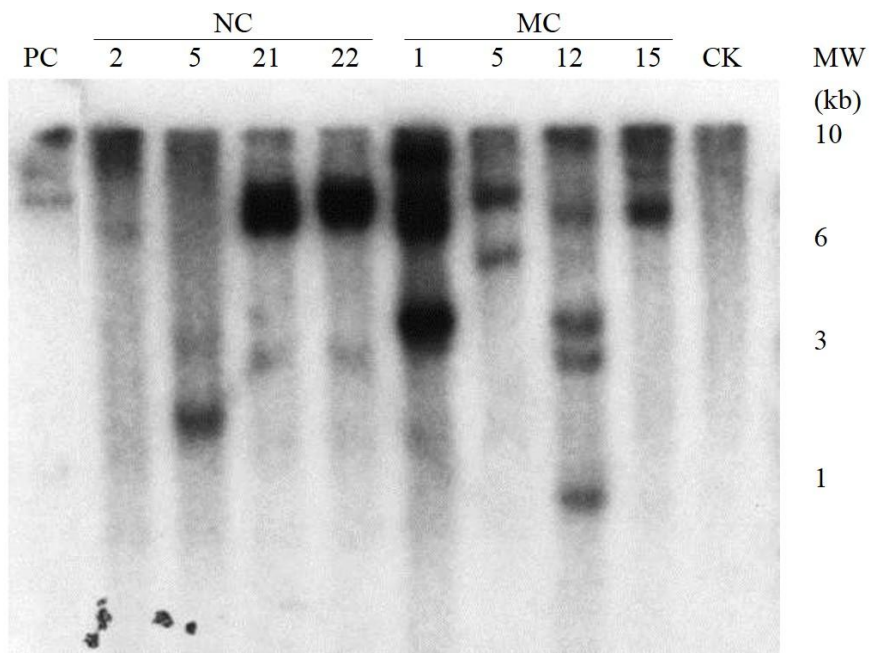
Figure III-II-5. Southern blot analysis of the RNAi plants

Southern blot analysis of transgenic plants using the *hph* coding sequence as a radiolabeled probe. Genomic DNA was digested with *HindIII*. CK, negative control, DNA from a non-transformed tall fescue plant; PC: positive control, 200 pg of plasmid (binary construct) DNA; MW: molecular markers in kb.

(A) Genomic DNA extracted from 8 NR plants; (B) Genomic DNA extracted from 3 MR plants; (C) Genomic DNA extracted from 4 NC plants and 4 MC plants



(C)



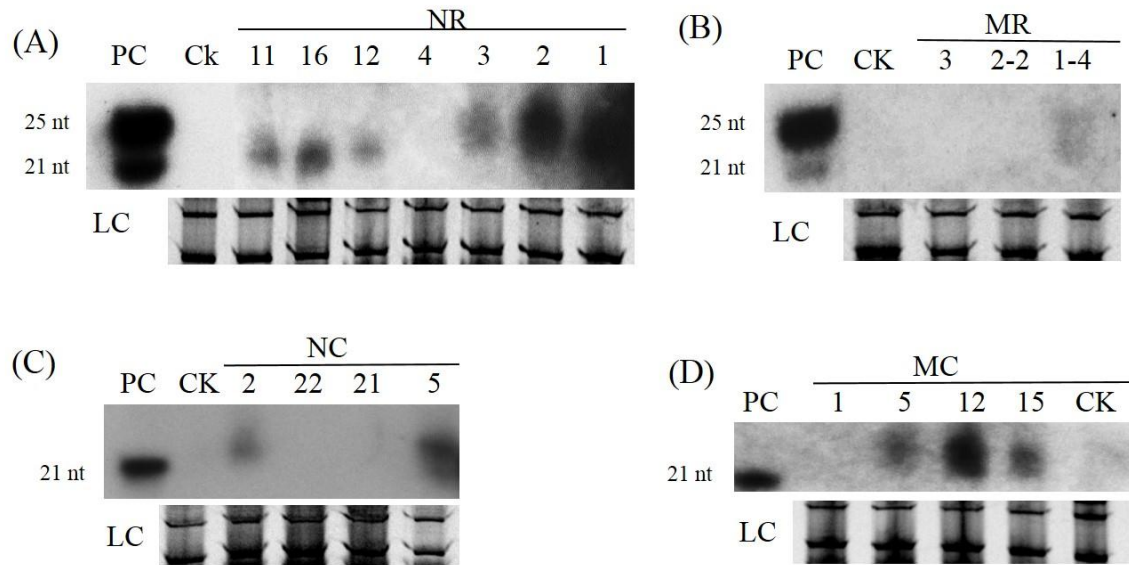


Figure III-II-6. RNA gel blot analysis of siRNA accumulation in RNAi plants

Total RNA was isolated from the leaves. For each gel blot, the sequence of the fused gene segments in the RNAi construct was labeled with ^{32}P and used to detect siRNA from their corresponding transgenic plants. For gel blots A and B, a 21 nt fragment and a 25 nt fragment complementary to the probe were used as a positive control and molecular weight marker; for gel blots C and D, only a 21 nt fragment complementary to the probe was used. CK: negative control, RNA extracted from wild type tall fescue. LC: stained rRNAs as a loading control.

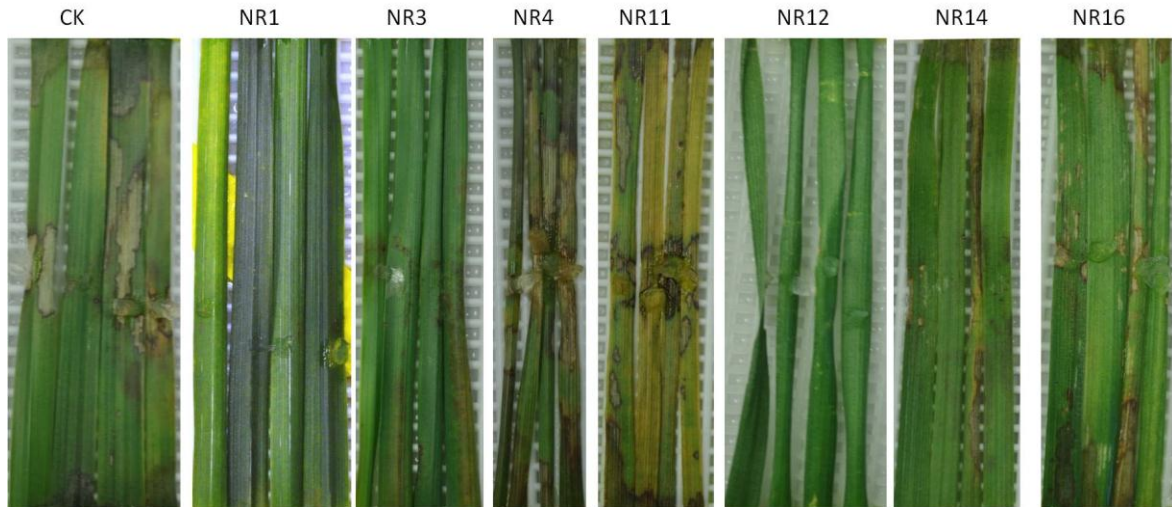


Figure III-II-7A. Inoculation results of NR plants

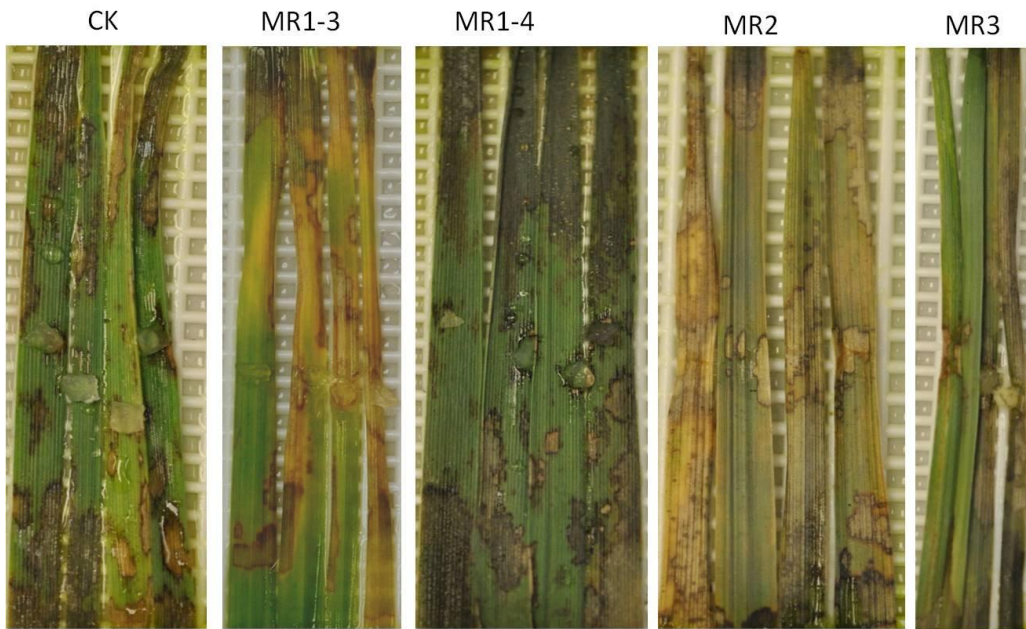


Figure III-II-7B. Inoculation results of MR plants

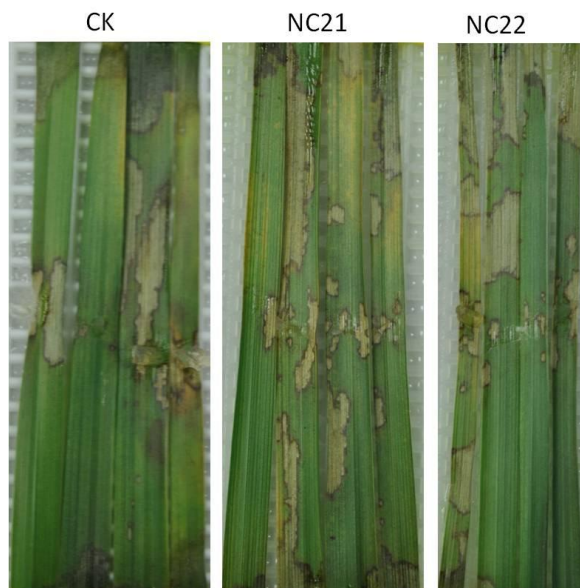


Figure III-II-7C. Inoculation results of NC plants

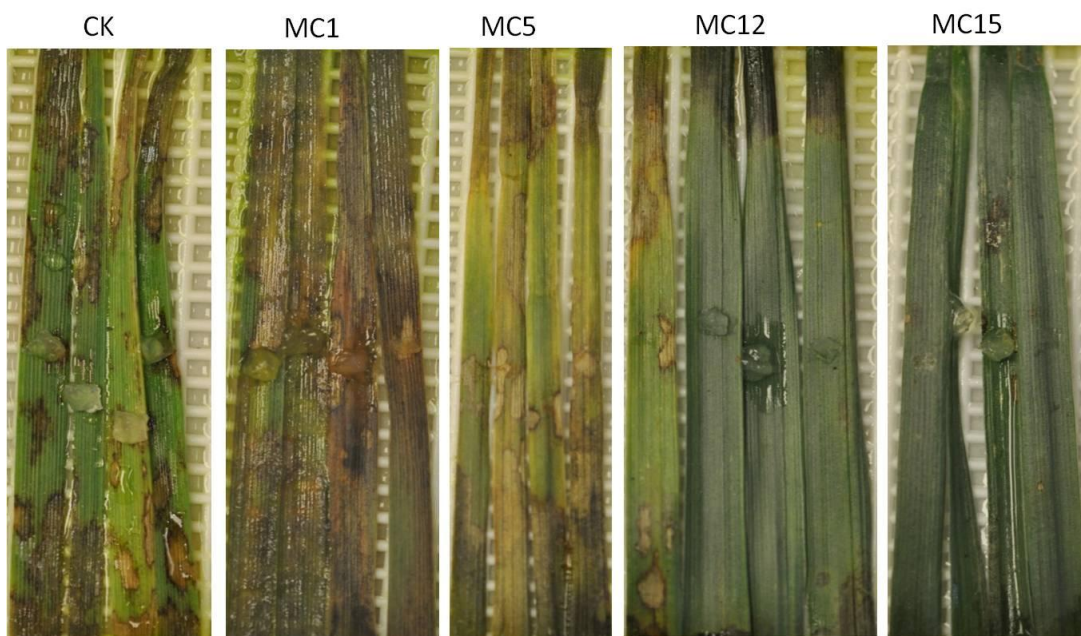
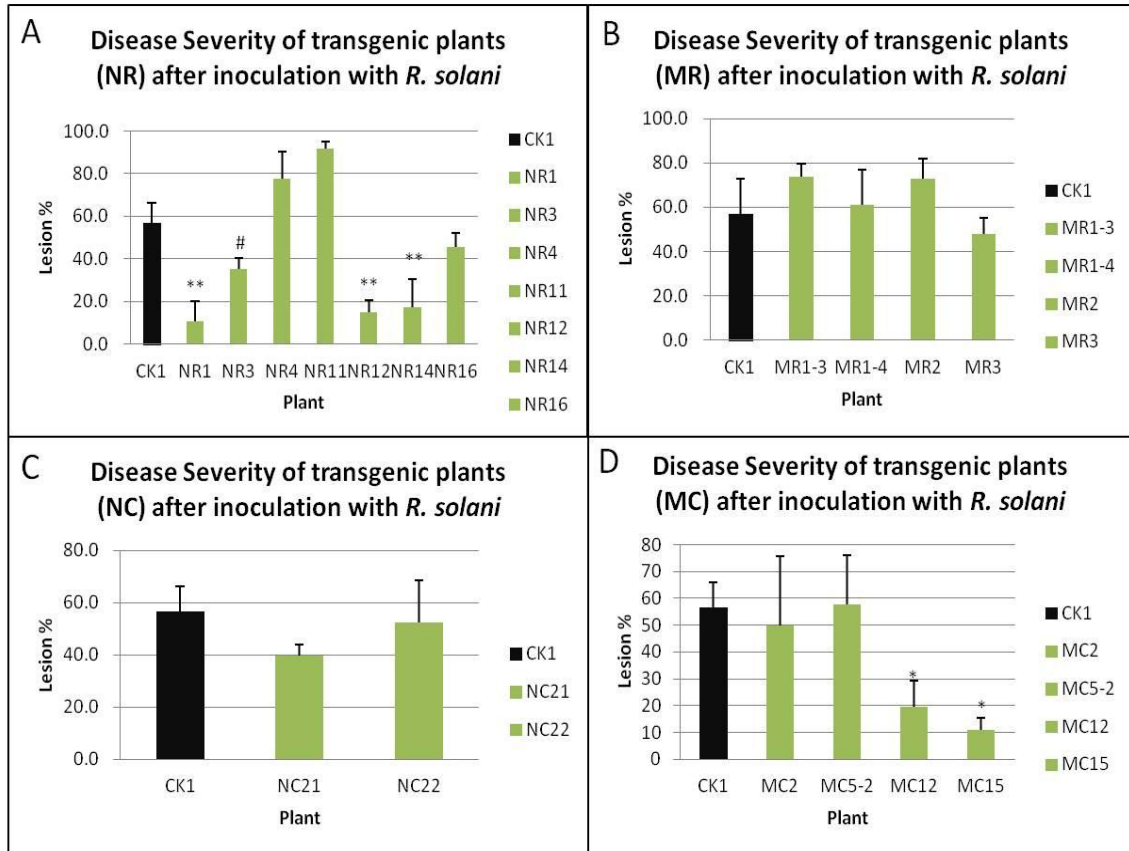


Figure III-II-7D. Inoculation results of MC plants

Detached leaves of transgenic tall fescue plants were inoculated with *Rhizoctonia solani*. Photos were taken four days after inoculation.

Figure III-II-8: Statistical analyses of *R. solani* resistance in RNAi plants

A: Compared to the wild type plant lesion percentage, 5 NR plants showed lower disease severity, 3 of them had highly significant resistance and 1 of them showed slight resistance. B and C: All MR and NC plants tested by inoculation showed no resistance. D: 2 MC plants showed significant resistance. E: Significance analysis result from SAS output. Only results with certain significant level are showed here. $P < 0.01$ indicates highly significant difference between transgenic plants and non-transformed plants, which is labeled with “***” in the figure A; $P < 0.05$ indicates significant difference between transgenic plants and non-transformed plants, which is labeled with “*” in figure D, and $P < 0.1$ indicates slight difference between transgenic plants and non-transformed plants, which is labeled with “#” in figure A.



E

Plant	Lesion%	Small RNA	Resistance significance P value
CK	56.8±9.4	/	/
NR1	10.7±9.2	yes	0.0008
NR3	35.2±4.9	yes	0.0661
NR12	15.0±5.7	yes	0.0017
NR14	17.3±13.0	/	0.0026
MC12	19.35 ± 9.8	yes	0.0431
MC15	11.0 ± 4.43	yes	0.0188