GENETIC ENGINEERING OF TURFGRASS FOR ENHANCED PERFORMANCE UNDER ENVIRONMENTAL STRESS

Man Zhou
Clemson University, mzhou@g.clemson.edu

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GENETIC ENGINEERING OF TURFGRASS FOR ENHANCED PERFORMANCE UNDER ENVIRONMENTAL STRESS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy
Genetics

by
Man Zhou
May 2012

Accepted by:
Dr. Hong Luo, Committee Chair
Dr. William R. Marcotte, Jr.
Dr. Guido Schnabel
Dr. Chinfu Chen
ABSTRACT

Turfgrass species are agriculturally and economically important perennial crops that are susceptible to biotic stress (e.g. fungal pathogens) and abiotic stress (e.g. salinity and drought). Every year, environmental stress significantly influences turfgrass quality and production causing economic loss globally. My research explores the feasibility of using two novel transgenes - *Penaeidin4-1* (*Pen4-1*) from shrimp (*Litopenaeus setiferus*) and *microRNA319a* (*miR319a*) from rice (*Oryza sativa*) to genetically engineer turfgrass for enhanced tolerance to environmental stress.

The antimicrobial peptide - *Pen4-1* has been reported to possess *in vitro* antifungal and antibacterial activities against various economically important pathogens. In this study, two DNA constructs were prepared containing either the coding sequence of a single peptide, *Pen4-1* or the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein translationally fused to *Pen4-1* coding sequence. Transgenic turfgrass plants containing different DNA constructs exhibited significantly enhanced resistance to dollar spot and brown patch, the two major fungal diseases in turfgrass. My results demonstrated the effectiveness of *Pen4-1* in a perennial species against fungal pathogens and may suggest a potential strategy for engineering broad-spectrum fungal disease resistance in crop species.

The miR319 family is one of the first characterized miRNA families in plants and it has been demonstrated to target *TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP)* genes encoding plant-specific transcription factors. Transgenic plants overexpressing the
rice miR319 gene, Osa-miR319a, exhibited dramatic morphological changes, including significantly decreased tiller numbers, wider and thicker leaves, larger stems, larger weight:area ratio and more total wax coverage. Overexpression of miR319 also led to enhanced drought and salt tolerance in transgenics, which might be attributed to the increased weight:area ratio and total wax coverage as well as less sodium uptake. Gene expression analysis in both wild-type and transgenic plants indicated that at least four putative miR319 target genes in turfgrass AsPCF5, AsPCF6, AsPCF8 and AsTCP14 were down-regulated in transgenic plants.

These results provide important information leading to the development of novel molecular strategies to genetically engineer crop species for enhanced performance under unfavorable environmental conditions, contributing to agriculture production.
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CHAPTER 1 - LITERATURE REVIEW

Part of the following contents has been published in “Methods and compositions for transgenic plants producing antimicrobial peptides for enhanced disease resistance. U.S. Patent Application No. 61/247,103” and SIVB conference abstracts “In Vitro Cellular & Developmental Biology 45 (issue abstract): 34-35” and “In Vitro Cellular & Developmental Biology 47 (issue abstract): 37”.

1.1 Agricultural Biotechnology for Crop Improvement

1.1.1 Challenges for current agriculture

By 2050, the world population will reach roughly 9 billion (Tester and Langridge, 2010). Increasing food production will have an important role in solving the problem of how to feed humans. However, it will be constrained by competing interests in land and resources, global climate change, increasing environmental stresses and current trends in land degradation (Godfray et al., 2010). Among these problems, environmental stresses are regarded as the most limiting factors for agricultural productivity (Cherry et al., 2000).

Environmental stresses can be divided into biotic stress and abiotic stress. The abiotic stress include drought, salinity, chilling, cold, freezing, heat, nutrient deficiency, sun burn, ozone and anaerobic stresses (Mittler and Blumwald, 2010). Take drought as an example, which is one of the most important environmental stresses affecting agriculture.
worldwide (Yang et al., 2010). There was a Europe-wide reduction of crop productivity due to heat and drought in 2003 (Ciais et al., 2005). Between 1980 and 2004 the US suffered an excess of $20 billion in damages due to drought solely and $120 billion due to the combination of drought and heat (Mittler, 2006; Mittler and Blumwald, 2010). The intergovernmental panel on climate change (IPCC) concludes that by the end of this century, the general drying of subtropics will cause drought stress worldwide due to the climate change and the increased accumulation of greenhouse gas (IPCC, 2007; Yang et al., 2010).

Salinity is another severe abiotic stress negatively impacting agricultural yields, because it is detrimental to plant growth and already affects a vast terrestrial areas of the world (Yamaguchi and Blumwald, 2005). Currently, it is estimated that at least 20% of irrigated land is under saline stress (Yamaguchi and Blumwald, 2005; FAO, 2008). Not only is the farmland experiencing serious soil salinization problems, but also are other ecosystems. Every year, vast natural ecosystems are transformed into farming systems. For example, in central Argentina, a large amount of natural woodland and grassland are converted to soybean fields. This expansion increased the groundwater levels and salinity, thus affecting long-term agricultural productivity (Jayawickreme et al., 2010). Besides the problems of soil salinization, the scarcity of fresh water resources forces farmers to use brackish water or salty water to irrigate. Therefore, it is important to develop crop cultivars to better adapt saline soil and inferior quality of irrigation water (Yamaguchi and Blumwald, 2005; Barrett-Lennard and Setter, 2010).
In addition to the abiotic stress challenges mentioned above, the incidence of biotic stress, such as pests and diseases, is another major limiting factor to worldwide agricultural productivity (Century et al., 2008; Varshney et al., 2011). The crop yields were reduced by 30% worldwide because of biotic stress, and this value is higher in developing countries (Christou and Twyman, 2004; Farre et al., 2010). Overcoming the biotic constraints reducing yields would be a desirable problem to solve.

1.1.2 Genetic engineering of crops to cope with environmental challenges

The objective of crop improvements for stress adaptation by agricultural biotechnology is to accumulate favorable alleles in genomes leading to better stress tolerance. Although conventional breeding has historically been playing an important role in crop improvements, it suffers several constraints (1) it takes longer time; (2) undesirable traits can also be transferred during the process; (3) the reproductive barriers limit the gene sources from various species; (4) complexity of the trait (Nelson et al., 2007; Yang et al., 2010; Varshney et al., 2011).

Agricultural biotechnology is a viable option, which can circumvent some of the constraints mentioned above. Generally there are two approaches for agricultural biotechnology, molecular breeding (MB) and genetic engineering (GE). Comparing to MB, GE has an advantage which has no barrier to transfer useful genes or alleles from different species even different kingdoms to target plants, indicating that it might be a
very applicable and efficient strategy to conduct crop improvements (Christou and Twyman, 2004; Varshney et al., 2011).

In recent years there are quite a few examples demonstrating that GE crops have significantly improved biotic stress tolerance (Tricoli et al., 1995; Ferreira et al., 2002; Castle et al., 2006) and abiotic stress tolerance under field conditions (Wang et al., 2005b; Hu et al., 2006; Nelson et al., 2007; Vanderauwera et al., 2007; Castiglioni et al., 2008; Oh et al., 2009; Wang et al., 2009; Xiao et al., 2009), not to mention tremendous examples tested under laboratory conditions (Oh et al., 2005; Sade et al., 2010; Gao et al., 2011b; Zhang et al., 2011; Zhou et al., 2011). For examples, transgenic rice plants with elevated accumulation of abscisic acid (ABA) have greater drought tolerance under field conditions (Xiao et al., 2009). Overexpression of a rice transcription factor AP37 elevated the transcription level of its target genes and increased the yield around 17%-56% under the field drought conditions (Oh et al., 2009). Transgenic rice plants overexpressing the stress-responsive NAC1 showed higher tolerance to salt and drought stress under field conditions (Hu et al., 2006). Transgenic maize plants with increased ZmNF-YB2 expression are more tolerant to drought (Nelson et al., 2007).

Compared with plants engineered for abiotic stress resistance, GE crops with enhanced biotic stress tolerance have gone farther and were commercialized more than a decade ago. For example, transgenic cotton and corn with Bt genes have been on the market for over 10 years with increased quality and quantity. Moreover, Bt corn has lower level of mycotoxin than non-Bt corn as a result of killing insects such as corn.
earworm carrying mycotoxin-containing fungi (Castle et al., 2006). Second generation of transgenic cotton entered the market in 2003 by Monsanto with the introduction of stacked genes (e.g. combined two Bt genes for broader spectrum of insects and herbicide resistance) (Castle et al., 2006). Several squash varieties resistant to various viruses developed by Asgrow Seed Company have also been commercialized (Tricoli et al., 1995; Castle et al., 2006). The most successful transgenic product is transgenic papaya engineered to be resistant to papaya ringspot virus. This transgenic product has saved the Hawaii papaya industry from collapse and made the expansion of papaya plantations to other areas (Ferreira et al., 2002; Castle et al., 2006).

In general, GE technology is becoming more popular, and has the potential to help address challenging problems in agriculture production. Although GE crops are not magic wands, which can free agriculture from all challenges and problems, many benefits have already been demonstrated. I support the view that genetic engineering is one of the most effective strategies to significantly enhance agricultural development (Christou and Twyman, 2004; Varshney et al., 2011). In this dissertation, I report research conducted to explore the use of agricultural biotechnology for improvement of turfgrass, an economically and environmentally important perennial crop species for enhanced performance under adverse environmental stress.
1.1.3 Novel sources of transgenes for genetic engineering of enhanced stress tolerance in transgenic plants

Using recombinant DNA and transgenic technologies, many genes from various sources have been used to genetically engineer enhanced stress tolerance in transgenic plants. Different levels of manipulation of target genes encoding either upstream regulatory proteins and signaling molecules or downstream functional proteins using either gain- or loss-of-function approaches have led to production of transgenic plants with enhanced tolerance to various environmental stresses (Mittler and Blumwald, 2010). In this dissertation research, I focus on genetic manipulation of two novel transgenes – antimicrobial peptide (AMP) and microRNA (miRNA) in transgenic perennial species to study their impact on plant response to environmental stress. I also conduct research to study physiological and molecular mechanisms of miRNA-mediated plant response to stress aiming at developing new biotechnology approaches to genetically improve crop plants with enhanced performance, significantly contributing to agriculture production.

Novel antimicrobial peptides

Pathogenic fungi are among the most important biotic stresses plants face and fight against. They are responsible for numerous crop diseases, and in some cases conventional chemical methods are insufficient to control them. Besides decreasing yields, they also produce mycotoxins which are stored in plant organs and negatively affect human health (Punja, 2001; Christou and Twyman, 2004). One important strategy to engineer plants with fungal resistance is to express molecules which can kill
pathogenic fungi or stop their growth. Pathogenesis-related (PR) proteins such as chitinases and glucanases have been engineered for over 10 years in at least 20 plant species including some agriculturally and economically important crops. These PR proteins can hydrolyze the major component of fungal cell walls thus inhibiting hypha growth. However, in most cases, the disease spreading may be restricted, but the overall effect on disease control is not ideal. New sources of transgenes for enhancing general defense responses to prevent fungal diseases still need to be further explored (Punja, 2001; Christou and Twyman, 2004).

Small peptides with anti-fungal activity have also been largely tested in laboratories to confer resistance to pathogenic fungi (Osusky et al., 2000; Sharma et al., 2000a). Here, in my dissertation, I provide evidence demonstrating the effectiveness of a new option of the small peptide Penaeidin4-1 (Pen4-1) from Atlantic white shrimp for engineering fungal disease resistance in transgenic turfgrass. The data obtained point to the great potential of using Pen 4-1 for engineering broad-spectrum fungal disease resistance in crop species.

MicroRNAs

Breeding for enhanced plant tolerance to drought and salinity has always been very challenging due to the complex nature of these stresses. For example, different timing, duration, intensity and frequency of the stress can significantly impact experimental quantification and repeatability (Varshney et al., 2011). The heterogenic field conditions are very different from laboratory settings, adding additional layers of
complexity (Mittler and Blumwald, 2010; Varshney et al., 2011). Promising greenhouse experimental results would not necessarily guarantee success under field conditions. Moreover, multiple molecular pathways are involved in controlling plant response to salt and drought stress, thus making the breeding task more challenging (Century et al., 2008).

In recent years, with the help of increasing genomic data and bioinformatics tools, light has been shed on the molecular regulatory networks controlling plant abiotic stress tolerance, and various upstream regulators have been identified as candidates for genetic manipulation. These include the ABA-dependent AREB/ABF regulon, the Snf1-related protein kinases, CBF/DREB regulon, the ABA-independent NAC/ZF-HD regulon, microRNAs and others, of which microRNAs have become increasingly attractive for study due to the important roles they play in plant responses to abiotic stress (Chen et al., 2008; Nakashima et al., 2009; Mittler and Blumwald, 2010). MiR398, 393, 395, and 399 have been shown to regulate gene expression during abiotic stresses including dehydration, salinity, cold, and oxidative stresses (Sunkar et al., 2006; Lu and Huang, 2008; Kawashima et al., 2009; Mittler and Blumwald, 2010; Gao et al., 2011a; Zhu et al., 2011). Here I report our novel finding that miR319 is also involved in plant response to abiotic stress, and try to answer a key question: how it functions in plants during abiotic stress.

In the following section, I will focus on AMPs and miRNAs, provide specific information about these two groups of novel transgenes, and explain how they can be employed in plant genetic engineering.
1.2 Plant Genetic Engineering Using Antimicrobial Peptides for Enhanced Biotic Stress Tolerance

1.2.1 General introduction to antimicrobial peptides (AMPs)

AMPs are short sequence peptides which play important roles in plant and animal immune systems serving as a first line of defense. They generally consist of fewer than 50 amino acid residues. They are widespread in nature with high selectivity against target organisms (Zasloff, 2002).

Based on the most important characteristic of AMPs-electrostatic charge, AMPs can be subdivided into two major groups (Vizioli and Salzet, 2002; Keymanesh et al., 2009). The larger group of AMPs is comprised of cationic molecules, while the much smaller group of AMPs is comprised of non-cationic molecules including aromatic peptides, anionic peptides and peptides derived from oxygen-binding proteins. The term “AMPs” often refers to cationic AMPs (Zasloff, 2002; Keymanesh et al., 2009).

Based on structural features, AMPs can be subdivided into three classes (Figure 1.1) (1) “linear peptides often adopting α-helical structures”; (2) “cysteine-rich open-ended peptides containing a single or several disulfide bridges”; and (3) “cyclopeptides forming a peptide ring” (Montesinos, 2007). All these AMPs share certain common structural characteristics such as (1) cationic and hydrophobic residues are most abundant in their amino acid composition; (2) amphipathicity; and (3) structures and conformations are diverse (Vizioli and Salzet, 2002; Keymanesh et al., 2009). In fact, the second
characteristic, amphipathicity, can facilitate interactions between AMPs and microbial membranes (Zasloff, 2002). Some AMPs are enriched in certain amino acids. For example, a large amount of cationic AMPs are rich in cysteines forming a single or several disulfide bridges (e.g., Ib-AMP4 from balsamine and Penaeidins from shrimp), which makes their structures more compact and stable against complicated cell biochemical conditions such as protease degradation. This group of AMPs is widely spread in nature and exhibits a remarkable sequence and structure diversity (Vizioli and Salzet, 2002; Hancock and Sahl, 2006).

AMPs can act potently towards pathogens but without damaging host cells. This selectivity may profit from the structure differences between host and target membranes (Yount and Yeaman, 2005). Although the details of how AMPs function towards target microorganisms are still remained further explored, it is generally accepted that the amphipathicity, cationic charge and small size of AMPs have played important roles to allow AMPs to attach to and insert into bacterial membrane bilayers. After insertion into the membrane, antimicrobial peptides act by either disrupting the membrane bi-layer physically or trans-locating themselves across membranes and inhibit certain internal targets (Yount and Yeaman, 2005; Hancock and Sahl, 2006).
The exceptionally broad range of microbial targets of antimicrobial peptides, and the absence of side effects to host organisms arouse interests in exploiting the great potential of these molecules in agricultural application (Hancock and Sahl, 2006).

1.2.2 Application of AMPs in agriculture

Transgenic technology accelerates genetic engineering of various crop species with AMP-encoding genes (Van der Biesen, 2001). It allows the transfer of antimicrobial genes into target plants from other species, thus overcoming the sexual barriers among different species, which limit the conventional breeding of disease resistance. In fact, agricultural uses of antimicrobial genes for plant improvement have been quite extensive, as demonstrated in tobacco, potato, tomato, oilseed crops and trees (Keymanesh et al., 2009). For example, expression of the mammalian antimicrobial peptide cecropin P1 with 31 amino acid residues conferred enhanced resistance to several phytopathogenic bacteria.
to transgenic tobacco (Zakharchenko et al., 2005). Expressing of Alfalfa antifungal peptide (alfAFP) from seeds of *Medicago sativa* in transgenic potato led to enhanced resistance to fungal diseases (Gao et al., 2000). Expression *Mj*-AMP1 from *Mirabilis jalapa* in transgenic tomato led enhanced resistance to early blight disease (Schaefer et al., 2005). Expression of cecropin B from *Bombyx mori*, in transgenic rice plants could confine lesion development of bacterial leaf blight (Sharma et al., 2000a).

However, there have been few reports on the introduction of antimicrobial genes into turfgrass plants for improving disease resistance. Recently, a truncated *dermaseptin SI* gene encoding a peptide with 18 amino acid residues was introduced into tall fescue (Dong et al., 2007). The resulted transgenic plants displayed normal morphology and greater resistance to gray leaf spot (*M. grisea*) and brown patch diseases (*R. solani*). This encouraging result (Dong et al., 2007), together with the above mentioned reports of transgenic tobacco, potato, tomato, oilseed crops and trees, suggest the potential of using AMPs to generate resistance to a wide range of pathogens in turfgrass.

### 1.2.3 Animal derived AMP- Pen4-1

Besides plant derived AMPs, animal derived AMPs have also been considered and demonstrated to be useful for plant genetic engineering, because they have already been shown to be inhibitory to plant pathogenic pathogens by *in vitro* and *in vivo* study (reviewed by Meng et al., 2010). For example, an animal derived AMP esculentin-1 with 46 amino acid residues present in the skin secretions of *Rana esculenta*, with the
substitution Met-28Leu, was introduced into tobacco. The transgenic plants exhibited resistance against bacterial and fungal phytopathogens (Ponti et al., 2003). Additionally, expression of the mammalian AMP cecropin P1 in transgenic tobacco resulted in increased resistance to several phytopathogenic bacteria (Zakharchenko et al., 2005). Moreover, some plant pathogens may have already evolved and gained tolerance to plant derived AMPs, thus decreasing the effects of the AMPs in vivo (Li et al., 2001).

Penaeidins, a family of AMPs originally isolated from the haemocytes of penaeid shrimp, are considered to be a source of AMPs which have the potential to apply in agriculture to deliver disease resistance to plants. Shrimp mainly rely on innate immune system (Cuthbertson et al., 2004), in which penaeidin antimicrobial peptides are one of the key elements (Cuthbertson et al., 2006). Penaeidins have a unique two-domain structure including an unconstrained proline-rich N-terminal domain (PRD) and a cysteine-rich domain (CRD) with a stable α–helical structure (Cuthbertson et al., 2005) (Figure 1.2). They primarily act against Gram-positive bacteria and fungi (Destoumieux et al., 1999), and are synthesized in granular haemocytes. Upon infection, penaeidins are released into the plasma and localized to tissues, eventually binding to cuticle surfaces (Destoumieux et al., 2000a; Destoumieux et al., 2000b; Muñoz et al., 2002). The complexity inherent in the multi-domain structure of the peptide may contribute to its broad range of microbial targets (Destoumieux et al., 2000a; Destoumieux et al., 2000b; Yang et al., 2003).
The penaeidin family is comprised of four classes, designated 2, 3, 4 and 5, and each class consists of different isoforms displaying a significant level of sequence diversity (Chen et al., 2004; Cuthbertson et al., 2006). Pen4-1, a class four isoform one penaeidin, isolated from Atlantic white shrimp (*Litopenaeus setiferus*). It contains 6 cysteine residues forming 3 disulfide bridges, and is the shortest isoform in penaeidin family with a length of 47 amino acids (Figure 1.2). It can inhibit multiple plant pathogenic fungal species, including the *Botrytis cinera*, *Penicillium crustosum*, and *Fusarium oxysporum* (Bachère et al., 2000). It is also effective at preventing growth of the Gram-positive bacteria including the species *Micrococcus luteus* and *Aerococcus*.
viridans, and it is inhibitory against the Gram-negative bacteria - *Escherichia coli* at relatively high concentrations (Cuthbertson et al., 2006). Notably, Pen4-1 can fight against multidrug-resistant fungi species: *Cryptococcus neoformans* (*Steroform A, Steroform B, Steroform C, Steroform D*) and *Candida spp* (*Candida lipolytica, Candida inconspicua, Candida krusei, Candida lusitaniae, Candida glabrata*) (Cuthbertson et al., 2006) (Table 1.1). Compared with other penaeidins, penaeidin class 4 has been shown to be highly effective against fungi (Cuthbertson et al., 2006). Additionally, the unusual amino acid composition of PRD Pen4-1 may confer resistance to proteases (Cuthbertson et al., 2006). These results suggest that Pen4-1 is an ideal candidate for genetic engineering of enhanced disease resistance in turfgrass.

<table>
<thead>
<tr>
<th>Filamentous fungi</th>
<th>Drug-resistant human fungal pathogens</th>
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<tr>
<td><em>Fusarium oxysporum</em></td>
<td><em>Cryptococcus neoformans</em></td>
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<tr>
<td><em>Botrytis cinerea</em></td>
<td><em>Steroform A</em></td>
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<tr>
<td><em>Penicillium crustosum</em></td>
<td><em>Steroform B</em></td>
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<td><strong>Gram-positive bacteria</strong></td>
<td><em>Steroform C</em></td>
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<tr>
<td><em>Aerococcus viridans</em></td>
<td><em>Steroform D</em></td>
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<tr>
<td><em>Micrococcus luteus</em></td>
<td><em>Candida spp.</em></td>
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<tr>
<td><em>Micrococcus coral 649</em></td>
<td><em>Candida albicans A39</em></td>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td><em>Candida lipolytica</em></td>
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<td><em>Bacillus epiphytus</em></td>
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<td><em>Bacillus cirroflagellosus</em></td>
<td><em>Candida krusei</em></td>
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<tr>
<td><em>Bacillus coral 644</em></td>
<td><em>Candida lusitaniae</em></td>
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<td><strong>Gram-negative bacteria</strong></td>
<td><em>Candida glabrata</em></td>
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<tr>
<td><em>Escherichia coli</em></td>
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Table 1.1 A range of microbial organisms Pen4-l can fight against. Adapted from Cuthbertson, et al. (2006)

Although the mechanism of how penaeidins act towards microorganisms has not yet been revealed, recent work with other proline-rich AMPs indicates that penaeidins might function through targeting certain cytosolic proteins (Otvos, 2000; Cuthbertson et al, 2004). For example, the proline-rich AMP apidaecin originally isolated from honey bee, can not only penetrate the microbial membrane but also internalize itself, and then target the heat-shock protein 70, an important molecular chaperone (Zasloff, 2002; Otvos 2000; Cuthbertson et al, 2004). It is hypothesized that a more complex mechanism rather than simple membrane disruption, i.e., targeting a specific microbial cytosolic component is adopted by Pen4-l (Otvos, 2000; Cuthbertson et al, 2004, Cuthbertson et al, 2006).

1.3 Genetic Engineering of MicroRNAs for Enhanced Abiotic Stress Tolerance

1.3.1 General introductions to microRNAs and miR319

The first glance in the world of small RNAs was the identification of microRNA (miRNA) genes, lin-4 and let-7 in nematodes. Lin-4 and let-7 control nematode development timing by regulating gene expression at post-transcriptional level. It was the first time people identified small RNAs and demonstrated that they negatively regulated
gene expression and were involved in development. (Slack et al., 2000). After the identification of a large number of miRNAs in other species, we now know that they are a universal element in animals, plants and humans. Although the studies on miRNAs have been more advanced in animals, for the past several years, our understanding and knowledge of miRNAs in plants have grown very rapidly. Since 2002, more than 180 genes and over 2500 genes encoding miRNAs in Arabidopsis and across all plant species respectively, have been annotated (miRBase).

MiRNAs are a class of 20 to 22 nt non-coding RNAs. They are encoded by miRNA genes and transcribed into primary transcript, which are then processed into pre and mature RNAs. The mature RNA can either direct cleavage or translational repression of its target genes. In plants, the main mode of action is direct cleavage by being recruited into RISC where many proteins are present (Jones-Rhoeades et al., 2006).

Identifying and annotating plant miRNAs are more complicated in plants than in animals not only because of the slightly different characteristics the plant miRNAs possess but also because of the fact that the plant miRNAs are only minorities in the large and complex populations of small RNAs, which is in contrast to the case in some of the vertebrates and flies where miRNAs are majority in the small RNA populations (Meyers et al., 2008). With the identification of hundreds of miRNAs, the challenge, then, is to understand each miRNA’s specific biological functions. To date, miRNAs have been shown to play a critical roles in development, abiotic and biotic stress response, and
nutrient homeostasis via interaction with their targets (Jones-Rhoades et al., 2006; Meyers et al., 2008).

My present project focuses on studying the regulatory roles of microRNA 319 (miR319) in controlling plant morphology and abiotic stress tolerance. The miR319 family is one of the first characterized and conserved miRNA families in plants, and it has been demonstrated to target *TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP)* genes encoding plant-specific transcription factors known to be largely involved in leaf development (Palatnik et al., 2003). MiR319 family is one of the most ancient and conserved miRNAs found in a large number of plant species (Axtell and Bowman, 2008; Cuperus et al., 2011). It has a very unique loop-first processing mechanism which makes its fold-back structure widely conserved in the evolution (Bologna et al., 2009). This may indicate its importance in controlling plant development and suggests that it could be a candidate target of genetic manipulation for plant trait modifications. Recently, an increasing number of microarray data demonstrated potential involvement of the miR319 genes in plant response to abiotic stress response (Sunkar and Zhu, 2004; Liu et al., 2008; Zhou et al., 2010a; Zhou et al., 2010b), but direct experimental proof about the contribution of the miR319 family to plant abiotic stress response is still lacking.

The impact of miR319 on plant morphogenesis has been studied in dicots. However, there has been no published data reporting similar research in monocots, especially in perennial grass species. Here, we use creeping bentgrass as an example to try to answer three questions which have not been addressed before:
(1) What impact the miR319 gene family may have in plant development in perennial grasses?

(2) Is miR319 involved in plant abiotic stress response in perennial grasses?

(3) What is the molecular mechanism of miR319-mediated plant tolerance to abiotic stress in perennial grasses?

1.3.2 Origin, biogenesis and modes of actions of plant miRNAs

1.3.2.1. General biogenesis of plant miRNAs

MiRNAs are fundamental and key post-transcriptional regulators of eukaryotic gene expression. Plant pri-miRNAs are transcribed by Pol II (RNA polymerase II) from miRNA genes mostly located far from protein-coding genes (Jones-Rhoades et al., 2006). As Figure 1.3 demonstrated, the RNA-binding protein DDL (DAWDLE) is presumed to stabilize pri-miRNAs for their later splicing. The conversion to pre-miRNA happened in D-bodies (nuclear processing centers) (Voinnet, 2009; Mateos et al., 2011). Here, three important components - SE (C2H2-zinc finger protein SERRATE), HYL1 (the double-stranded RNA-binding protein HYPONASTIC LEAVES1) and DCL1 (Dicer-like 1) function coordinately (Voinnet, 2009; Mateos et al., 2011). Pre-miRNAs are then cut into mature miRNAs duplexes - miRNA/miRNA* by DCL1 (miRNA is the guide strand, and miRNA* will be degraded), and methylated by HEN1, which is an action to prevent them from being degraded by SDN (SMALL RNA DEGRADING NUCLEASE) class of exonucleases. Mature miRNAs are then exported to the cytoplasm possibly by plant
exportin 5 ortholog HASTY and other unknown factors. The guide strand is then incorporated into ARGONAUTE (AGO) proteins to carry out their silencing function (Xuemei, 2005; Voinnet, 2009; Mateos et al., 2011) (Figure 1.3).
Figure 1.3 Biogenesis of plant miRNAs. Adapted from Voinnet (2009).
1.3.2.2. Mechanisms of plant miRNA precursor processing

A common core step in miRNA biogenesis is precursor processing. The precision of this process is responsible for the miRNA specificity and ultimately for their correct function (Mateos et al., 2011). In animals, DROSHA cuts at approximately 11 nt from “the joint between the single-stranded RNA and double-stranded stem” to produce pre-miRNA (Han et al., 2006). After exporting pre-miRNA to nucleus, Dicer performed the second cut at around 21 nt from the end of the pre-miRNA to release the miRNA/miRNA* duplex (Carthew and Sontheimer, 2009; Mateos et al., 2011). Slightly different from animal pri-miRNAs which are normally homogenous fold-back structures, plant pri-miRNAs are a collection of diverse stem-loop structures with different sizes and shapes, thus making it more difficult to define its mechanism (Mateos et al., 2011).

Recent studies indicate that there are two processing mechanisms in plant miRNAs - “base-to-loop” and “loop-to-base” (Bologna et al., 2009; Mateos et al., 2011). The majority of the plant miRNAs adopts the “base-to-loop” processing mechanism, which is a mode more similar to animal precursor processing (Addo-Quaye et al., 2009; Bologna et al., 2009; Werner et al., 2010). In this model, the precursor is comprised of 3 regions: (1) a partially structured 15-nt lower stem, (2) miRNA/miRNA* duplex, and (3) a terminal loop (Werner et al., 2010; Mateos et al., 2011). The lower stem located below the miRNA is first recognized and cut. Then a second cleavage is followed to release the mature miRNA (Figure 1.4a). The whole processing appears to depend on lower stem, and even a single change in this region is able to fail its processing (Werner et al., 2010).
However, the family of miR319/159 is an exception of this canonical model. They have large fold-back sequences consisting of 4 parts (1) lower stem (LS), (2) miRNA/miRNA* (duplex M/M*), (3) upper stem (US) and (4) terminal loop (TL) (Addo-Quaye et al., 2009; Mateos et al., 2011). Unlike other plant miRNAs, the processing begins with a first cleavage to release TL instead of LS. Next, 3 more dicing events occur until the duplex M/M* is finally liberated (Addo-Quaye et al., 2009) (Figure 1.4b). In contrast to “base-to-loop” mechanism, LS is not important in this model. Actually, a miR319a precursor without LS was processed efficiently in vivo. However, this processing is extremely sensitive to modifications in US and TL (Addo-Quaye et al., 2009; Bologna et al., 2009). In addition, the bulges present in the fold-back structure help the accumulation of miRNAs instead of other small RNAs. This unique “loop to base” processing mechanism of miR319 is conserved in Arabidopsis and moss, indicating that this mechanism is evolutionarily ancient (Addo-Quaye et al., 2009; Bologna et al., 2009).
1.3.2.3. MicroRNA-directed mRNA cleavage

MicroRNAs regulate gene expression by two basic mechanisms: RNA cleavage and translational repression. It has been indicated that the complementarity of target sites between miRNAs and their target mRNAs are key determinants of which mode to be adopted. Perfect complementarity may lead to the mechanism of slicing, whereas mismatch in the central area of the target site is supposed to trigger translational inhibition (Hutvágner and Zamore, 2002; Song et al., 2004). Unlike animal miRNAs, plant miRNAs show perfect or near-perfect match with their targets, and they are believed to adopt the slicing mode more frequently (Brodersen et al., 2008). In slicing mode, miRNA guides AGO to cleave a single phosphodiester bond within target mRNA molecules. After the direct cleavage, AGO is freed to carry out other slicing (Jones-Rhoades et al., 2006).
However, by 2007 there are already several examples demonstrating the existence of translational expression - miR172 as a translational repressor of AP2 in Arabidopsis flower development and miR156/157 as an inhibitor of SP13 translation (Chen, 2004; Gandikota et al., 2007). In 2008, a breakthrough was made by Brodersen et al. demonstrating that “plant miRNA-guided silencing has a widespread translational inhibitory component that is genetically separable from endonucleolytic cleavage” (Brodersen et al., 2008).

In 2009, combining all the data, a general model was proposed to explain the mode of miRNA actions (Brodersen et al., 2008; Voinnet, 2009) (Figure 1.5). In this model, it is suggested that slicing was predominantly adopted by plant miRNAs and suited to produce irreversible switches (Figure 5, left). Nonetheless, when a reversible switch was needed, translational repression would be suited, for instance, to coordinate and reset stress-responsive gene expression (Figure 5, middle) (Voinnet, 2009). These two layers of regulation could be brought together by plants, yet they may not coincide spatially or temporally (Figure 5, right) (Voinnet, 2009).
Figure 1.5 Modes of miRNA actions (a) Slicing (b) Translational inhibition (c)Translational inhibition and slicing. Adapted from Voinnet (2009).

1.3.3 Regulatory roles of miRNA-target nodes

1.3.3.1. Concept of miRNA-target nodes

A miRNA node is “an interface that comprises the regulatory relationship between a specific locus from a miRNA family and the direct targets under its control” (Rubio-Somoza et al., 2009; Rubio-Somoza and Weigel, 2011). MiRNA nodes can either infer to a certain specific case such as the relationship between one miRNA locus and one target (e.g. miR319a-TCP2 node) or the relationship between all miRNA loci of a family and all its targets (e.g. miR319-TCP) (Rubio-Somoza and Weigel, 2011). Many predicted miRNA targets encode regulatory proteins such as transcription factors, suggesting a role of miRNAs as master regulators and pointing miRNA-target nodes to be the core of gene regulation networks (Jones-Rhoades et al., 2006).
1.3.3.2. Identification of miRNA-target nodes

To identify the miRNA-target nodes in plant regulatory work, the first two things needed to do are to identify novel miRNA genes and their targets.

(1) Identification of plant miRNA genes

The following 3 methods are most commonly used to identify novel plant miRNA genes (Jones-Rhoades et al., 2006).

i) Direct cloning and sequencing

Directly isolate small RNAs from plant samples, ligate them to adaptor oligonucleotides, and do reverse transcription, amplification, and sequencing (Jones-Rhoades et al., 2006). The early sequencing method is conventional sanger sequencing (Reinhart et al., 2002; Sunkar and Zhu, 2004), which generates a lot of false positives (Jones-Rhoades et al., 2006; Jones-Rhoades, 2011). Later the emergence of deep sequencing first in 2005 (Lu et al., 2005) has much facilitated our understanding on identification and annotating plant miRNAs.

ii) Forward genetics

Although miRNAs were first discovered by forward genetic screens in *C. elegans* (Lee et al., 1993), few plant miRNAs were discovered by this method (Jones-Rhoades, 2011). This is mainly because of function redundancy among different members of a miRNA family and small target sizes for mutagenesis (Jones-Rhoades, 2011).
iii) Bioinformatics predictions followed by experimental confirmation

Annotate homologs of miRNA discovered by cloning using bioinformatics tools. For example, a genome is sequenced and assembled. Except some evolutionarily young miRNAs, it is convenient to annotate the homologs of known miRNAs by analyzing conservation in sequence and secondary structure (Jones-Rhoades et al., 2006; Meyers et al., 2008).

(2) Identification of miRNA targets

Because of high complementarity between targets and a microRNA mature sequence is required for the interactions, target identification in plants is much easier compared with animals (Schommer et al., 2008). This also facilitates the prediction of targets by algorithm based on homology query.

Based on high complementarity, a set of miRNA targets can be identified by bioinformatics tools, especially for those miRNA-target pairs conserved across species (Reinhart et al., 2002; Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Jones-Rhoades, 2011). A set of experimental techniques can then be used to corroborate the miRNA-target relationship. Another strategy to identify miRNA targets is to do mRNA expression arrays in genome-wide screens (Jones-Rhoades et al., 2006). For example, expression array data demonstrated that the expression of 5 TCP genes was downregulated in Arabidopsis plants overexpression miR319 (Palatnik et al., 2003; Jones-Rhoades et al., 2006).
1.3.3.3. The scope of diverse regulatory roles of miRNA nodes

(1) MiRNA networks and plant development

Two reverse genetic strategies are commonly used to investigate the function of particular miRNA nodes. The first is an overexpression strategy to potentially downregulate all possible target genes controlled by the miRNA. The second strategy is to make transgenic plants expressing a modified version of targets which are resistant to its corresponding miRNA (Palatnik et al., 2003; Jones-Rhoades et al., 2006).

By using these strategies, functions of several miRNA nodes have been identified. Later the network of these nodes is revealed gradually, demonstrating that plant miRNA notes play a pivotal role in plant development such as phase change, architecture and stress response (Rubio-Somoza and Weigel, 2011). Several miRNA nodes participating in different developmental events have been characterized (Rubio-Somoza and Weigel, 2011) and there are several examples described in the following paragraphs.

Currently, the most known mechanisms of leaf senescence involve two miRNA nodes including miR319-TCP and miR164-NAC. Expression of miR164 declines as leaf aging thus leading to increased expression of its targets NAC1, ORE1 which promote leaf senescence. Accordingly, miR164 ectopic or constitutive expression would promote leaf longevity (Kim et al., 2009; Rubio-Somoza and Weigel, 2011). MiR319 actively participates in many plant developmental events, among which is leaf senescence (Rubio-Somoza and Weigel, 2011). Ectopic expression of miR319 would make *Arabidopsis* leaves stay greener, and delay the onset of leaf senescence compared with wild type leaves. Its target TCP4 gene was demonstrated to be active in many old leaves. It is
proposed that the down-regulation of TCP genes by miR319 leads to low expression level of LOX2 (LIPOXYGENASE2) gene, which is positively regulated by TCP transcription factors and is one the most important JA (Jasmonic acid) synthesis enzymes. Since JA negatively regulates leaf senescence, low levels of JA promote leaf longevity in plants with impaired miR319-TCPs activity (Schommer et al., 2008). Although these 2 nodes are all involved in the similar process, the problem whether they cross talk still remains to be addressed (Rubio-Somoza and Weigel, 2011).

The large number of amazing variety of leaf shapes is a mystery which fascinated people for centuries till the discovery of miRNAs. It is first unrevealed in Arabidopsis that specific and systematic regulation by miRNAs play the primary role on leaf patterning responsible for the majority of phenotypes (Todesco et al., 2010; Rubio-Somoza and Weigel, 2011). Regulating leaf morphogenesis mainly includes three aspects, organ polarity, cell proliferation, and vascular development, in which various miRNA nodes are involved. For example, At least four miRNA nodes (miR164, miR319, miR396 and miR390) are involved in regulation of the cell proliferation (Rubio-Somoza and Weigel, 2011), which will be discussed in more detail in 1.3.4. Organ polarity including abaxial/adaxial differentiation consists of at least miR165/166 and miR390. For vascular development, miR165/166 and miR159 nodes play essential roles (Donner et al., 2009; Rubio-Somoza and Weigel, 2011).
(2) Regulatory roles of miRNAs in plant stress response

The exploration of the relationship between miRNA expression and stress responses has just started. It is discovered that reduced expression of miR398 leads to improved tolerance to oxidative stresses through regulation of its target genes (Sunkar et al., 2006). Additionally, expression of miR395 increases upon sulfate starvation (Jones-Rhoades and Bartel, 2004). MiR399 down-regulates its target mRNA accumulation upon Pi starvation (Fujii et al., 2005). These data suggest that miRNAs have functional roles for plants to cope with various environmental stresses.

There are also several research groups performing high-throughput profiling of the expression of the known miRNAs under different environmental stresses (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Zhao et al., 2007; Yang et al., 2008; Ding et al., 2009). Sequencing of a library of small RNAs isolated from Arabidopsis seedlings exposed to various stresses identified several stress-related miRNAs. MiR393, miR397b and miR402 that were up-regulated in response to cold, dehydration, salinity and ABA (Sunkar and Zhu, 2004). Later, 14 stress-inducible miRNAs were identified in Arabidopsis by microarray analysis and confirmed by RT-PCR. Of the 14 miRNAs identified, 10, 4 and 10 microRNAs are regulated by high-salinity, drought and cold respectively. This showed that some of these 14 miRNAs were prone to being induced by not only one treatment, indicating a cross talk between miRNAs and different stressors (Yang et al., 2008). In other eudicots, genome-wide high-throughput sequencing was also performed to identify stress-responsive miRNAs. For instance, in Medicago truncatula
32 known miRNAs and 8 new miRNAs were identified to be responsive to drought stress (Wang et al., 2011).

Early studies in monocots have also demonstrated that miRNAs contribute to plant adaptation to stresses. For example, microarray analysis examining miRNA expression during drought stress in rice showed that miR169g was up-regulated upon drought (Zhao et al., 2007). Later miR169g and miR169n were found to be induced by high salinity in rice (Zhao et al., 2009). In maize, microarray hybridization revealed that the expression of 98 miRNAs from 27 families were significantly affected after salt treatment (Ding et al., 2009). MiR474 which targets proline dehydrogenase (PDH) was shown to be up-regulated during drought stress in maize (Wei et al., 2009).

In addition, microRNAs are also reported to be responsive to cold stress, oxidative stress, hypoxia stress, UV-B radiation and nutrient stress (Martin et al., 2010; Sunkar, 2010). Furthermore, a comparative analysis using an array-based approach between a salt tolerant and salt sensitive maize variety indicated differential responses of miRNAs during salt stress (Ding et al., 2009). Stem-loop RT-PCR analysis in sugarcane also indicated differential responses of miRNAs between cold-tolerant and cold-sensitive cultivars upon cold stress (Thiebaut et al., 2011). Identification of relevant traits in stress-tolerant relatives could provide insights into strategies that can be used for future improvement of crops for their responses to stress.

In general, miRNAs play an important role in plant stress response regulatory networks. The discovery of stress responsive miRNAs is just a start, it would be more
challenging to unravel the detailed regulatory role each miRNA plays and how they coordinate together to form a network. In the future, miRNA networks and their targets represent an important genetic reservoir that can be exploited to better understand the molecular mechanisms of plant stress response and further applied in genetic engineering for developing new cultivars with greater stress tolerance (Sunkar, 2010).

1.3.4 Current understanding of miR319-mediated plant morphogenesis and abiotic stress response

1.3.4.1. miR319 family is highly conserved among different species

Although the majority of the plant miRNAs are family- or species-specific, a minority of the miRNA families is conserved among different plant families. In particular, miR319 family is extremely conserved not only in angiosperms but also in gymnosperms, lycopods and bryophytes as shown in Figure 1.6 (Axtell and Bowman, 2008; Cuperus et al., 2011). This high conservation among different land plant species indicates that it has important biological functions.
Figure 1.6 MiR 319 family presented in various land species. In each species, the study of miR319 was scored depending on the accumulation of experimental support. Abbreviations: ath, Arabidopsis thaliana; bna, Brassica napus; mtr, Medicago truncatula; osa, Oryza sativa; ppt, Physcomitrella patens; pta, Pinus taeda; ptc, Populus trichocarpa; smo, Selaginella moellendorfii; tae, Triticum aestivum; vti, Vitis vinifera; zma, Zea mays. Aadapted from Axtell and Bowman (2008).

1.3.4.2. miR319-mediated plant morphology change

Although a vast majority of microRNAs were discovered by sequencing, the first plant miRNA mutant jawD which overexpresses Ath-miR319a was discovered through experimentation instead of deep sequencing (Palatnik et al., 2003). In addition, the target genes of miR319 were first identified and confirmed by experimental analysis (Palatnik et al., 2003; Schommer et al., 2008). The TCP gene family is a group of plant-specific transcription factors which were known to be largely involved in plant leaf development. All the members in the TCP gene family share a conserved TCP domain region which adopts a basic helix-loop-helix structure (bHLH). TB from maize, CYC from antirhium
and PNCA from rice are the founding members of the TCP family. It is suggested that they negatively regulate plant development by observing their mutant phenotypes. In Arabidopsis, 5 target genes of miRNA319 are TCP2, TCP3, TCP4, TCP10, and TCP24, which belong to class 2 of the TCP family. This group of TCP genes is represented by CIN in Antirrhinum (Nath et al., 2003; Schommer et al., 2008; Nag et al., 2009).

Leaves are emerged from shoot apical meristem (SAM) where a pool of undifferentiated cells is. With the regulation of auxin, the leaf primordia are formed. Leaf complexity is determined by cell proliferation at the leaf margin, which can decide the margins being dissected, entire or formation of compound leaves. The boundary between undifferentiated cells and the cells for differentiation is established by the miR164 targeting CUC1 (CUP-SHAPED COTYLEDON1) and CUC2 (CUP-SHAPED COTYLEDON2) (Palauqui et al., 1997; Rubio-Somoza and Weigel, 2011). Fused cotyledons occur when miR164-CUC activity is compromised in agreement with phenotypes when miR319-TCP regulation is compromised (Palauqui et al., 1997). Furthermore, TCPs can directly bind to the regulatory sequences of miR164a and modulate its expression level, suggesting miR164 node is ultimately impacted by miR319 node (Rubio-Somoza and Weigel, 2011) (Figure 1.7).
Figure 1.7  Coordinated action of miRNA nodes in regulating cell proliferations in plant leaves. Modified from Rubio-Somoza and Weigel (2011).

In Arabidopsis, the absence of miR319-TCPs or miR164a-CUC2 causes leaf margins to be excessively proliferated leading to crinkled and more serrated leaves (Figure 1.8a, b). Overexpression of a miR319-resistant form of TCP2 fully restores Jaw mutants (Figure 1.8c) (Palatnik et al., 2003; Koyama et al., 2007). Cin (CINCINNATA) mutants have crinkly leaves too. The snapdragon CIN gene, which belongs to TCP family, is required for forming a flat leaf by differential regulation of cell division on different stages of leaf morphogenesis (Nath et al., 2003). For compound leaf formation, the escape of TCP protein LA (LANCEOLATE) by miR319 also had a significant impact on leaf morphology. In la homozygous individuals, only simple leaves would be produced.
because of too strong TCP activity leads to cell proliferation arrest (Figure 1.8d) (Ori et al., 2007; Rubio-Somoza and Weigel, 2011).

**Figure 1.8** MiR319 mediated plant leaf morphology change. (a) The phenotypic change of miR-JAW Arabidopsis mutant. (b) The morphology change of tomato leaves by overexpressing miR319. (c) The phenotype in Arabidopsis caused by overexpression of miR319 resistant version of TCP2. (d) MiR319 regulated La gene in dose-dependent manner, leading to reduced leaf complexity and transformation from compound leaf to simple leaflet. Adapted from Palatnik et al. (2003), Ori et al. (2007). Adapted by permission from Macmillan Publishers Ltd: [Nature] (Palatnik et al.), copyright (2003) and permission from Macmillan Publishers Ltd: [Nature Genetics] (Ori et al.), copyright (2007).

Although miR319 regulated TCP activity has been well studied in dicot species, there have been no published data on this in monocot species. Our study in turfgrass would fill this blank.
1.3.4.3. MiR319-mediated plant abiotic stress response

In Arabidopsis miR319 was up-regulated in response to cold stress (Sunkar & Zhu 2004, Liu 2008). Theibaut et al. conducted research studying in more detail about how miR319 was involved in the regulation of cold stress. Their data showed a complicated behavior of miR319 in response to cold stress. MiR319 was up-regulated first after 24 hours of treatment in a cold stress environment and then down-regulated after a 48-hour treatment. When they compared the miR319 expression in two sugarcane cultivars - cold sensitive and cold tolerant, they found the induction of miR319 was delayed and weaker in a cold-tolerant cultivar compared with a cold-sensitive cultivar. The change in expression level of their putative targets PCF6 and GAMyb upon cold stress is complicated. Although they decreased after a 48-hour treatment of cold stress, PCF6 was up-regulated after 24h. When using other cultivars, PCF6 was still up-regulated after 48h, but GAMyb was down-regulated. These genes are regulated not only by miR319, but also by some other signals, for example ABA. ABA can be triggered by cold stress, and some Myb genes such as GAMyb can be induced by ABA. So the expression level would be a balance of ABA induction and miR319 cleavage. In fact, the expression level of target genes did not decrease even when the miRNA cleavage of target mRNA occurred (Thiebaut et al., 2011).

It has also been reported that a single miRNA has the potential to regulate multi-stress related target genes (Yang et al., 2008). MiR319 was up-regulated 1.81 fold upon 300 mM of NaCl treatment and 1.54 fold upon cold stress (Yang et al., 2008). Moreover analysis of the stress-relevant cis-elements in the promoter regions of miRNA genes

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provides additional evidence that the miRNAs are very likely to be involved in plant response to abiotic stress. In the upstream regions of miRNA 319 genes in *Aradidopsis*, there are two TC-rich repeats (defense and stress), two AREs (stress), MBS (drought), HSE (heat stress), TGA element (auxin), AuxRR core (auxin), Box W1 (fungal elicitor) (Yang et al., 2008).

Although the current microarray data and promoter analysis all suggest that miR319 may be correlated with plant stress response, *in vivo* experimental evidence demonstrating how miR319 regulates this process is still lacking. My present study will provide information allowing more understanding on how miR319 impacts plant adaptation to salt and drought stress in monocot perennial grasses.

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CHAPTER 2 - EXPRESSION OF A NOVEL ANTIMICROBIAL PEPTIDE PENAEDIN4-1 IN CREEPING BENTGRASS (AGROSTIS STOLONIFERA L.) ENHANCES PLANT FUNGAL DISEASE RESISTANCE

Man Zhou¹, Qian Hu¹, Zhigang Li¹, Dayong Li¹, Chin-Fu Chen¹,², Hong Luo¹*
¹Department of Genetics and Biochemistry, Clemson University, 100 Jordan Hall, Clemson, SC 29634, USA
²Current address: Center for Molecular Studies, Greenwood Genetic Center, Greenwood, SC 29646, USA
*For correspondence (fax 864 656 0393; phone 864 656 1746; e-mail hluo@clemson.edu)
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Keywords: antimicrobial peptide, Penaeidin4-1, perennial turfgrass, plant disease resistance, Rhizoctonia solani, Sclerotinia homoeocarpa.

Abstract

Background: Turfgrass species are agriculturally and economically important perennial crops. Turfgrass species are highly susceptible to a wide range of fungal pathogens. Dollar spot and brown patch, two important diseases caused by fungal pathogens Sclerotinia homoeocarpa and Rhizoctonia solani, respectively, are among the most severe turfgrass diseases. Currently, turf fungal disease control mainly relies on fungicide treatments, which raises many concerns for human health and environment. Antimicrobial peptides found in various organisms play an important role in innate immune response.

Methodology/Principal Findings: The antimicrobial peptide - Penaeidin4-1 (Pen4-1)
from the shrimp *Litopenaeus setiferus* has been reported to possess *in vitro* antifungal and antibacterial activities against various economically important fungal and bacterial pathogens. In this study, we have studied the feasibility of using this novel peptide for engineering enhanced disease resistance into creeping bentgrass plants (*Agrostis stolonifera* L., cv. Penn A-4). Two DNA constructs were prepared containing either the coding sequence of a single peptide, Pen4-1 or the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein translationally fused to Pen4-1 coding sequence. A maize ubiquitin promoter was used in both constructs to drive gene expression. Transgenic turfgrass plants containing different DNA constructs were generated by *Agrobacterium*-mediated transformation and analyzed for transgene insertion and expression. In replicated *in vitro* and *in vivo* experiments under controlled environments, transgenic plants exhibited significantly enhanced resistance to dollar spot and brown patch, the two major fungal diseases in turfgrass. The targeting of Pen4-1 to endoplasmic reticulum by the transit peptide of AP24 protein did not significantly impact disease resistance in transgenic plants.

**Conclusion/Significance:** Our results demonstrate the effectiveness of Pen4-1 in a perennial species against fungal pathogens and may suggest a potential strategy for engineering broad-spectrum fungal disease resistance in crop species.
Introduction

Turfgrasses, agriculturally and economically important crop species, are used worldwide for lawns of buildings, roadsides, athletic and recreational fields providing numerous benefits including reducing soil erosion, trapping dust and pollutants, moderating temperature, safer playing grounds and beautifying the environment (The National Turfgrass Research Initiative, 2003; Haydu et al., 2006). There are more than 50 million acres of turfgrass and 16,000 golf courses in the US alone, and the turfgrass industry is a business with multibillion dollars annually (2003; Haydu et al., 2006). Turfgrass species are highly susceptible to a wide spectrum of fungal pathogens. Dollar spot and brown patch, two important diseases caused by fungal pathogens Sclerotinia homoeocarpa and Rhizoctonia solani respectively, are two of the most severe and common diseases on turfgrass lawns (Chai et al., 2002; Guo et al., 2003). Currently, fungicides are commonly applied to control fungal diseases. This raises concerns about the potential emergence of new pathogen strains as a result of intensive use of chemicals (Damicone and Smith, 2009; Keymanesh et al., 2009; Young and Patton, 2010). Resistance to some major classes of fungicides such as benzimidazoles, demethylation inhibitors (DMIs), QoI respiration inhibitors (QoIs) and dicarboximides (DCFs) has been detected in many phytopathogenic fungi species (Ma and Michailides, 2005). For example, large scale agricultural use of DMIs since 1970s has led to the emergence of resistant genotypes of several phytopathogenic fungi impacting different crop and fruit species including turfgrass (Brent and Federation, 1995; Schnabel and Jones, 2001; Ma and Michailides,
2005; Young and Patton, 2010). Similarly, benzimidazole-resistant genotypes were also identified in *Monilinia fructicola*, *Penicillium expansum*, *Botrytis cinerea*, *Helminthosporium solani* and *sclerotinia homoeocarpa*. (Luck and Gillings, 1995; Mkay and Cooke, 1997; Bartlett et al., 2002; Ma et al., 2003; Ma and Michailides, 2005; Young and Patton, 2010). Therefore, the problem of emergent new resistant pathogen strains and the negative long-term impacts of fungicides on human health and the environment have both driven the search for new alternatives for the currently used chemicals (Rekha et al., 2006; Keymanesh et al., 2009). It is desirable that new cultivars be developed that present sustainable resistance to a broad range of pathogens and are safe for the environment or human consumption (Zasloff, 2002; Keymanesh et al., 2009).

Antimicrobial peptides (AMPs) found in various organisms play an important role in innate immune response (Boman, 1995; Rao, 1995; Broekaert et al., 1997; Hancock and Chapple, 1999; Zasloff, 2002), providing good candidates for use in plants for enhanced disease resistance. AMPs are short sequence peptides with generally fewer than 50 amino acid residues, most of which have antimicrobial activity against a broad spectrum of pathogens. They are a first line of defense in plants and animals and resistance against them is much less observed compared with current antibiotics (Zasloff, 2002). AMPs from various sources have been demonstrated to confer resistance against fungal and bacterial pathogens in an array of genetically engineered plant species, including *Arabidopsis* (Lee et al., 2008), tobacco (Jaynes et al., 1993; Florack et al., 1995; Terras et al., 1995; Huang et al., 1997; DeGray et al., 2001; Li et al., 2001; Chakrabarti et al., 2003; Yevtushenko et al., 2005; Yevtushenko and Misra, 2007; Yang et al., 2008), rice
(Sharma et al., 2000b; Coca et al., 2004; Coca et al., 2006; Patkar and Chattoo, 2006; Imamura et al., 2010; Jha and Chattoo, 2010), potato (Allefs et al., 1995; Arce et al., 1999; Gao et al., 2000; Osusky et al., 2000; Osusky et al., 2004; Yi et al., 2004; Osusky et al., 2005), tomato (Alan et al., 2004), cotton (Rajasekaran et al., 2005), pear (Reynoird et al., 1999), banana (Chakrabarti et al., 2003), ornamental crops, geranium (Pelargonium sp.) (Bi et al., 1999), American elm (Newhouse et al., 2007) and hybrid poplar (Liang et al., 2002; Mentag et al., 2003).

Penaeidins, a family of AMPs originally isolated from the haemocytes of penaeid shrimp, is considered to be a source of compounds, which have the potential to be applied in agriculture to deliver disease resistance to plants. Unlike vertebrates possessing the adaptive immune system, shrimp only have an innate immune system, among which are penaeidin antimicrobial peptides (Cuthbertson et al., 2004; Cuthbertson et al., 2006). Upon pathogen challenge to the host, the peptides are released from granular haemocytes to the plasma and attached to cuticles fighting microbial infection (Destoumieux et al., 2000a; Destoumieux et al., 2000b; Muñoz et al., 2002; Cuthbertson et al., 2004). Penaeidins have a unique two-domain structure including an unconstrained proline-rich N-terminal domain (PRD) and a cysteine-rich domain (CRD) with a stable α-helical structure (Yang et al., 2003; Cuthbertson et al., 2004; Cuthbertson et al., 2005). The complexity inherent in this unique structure might have contributed to its broad range of microbial targets, including primarily Gram positive bacteria and fungi (Destoumieux et al., 1999; Destoumieux et al., 2000a; Yang et al., 2003; Cuthbertson et al., 2004).

The penaeidin family is divided into four classes, designated as 2, 3, 4 and 5.
Each class displays a remarkable level of primary sequence diversity (Chen et al., 2004; Cuthbertson et al., 2006). Pen4-1, an isoform within the class number 4 penaeidins (isoform number 1) is isolated from Atlantic white shrimp (*Litopenaeus setiferus*). It contains 6 cysteine residues forming 3 disulfide bridges and is the shortest isoform in penaeidin family with a length of 47 amino acids. It can inhibit multiple plant pathogenic fungal species, including the *B. cinera*, *P. crustosum* and *F. oxysporum* (Cuthbertson et al., 2004). It is also effective against Gram-positive bacteria species including *M. luteus* and *A. viriduans*, and inhibitory against Gram-negative bacteria, *E. coli*, at relatively high concentrations (Cuthbertson et al., 2006). Notably, Pen4-1 can inhibit the growth of multidrug-resistant fungi species: *Cryptococcus neoforman* (*Steroform A, Steroform B, Steroform C, Steroform D*) and *Candida spp.* (*Candida lipolytica, Candida inconspicua, Candida krusei, Candida lusitaniae* and *Candida glabrata*) (Cuthbertson et al., 2006). Compared to other classes of penaeidins, penaeidin class 4 has shown a high level of potency against fungi (Cuthbertson et al., 2006). Additionally, the unusual amino acid composition of Pen4-1, especially in the proline-rich domain, may confer resistance to proteases (Cuthbertson et al., 2006). These results suggest that Pen4-1 is a good candidate for genetic engineering of enhanced disease resistance in plants. The present study investigates the feasibility of using the plant-optimized nucleotide sequences encoding Pen4-1 from *L. setiferus* for engineering fungal pathogen resistance into perennial turfgrass plants. We report the development of transgenic lines of a commercial creeping bentgrass (*Agrostis stolonifera* L.) cultivar, cv. Penn A-4 with enhanced resistance to two important fungal pathogens, *Sclerotinia homoeocarpa* and *Rhizoctonia solani* as a result of
expression of a synthetic peptide gene, *Pen4-1*.

**Results**

**Production and molecular characterization of transgenic creeping bentgrass plants harboring the *Pen4-1* gene**

To generate transgenic plants expressing *Pen4-1* and study the role *Pen4-1* plays in plant disease resistance, two chimeric DNA constructs were prepared containing either the coding sequence of a single peptide *Pen4-1* (Figure 2.1a) or the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein translationally fused to *Pen4-1* coding sequence (Figure 2.1b). A corn ubiquitin (*ubi*) promoter was used in both constructs to drive *Pen4-1* expression and an herbicide resistance conferring gene named *bar* driven by the CaMV 35S promoter was included as a selectable marker for plant transformation. The original nucleotide sequences of *Pen4-1* were modified for plant-optimized codon usage, and chemically synthesized for use in chimeric gene construction (Figure 2.2).
Figure 2.1 Generation and molecular analysis of the transgenic lines expressing Pen4-1. (a) Schematic diagram of the Pen4-1 expression chimeric gene construct, p35S-bar/Ubi-Pen4-1. Pen4-1 gene is under the control of the maize ubiquitin promoter (Ubi) and linked to the herbicide resistance gene, bar, driven by the CaMV 35S promoter. (b) Schematic diagram of the AP24::Pen4-1 expression chimeric gene construct, p35S-bar/Ubi-AP24::Pen4-1, in which the AP24::Pen4-1 fusion gene is under the control of the maize Ubi promoter. The CaMV35S promoter-driven bar gene is included for herbicide resistance. (c) Example of Southern blot analysis of Pen4-1 expression transgenics. Twenty micrograms of BamHI-digested genomic DNA was probed by a 440 bp ³²P-labelled bar gene fragment. Hybridization bands were indications of copy numbers of transgene insertion. Lanes 1-6 were DNAs from representative transgenic creeping bentgrass plants. WT (wild-type) was DNA from non-transformed creeping bentgrass plants. (d) Example of Northern blot analysis of Pen4-1 expression transgenics.
Lanes 1-6 were total RNA from representative transgenic creeping bentgrass plants. Twenty micrograms of the total RNA extracted from young leaves were probed with a $^{32}$P-labelled Pen4-1 gene fragment. WT (wild-type) was total RNA from non-transformed creeping bentgrass plants.

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**Figure 2.2** Nucleotide and deduced amino acid sequences of the Pen4-1 gene. The original nucleotide sequences of Pen4-1 were modified for plant-optimized codon usage. The predicted single-letter amino acids are shown above the coding sequence. The added translation stop codon is also indicated.

Using *Agrobacterium*-mediated transformation of embryogenic callus derived from mature seeds and phosphinothricin selection, we separately introduced the two chimeric gene constructs (Figure 2.1a, 2.1b) into a creeping bentgrass (*A. stolonifera* L.) cultivar, Penn A-4, producing a total of 25 independent T₀ transgenic lines transformed with the construct, p35S-bar/Ubi-Pen4-1, and 5 with the construct, p35S-bar/Ubi-AP24::Pen4-1. PCR amplification of foreign genes using genomic DNA from transgenic plants confirmed the presence of transgenes (data not shown). Southern hybridization with a bar-specific probe revealed that all the transgenic events contained one or two copies of transgene integration, most of which carried single copy insertions (see
example in Figure 2.1c). No significant difference in general plant morphology, root and shoot development as well as overall plant biomass was observed between transgenic and control plants without Pen4-1 gene.

**Pen4-1 expression in transgenic plants of creeping bentgrass**

Transgenic plants were further analyzed for Pen4-1 expression by Northern blot analysis. Hybridization of RNA samples from leaves revealed detectable Pen4-1 transcript, indicating transgene expression in all the transgenic plants (see examples in Figure 2d). Moreover, all transgenic lines, regardless of Pen4-1 alone or AP24::Pen4-1 fusion gene being expressed in plants, did not appear to show significant differences from each other for Pen4-1 mRNA accumulation (data not shown). Our efforts in detecting Pen4-1 protein in plant extracts from turfgrass transgenic lines using a polyclonal antibody raised against the selected region of Pen4-1 protein was unsuccessful (data not shown). This difficulty in detecting Pen4-1 protein in turfgrass plants was also encountered when analyzing Pen4-1 production in Arabidopsis transgenic lines expressing the Pen4-1 gene (data not shown). Many attempts in improving protein extraction and immunoblotting using currently available methodology and published procedures did not result in satisfactory results. This difficulty in Western assay with Pen4-1 may result from poor retention of the protein by blotting membranes due to its small size and a highly positive charge. Protease degradation of Pen4-1 during protein extraction could be another possibility, but is unlikely given the unusual amino acid composition of PRD Pen4-1 conferring resistance to proteases (Cuthbertson et al., 2006).
The same problem had been reported previously for other plant-expressed small AMPs (Li et al., 2001; Coca et al., 2004; Osusky et al., 2005; Rajasekaran et al., 2005).

Four representative transgenic lines harboring p35S-bar/Ubi-Pen4-1 and 5 containing the construct, p35S-bar/Ubi-AP24::Pen4-1 were used in the subsequent pathogen test experiments, all of which contained a single copy integration of the transgene. These transgenic lines were clonally multiplied by vegetative propagation. Evaluation of these plants under greenhouse conditions showed that they performed very similarly in growth. Three groups of control plants were used for comparison with the Pen4-1-expressing transgenic lines in our pathogen test experiments. They were untransformed plants either derived from seeds or regenerated from tissue culture and transgenic lines harboring the same expression vector without the Pen4-1, but with a different foreign gene, which was not associated with plant response to pathogen attack. All control plants, regardless of their origins, did not show significant differences in morphology and growth as well as response to pathogen infection.

In planta antifungal assays with R. solani

R. solani is a soil-borne fungus that causes brown patch disease, one of the most severe diseases on turfgrass lawns. To examine the impact of Pen4-1 on plant response to infection with R. solani, we conducted experiments investigating plant disease resistance by both in vitro and in vivo assays using detached leaves and whole plants, respectively.

The detached leaves from T₀ transgenic lines expressing Pen4-1 and the control plants were placed on 1% of agar in Petri dishes, and challenged by the pathogen using
agar plugs infested with mycelium of *R. solani* isolate obtained from infected creeping bentgrass plants. Disease symptoms measured as lesion size were documented at various times after inoculation. Compared to control plants, transgenic lines harboring either p35S-bar/Ubi-*Pen4-1* or p35S-bar/Ubi-AP24::*Pen4-1* exhibited dramatically enhanced disease resistance with a reduction in lesion length by 42% to 48% fourteen days after inoculation (Figure 2.3a, b). Statistical analysis of Tukey’s Hornesly Significant Difference (Tukey’s HSD) indicated that the lesion size reduction in *Pen4-1*-expressing transgenic plants was significant (*P*<0.01); whereas, no significant difference in lesion size was observed among *Pen4-1*-expressing transgenic lines (*P*>0.05) (Figure 2.3b).

**Figure 2.3** Response of transgenic creeping bentgrass plants expressing *Pen4-1* to *R. solani* infection - *in vitro* plant leaf inoculation assay. (a) The detached second expanded leaves from the top of plant stolons were used for pathogen inoculation test. The image shows example of representative leaves from all tested *Pen4-1*-expressing transgenic plants with a single transgene insertion (TG, on the right) and wild-type control plants (WT, on the left) 14 days post-inoculation (DPI). Transgenic plants exhibited significant resistance to *R. solani* in comparison to wild-type controls. (b) The development of brown patch disease was rated by measuring the lesion length of the infected leaves 2, 8 and 14 DPI. Statistical analysis of *R. solani* inoculation test was conducted on wild-type
control plants (WT) and various transgenic lines harboring either p35S-bar/Ubi-Pen4-1 (TG1 and TG2) or p35S-bar/Ubi-AP24::Pen4-1 (TG3 and TG4). Data are presented as means ± SE (n=10), and error bars represent standard errors. Asterisks (** or *) indicate a significant difference between Pen4-1-expressing transgenic and control plants at $P < 0.01$ or $P < 0.05$ by Tukey’s HSD test using JMP 9.0.0.

Plant performance in response to *R. solani* infection was further evaluated by *in vivo* essays using the whole plants grown in pots. Control plants without Pen4-1 and transgenic lines harboring either p35S-bar/Ubi-Pen4-1 or p35S-bar/Ubi-AP24::Pen4-1 were both challenged by the pathogen in replicated experiments under a controlled environment. Plants in each pot were inoculated with 3 grams of rye seeds colonized by *R. solani*. Pen4-1-expressing transgenic lines all exhibited high resistance against pathogen infection with a reduced lesion diameter by 30% - 43% compared to control plants 14 days after inoculation (Figure 2.4a, b and c). Statistical analysis of Wilcoxon test indicated that the disease symptoms among the different Pen4-1-expressing transgenic lines were not significant ($P>0.05$) (Figure 2.4c), whereas a significant difference in disease development between control and Pen4-1-expressing transgenic plants was observed ($P<0.01$) (Figure 2.4c).
Figure 2.4  Response of transgenic creeping bentgrass plants expressing Pen 4-1 to *R. solani* infection - *in vivo* direct plant inoculation bioassays with lower dose of *R. solani*.  
(a) The fully developed transgenic (independent events TG1 to TG4) and wild-type (WT) plants clonally propagated from individual tillers were grown and maintained in pots (15 cm x 10.5 cm) and inoculated with 3 g of rye seeds colonized by *R. solani*. The image on the upper panel shows plants before pathogen infection. Example of plants from wild-type (WT) and representative transgenic lines harboring either p35S-bar/Ubi-Pen4-1 (TG1, TG2) or p35S-bar/Ubi-AP24::Pen4-1 (TG3 and TG4) two weeks after pathogen inoculation (14 DPI) are shown on the bottom panel. Transgenic plants exhibited less severe disease symptom than wild-type controls. (b) A closer look of infected plants showing the different lesion size of WT and TG. (c) The development of brown patch disease was rated by measuring the lesion diameters of the infected leaves 14 DPI. Statistical analysis of *R. solani* inoculation test was conducted on WT and various TG lines. Data are presented as means ± SE (n=6), and error bars represent standard errors. Asterisks (**) or *) indicate a significant difference between transgenic plants and wild-type controls at $P < 0.01$ or $P < 0.05$ by Wilcoxon test using JMP 9.0.0.

When exposed to a second dose of pathogen infection, *i.e.* plants in each pot were inoculated with additional 3 grams of rye seeds colonized by *R. solani* 14 days after the first inoculation, the control plants suffered severe damage with 75% to 95% of them in
the pots being affected two weeks after inoculation, whereas Pen4-1-expressing transgenic lines were much less impacted with only around 25% of plants in the pots being infected (Figure 2.5a, b). The disease ratings of the Pen4-1-expressing transgenic lines were reduced by 41% to 44% compared to that of the control plants (Figure 2.5c). Statistical analysis of Wilcoxon test indicated that the disease development among the different Pen4-1-expressing transgenic lines was not significant (P>0.05) (Figure 2.5c).

Figure 2.5  Response of transgenic creeping bentgrass plants expressing Pen 4-1 to R. solani infection - in vivo direct plant inoculation bioassays with higher dose of R. solani. (a) Transgenic (TG) and wild-type (WT) plants were inoculated with a second dose of R. solani (3g of rye seeds colonized by the pathogen) 14 days after the first inoculation with 3 g of rye seeds colonized by R. solani. The image shows example of plants from wild-type (WT) and representative transgenic lines harboring either p35S-bar/Ubi-Pen4-1 (TG1) or p35S-bar/Ubi-AP24::Pen4-1 (TG3 and TG4) two weeks after the second pathogen inoculation. Transgenic plants exhibited much less severe disease symptom than wild-type controls. (b) A closer look of infected plants showing the different lesion size of WT and TG. (c) The development of brown patch disease was rated by visual estimation of the lesion percentage of the infected leaves 14 DPI using the Horsfall Barrett scale. Statistical analysis of R. solani inoculation test was conducted on WT and various TG lines. Data are presented as means ± SE (n=6), and error bars represent standard errors. Asterisks (**) or (*) indicate a significant difference between
transgenic plants and wild-type controls at $P < 0.01$ or $P < 0.05$ by Wilcoxon test JMP 9.0.0.

**In planta antifungal assays with *S. homoeocarpa***

Transgenic plants expressing Pen4-1 were also evaluated for their resistance to dollar spot, another important turfgrass disease caused by *S. homoeocarpa* (Couch, 1995; Walsh et al., 1999). Both *in vitro* and *in vivo* assays were conducted to examine the impact of Pen4-1 on plant response to infection with *S. homoeocarpa*.

*In vitro* assays were conducted using leaves from T₀ Pen4-1-expressing transgenic lines and control plants. The detached leaves were placed on 1% of agar in Petri dishes, and challenged by the pathogen using *S. homoeocarpa*-infested agar plugs. Disease symptoms measured as lesion size were documented at various times after inoculation. Compared to control plants, transgenic lines harboring either p35S-bar/Ubi-Pen4-1 or p35S-bar/Ubi-AP24::Pen4-1 all exhibited dramatically enhanced disease resistance with a reduction in lesion length by 40% to 47% seven days after inoculation (Figure 2.6a, b). Statistical analysis of Tukey’s HSD indicated that the lesion size reduction in Pen4-1-expressing transgenic lines was significant ($P<0.05$), whereas no significant difference in lesion size was observed among Pen 4-1-expressing transgenic lines ($P>0.05$) (Figure 2.6b).
Figure 2.6  Response of transgenic creeping bentgrass plants expressing Pen 4-1 to *S. homoeocarpa* infection - *in vitro* plant leaf inoculation assay. (a) The detached second expanded leaves from the top of plant stolons were used for pathogen inoculation test. The image shows example of representative leaves from all tested Pen 4-1-expressing transgenic plants with a single transgene insertion (TG, on the right) and wild-type control plants (WT, on the left) 7 days post-inoculation (DPI). Transgenic plants exhibited significant resistance to *S. homoeocarpa* in comparison to wild-type controls. (b) The development of dollar spot disease was rated by measuring the lesion length of the infected leaves 2, 4 and 7 DPI. Significant resistance to *S. homoeocarpa* by transgenic plants was observed 7 DPI when compared to wild-type controls. Statistical analysis of *S. homoeocarpa* inoculation test was conducted on wild-type control plants (WT) and various transgenic lines harboring either p35S-bar/Ubi-Pen4-1 (TG1 and TG2) or p35S-bar/Ubi-AP24::Pen4-1 (TG3 and TG4). Data are presented as means ± SE (n=10), and error bars represent standard error. Asterisks (**) or (*) indicate a significant difference between transgenic plants and wild-type controls at *P* < 0.01 or *P* < 0.05 by Tukey’s HSD test using JMP 9.0.0.

Plant performance in response to *S. homoeocarpa* infection was further evaluated by *in vivo* assays using the whole plants grown in big pots. Control plants and transgenics harboring either p35S-bar/Ubi-Pen4-1 or p35S-bar/Ubi-AP24::Pen4-1 were both challenged by the pathogen in replicated experiments under a controlled environment. Pen4-1-expressing transgenic lines all exhibited high resistance against pathogen infection with disease ratings reduced more than 50% compared to various control plants 9 days after inoculation (Figure 2.7a, b). Statistical analysis of the Wilcoxon test
indicated that disease development in all the Pen4-1-expressing transgenic lines was significantly delayed (Figure 2.7b) and in the recovery phase, transgenic lines performed much better than control plants ($P<0.05$). However, no significant difference in disease resistance among Pen4-1-expressing transgenic lines was observed ($P>0.05$) (Figure 2.7b).
Figure 2.7  Response of transgenic creeping bentgrass plants expressing Pen 4-1 to *S. homoeocarpa* infection - *in vivo* direct plant inoculation bioassays with higher dose of *S. homoeocarpa*. (a) The fully developed transgenic (TG) and wild-type (WT) plants clonally propagated from individual stolons were grown and maintained in pots (15 cm x 10.5 cm) and inoculated with 0.5 g of rye seeds colonized by *S. homoeocarpa*. The image shows example of plants from wild-type (WT) and representative transgenic lines harboring either p35S-bar/Ubi-Pen4-1 (TG1) or p35S-bar/Ubi-AP24::Pen4-1 (TG3) 9 days after pathogen inoculation (9 DPI). The plants in the front row are uninfected.
controls. Transgenic plants exhibited significant disease resistance compared to wild-type controls. (b) The development of dollar spot disease was rated by visual estimation of the lesion percentage of the infected leaves 3, 5, 7, 9 DPI, and 21 days post-recovery (DPR) using the Horsfall Barrett scale. Statistical analysis of S. homoeocarpa inoculation test was conducted on WT and various TG lines. Data are presented as means ± SE (n=6), and error bars represent standard error. Asterisks (** or *) indicate a significant difference between transgenic plants and wild-type controls at $P < 0.01$ or $P < 0.05$ by Wilcoxon test using JMP 9.0.0.

**Discussion**

The results reported herein show that Pen4-1, one of the penaeidin proteins isolated from Atlantic white shrimp (*Litopenaeus setiferus*), when expressed in transgenic perennial grass plants, confers antifungal traits. Transgenic creeping bentgrass plants expressing Pen4-1 exhibited significantly enhanced resistance to dollar spot and brown patch, the two major fungal diseases in turfgrass caused by *S. homoeocarpa* and *R. solani* respectively. To our knowledge, this is the first report of genetically engineering an economically and environmentally important perennial grass species with a gene encoding an AMP from the class four isoform of the shrimp penaeidin family for enhanced resistance against two fungal pathogens. There was only one recent study reporting the use of penaeidin protein for plant disease resistance (Wei et al., 2011). In that study, Np3 and Np5, the two AMPs belonging to class 3 and 5 of the penaeidin family from Chinese shrimp (*Fenneropenaeus chinensis*) (Kang et al., 2004; Kang et al., 2007) were engineered into rice and the four transgenic lines generated were reported to show enhanced resistance to bacterial blight (*Xanthomonas oryzae*).

In the present study, the *Pen4-1* gene with plant-preferred codon usage was
chemically synthesized for chimeric gene construction and plant transformation. The 30 transgenic turfgrass lines constitutively expressing either the Pen4-1 gene (25) or the AP24::Pen4-1 fusion gene (5) all contained one or two copies of the integrated transgene and were normal in morphology and development. Pen4-1 expression was confirmed at the transcription level (Figure 2.2d). In planta disease resistance assays to compare transgenic lines expressing Pen4-1 and control plants without Pen4-1 for their response to two important turfgrass fungal pathogens clearly demonstrated the effectiveness of this novel AMP in rendering transgenic plants with significantly enhanced resistance to both brown patch and dollar spot diseases. It is unlikely that the observed results would be attributed to disrupted genes or regulatory sequences at the transgene integration site(s) since Pen4-1-expressing transgenic lines from independent transformation events all show similar phenotypes and confer increased resistance to fungal pathogens, whereas transgenic control plants that contain the same expression vector, but without Pen4-1, do not exhibit enhanced performance when subjected to pathogen infection. It also should be noted that in the current research, T₀ transgenic plants were clonally propagated and used for pathogenicity assays as previously reported in other studies on perennial grasses (Qu et al.). Our earlier work studying transgene expression and transmission using the selectable marker, herbicide resistance conferring gene bar in creeping bentgrass had demonstrated that Agrobacterium-mediated transformation of creeping bentgrass led to a high frequency of a single-copy transgene insertion that exhibited stable inheritance patterns. The inheritance and stability of transgene were demonstrated in both greenhouse and field conditions (Luo et al., 2004a; Luo et al., 2004b; Luo et al., 2005; Li et al. 2010).
Currently, we are also conducting experiments studying stable transmission of Pen4-1 into next generations and inheritance of the enhanced disease resistance trait by the progeny of the primary transgenic plants. Data from this research will provide further support facilitating large-scale application of Pen4-1 in turf species for plant protection.

Since Pen4-1 originates from shrimp, the successful use of this protein in agricultural biotechnology requires that it be efficiently produced when Pen4-1 gene is introduced into the plant host genome, and that its biological activity maintained when produced in transgenic plants. Although the antifungal activity of Pen4-1 has been demonstrated by *in vitro* test of the synthesized protein (Cuthbertson et al., 2004; Cuthbertson et al., 2005; Cuthbertson et al., 2006), it remains to be determined whether or not high-level gene expression, efficient protein production, and correct folding or processing of this protein could be achieved *in planta*. We therefore modified the coding sequence of Pen4-1 for monocot plant-preferred codon usage. The same strategy has been used previously when introducing other non-plant-derived AMP genes in plants (Coca et al., 2004). In our study, a RNA transcript of the codon-optimized Pen4-1 gene was detected in all the transgenic lines. Although attempts in detecting protein products of the Pen4-1 in transgenic lines were unsuccessful, transgenic plants all displayed significantly enhanced resistance to the two major turfgrass fungal diseases compared to wild-type controls indicating that Pen4-1 was successfully produced in transformed creeping bentgrass plants. It should be noted that native AMP genes have also been reported to be expressed and function in other plant systems. For example, when introducing *Aspergillus giganteus* antifungal protein AFP into rice plants, the translational efficiencies
of transcripts originating from the native and codon-optimized \textit{AFP} gene appeared similar in transgenic plants (Coca et al., 2004). Similarly, the introduction of \textit{np3} and \textit{np5}, two other AMP genes from Chinese shrimp in their native forms into rice led to enhanced plant resistance to bacterial blight. This suggested the production of active AMPs in transgenic plants although the presence of the protein products in transgenic plants was not demonstrated (Wei et al., 2011). Further transgenic studies to compare the original and codon-optimized forms of \textit{Pen4-1} gene for their protein translation efficiencies would facilitate its use in other plant systems to achieve enhanced disease resistance.

All penaeidins possess a unique two-domain structure including an unconstrained proline-rich N-terminal domain, PRD and a disulfide bond-stabilized cysteine-rich domain, CRD (Cuthbertson et al., 2005). To ensure efficient disulfide bond formation of the Pen4-1 produced in transgenic creeping bentgrass plants, we prepared a chimeric gene encoding a fusion protein in which the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein was translationally fused to the Pen4-1 coding sequence. Transit signal peptides, such as the one from AP24, are known to be capable of directing proteins into the endoplasmic reticulum, facilitating the formation of disulfide bonds (Coca et al., 2004). Therefore, the AP24::Pen4-1 fusion protein produced in plant cells should lead to mature Pen4-1 that is more likely to be correctly folded than the Pen4-1 protein alone produced in transgenic plants. However, in the present study, we did not observe dramatic differences in enhanced plant resistance to the two turfgrass fungal pathogens between transgenic turfgrass lines expressing \textit{AP24::Pen4-1} fusion gene and those expressing \textit{Pen4-1} gene alone. One possible explanation could be that a
minimal protein activity was enough to inhibit pathogen infection; therefore, the methods for pathogenicity assays used in the present study could not detect the real difference in protein activities between the Pen4-1 alone and the AP24::Pen4-1 fusion protein expressed in transgenic plants. Another possibility would be that although the mechanism of protein secretion is highly conserved through the living world (von Heijne and Abrahams n, 1989), signal peptides from one organism do not always function efficiently when expressed in another organism (Smith et al., 1985; Chang et al., 1987; von Heijne and Abrahams n, 1989). The correct choice of the signal peptide would have a great effect on the production of the AMPs.

The multi-domain structure and the feature of protease resistance of this peptide may also play important role in bestowing more flexibility to protein processing and determining protein activities (Cuthbertson et al., 2006). In most cases the presence of both the CRD and the PRD are important to confer the maximal antimicrobial activity. However, it has been demonstrated that the single Pen4 PRD alone exhibited a similar level of antimicrobial activity to that of the full-length Pen4 (Cuthbertson et al., 2004; Cuthbertson et al., 2005). This implies that the disulfide bond formation in Pen4 may not play a critical role in its antimicrobial ability. Therefore, specific targeting of Pen4-1 to endoplasmic reticulum by the AP24 signal peptide did not seem to result in enhanced protein activity. It is also possible that pathogen attack would lead to disruption of the plant cells, releasing the peptides, which could then be oxidized in the extracellular space to form the disulfide bond. Further studies comparing Pen4 PRD alone and the full-length Pen4 in transgenic plants for their antimicrobial activities would help better understand
the role disulfide bonds play in determining the overall activity of Pen4 proteins.

The *in vitro* tests of Pen4-1 have revealed its resistance to a wide range of phytopathogens, of which many infect plant species including rice, wheat, wine grapes, strawberry and other crop plants (Cuthbertson et al., 2006). The current study with creeping bentgrass as a target species provides the very first example of using Pen4-1 for genetic engineering of enhanced disease resistance in transgenic crop plants, pointing to the great potential of implementing similar strategies in other plant systems, especially in food crops for improvement of plant biotic stress resistance.

**Materials and Methods**

**Synthesis of Pen4-1 gene**

The full sequence of *Pen4-1* gene was obtained from PenBase (Gueguen et al., 2006). The original nucleotide sequences of *Pen4-1* encoding the mature Pen4-1 protein (47 amino acids) were modified for plant-optimized codon usage. A stop codon (TAG) was added to the 3’ end of the coding sequence (Figure 2.1). The modified full sequence of *Pen4-1* was chemically synthesized by Integrated DNA Technology (Coralville, IA, USA), cloned in pZErO-2 (Invitrogen, Carlsbad, CA, USA) and verified by sequencing.

**Construction of plant expression vectors**

To generate transgenic plants expressing Pen4-1 and study the role Pen4-1 plays in plant disease resistance, two chimeric DNA constructs were prepared containing either
the coding sequence of a single peptide Pen4-1 (Figure 2.2a) or the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein translationally fused to Pen4-1 coding sequence (Figure 2.2b). Both constructs were prepared using a pSB11-based Agrobacterium binary vector that contains a selectable marker gene conferring antibiotic spectinomycin resistance for bacterial transformation (Komari et al., 1996).

The two plant expression vectors, p35S-bar/Ubi-Pen4-1, and p35S-bar/Ubi-AP24::Pen4-1 constructed in this work are presented in Figures 2.2a and b. Plasmid p35S-bar/Ubi-Pen4-1 (Figure 2.2a) contained only the single peptide sequence of the codon-optimized Pen4-1 gene, whereas plasmid p35S-bar/Ubi-AP24::Pen4-1 (Figure 2.2b) contained a chimeric Pen4-1 gene with the DNA sequence coding for the transit signal peptide of the secreted tobacco AP24 protein (Melchers et al., 1993) being translationally fused to Pen4-1 coding sequence. For their expression in turfgrass, both Pen4-1 and AP24::Pen4-1 were cloned between the maize ubi promoter and the nos terminator. An herbicide resistance conferring gene named bar driven by the CaMV 35S promoter was included in both plasmids as selectable marker for plant transformation.

To prepare p35S-bar/Ubi-Pen4-1, the synthesized Pen4-1 coding sequence (with added stop codon) was PCR amplified from pZEro-2:Pen4-1 by primers Pen4-ATG: 5’-CGCGGATCCATGCACTCTCCGGCTACACC-3’ (a BamHI restriction site and a start codon, ATG added in the 5’ end were underlined and in italic respectively) and Pen4R: 5’-CGCGCATGCGAGCTCTAGAGGTGGCAGCAGTCG-3’ (an SphI and an SacI restriction sites added in the 5’ end were underlined). The amplified fragment was
digested with *Bam*HI and *Sac*I enzymes and ligated into the corresponding sites of p35S-
*bar*/Ubi-*GUS* (Luo, unpublished results) to replace the *gusA* coding sequence. To prepare
p35S-*bar*/Ubi-*AP24::Pen4-1* construct, the PCR amplified fragment of *Pen4-1* using
*Pen4F* (5’-CACTCCTCCGGCTACACC-3’) and *Pen4R* primers was treated with DNA
polymerase I, large (Klenow) fragment (New England Biolabs, Beverly, MA, USA) in
the absence of dNTP, then digested with *Sph*I and ligated into the *Nco*I (blunt-ended with
Klenow in the presence of dNTP)-*Sph*I sites of the plasmid pGEM-T-*AP24* (Coca et al.,
2004), resulting in pGEM-T-*AP24::Pen4-1* (data not shown). Upon verification of the
correct sequence of the amplified *Pen4-1* and its in-frame fusion to the *AP24* signal
sequence, the *AP24::Pen4-1* chimeric gene was released from pGEM-T-*AP24::Pen4-1*
by *Bam*HI and *Sac*I digestions and ligated into the corresponding sites of p35S-*bar*/Ubi-
*GUS* to replace the *gusA* coding sequence. The two constructs were transformed into
*Agrobacterium tumefaciens* strain LBA4404 by electroporation for subsequent plant
transformation.

**Production, propagation and maintenance of transgenic turfgrass plants**

A commercial genotype of creeping bentgrass (*A. stolonifera* L.) Penn A-4 was
used for plant transformation. Transgenic creeping bentgrass lines harboring either p35S-
*bar*/Ubi-*Pen4-1* or p35S-*bar*/Ubi-*AP24::Pen4-1* were produced by *Agrobacterium-
mediated transformation of embryonic callus initiated from mature seeds essentially as
previously described (Luo et al., 2004a). General procedures of plant propagation and
maintenance were followed by a previous protocol (Li et al. 2010). Transgenic plants
were grown in commercial potting mixture soil (Fafard 3-B Mix, Fafard Inc., Anderson, SC, USA) and maintained in the greenhouse under a 16-hour photoperiod with supplementary lighting at 27°C in the light and 25°C in the dark. Plants from individual transformation events were clonally propagated from tillers and grown in pots (15 cm x 10.5 cm, Dillen Products, Middlefield, OH, USA) using commercial potting mixture soil. Propagated plants were maintained in greenhouse for 4 to 6 months with regular fertilization, mowing and irrigation, and used for further analysis.

**Plant DNA isolation and southern blot analysis**

Plant genomic DNA was extracted as previously described using the cetyltrimethyl ammonium bromide (CTAB) method (Luo et al., 2005; Li et al., 2010). After digestion of the DNA with *Bam*HI according to supplier’s instruction (New England Biolabs, Beverly, MA, USA), DNAs were electrophoresed on 0.8% agarose gels, transferred onto nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and hybridized to $^{32}$P-labelled DNA probes of *bar*. Hybridization was carried out in modified Church and Gilbert buffer at 65°C following the standard protocol (Sambrook et al., 1989). Hybridizing fragments were detected by exposure of the membrane on a phosphor screen at room temperature overnight, and scanning on a Typhoon 9400 phosphorimager.

**RNA isolation and northern blot analysis**

Total RNA was isolated from the leaves of transgenic and wild-type control plants
using Trizol reagent (Invitrogen). RNAs were subjected to formaldehyde-containing agarose gel electrophoresis, and transferred onto Hybond-N+ filters (GE Healthcare Bio-Sciences Corp.). The DNA fragment coding for the Pen4-1 gene was used as probe. Hybridization and membrane wash were performed following the standard protocol (Sambrook et al., 1989; Li et al., 2010).

**Western blot Analysis**

Total proteins were extracted from leaves following two different procedures. The first was essentially as described by Fu *et al.* (Fu *et al.*, 2007b). Two hundred mg of leaf tissue was ground, then suspended in 500 μl of protein extraction buffer [1× PBS (pH 7.4), 10 mM EDTA, 1 mM PMSF, 6 μl protease inhibitor cocktail for plant cell and tissue extracts (sigma), 1% (v/v) β-mercaptoethanol, and 0.1% (v/v) Triton X-100]. The second protocol was as described by Coca *et al.* (Coca *et al.*, 2004). Four hundred mg of plant material was ground in liquid nitrogen and homogenized in SDS PAGE loading buffer without 2-mercaptoethanol and incubated at 95°C for 10 min. After centrifugation, the supernatant was precipitated with 4 volumes of acetone at -20°C for 30 min. Proteins were pelleted, dried and dissolved in SDS-PAGE loading buffer containing 5% 2-mercaptoethanol. For protein analysis, 30 μg of protein sample was loaded onto a 16% Tricine SDS-PAGE gel. SDS-PAGE was performed as described previously (Schägger, 2006). For western blot, protein was transferred from the SDS-PAGE gel onto Protran BA76 Nitrocellulose media (Whatman Inc., Piscataway, NJ, USA) using an electrophoresis blotting system (Bio-Rad, Hercules, CA, USA). The Protein transfer
efficiency was verified by using Poceau S, and incubated with 5% carnation nonfat dry milk in TBST overnight. The blots were then probed with anti-Pen4-1 antibody developed by YenZym Antibodies, LLC (Burlingame, CA, USA), using the peptide HSSGYTRPLRKPSRC, followed by adding the HRP-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and incubation for 1 hour at room temperature with shaking. The signals were detected by incubation of membrane for 30 minutes at room temperature in the substrate working solution (4-Chloro-1-naphthol). After stopping the reaction by rinsing the membrane with water, the membrane was photographed immediately.

**In vitro plant leaf inoculation with R. solani and S. homoeocarpa**

Transgenic plants were challenged with *R. solani* and *S. homoeocarpa*, which respectively cause brown patch and dollar spot, the two most common fungal diseases in creeping bentgrass. Following procedures modified from the previous reports (Osusky et al., 2000; Dong et al., 2007; Dong et al., 2008; Williams et al., 2011), we grew the *R. solani* and *S. homoeocarpa* cultures on potato dextrose agar at 25°C for 3 days prior to inoculation of detached leaves under aseptic conditions. The second expanded leaves from the top of plant stolons were used for inoculation. Ten leaves from each of the transgenic lines and wild-type control plants were randomly chosen for study. The leaves cut from plants were first washed with 70% ethanol, and then rinsed with sterilized water. The leaves were put on 1% of agar in Petri dishes (150 x 15 mm). An agar plug (d=3mm) infested with mycelium of *R. solani* or *S. homoeocarpa* was placed on the bottom of the
midrib of each detached leaf for inoculation. The Petri dishes were put in a lighted growth chamber under a 14/10 h (day/night) photoperiod. Temperature and relative humidity (RH) in the growth chamber were 28°C and 70%. The development of brown patch disease was rated by measuring the lesion length of the infected leaves 2 days, 8 days and 14 days post-inoculation. The development of dollar spot disease was rated by measuring the lesion length on the infected leaves 2 days, 4 days and 7 days post-inoculation. The experiment was repeated three times.

In vivo direct plant inoculation with S. homoeocarpa and R. solani

The preparation of the S. homoeocarpa and R. solani cultures and the in vivo plant inoculation with pathogens were conducted based on the previously reported procedures (Liu et al., 1998; Chai et al., 2002; Wang et al., 2003b). Selected Pen4-1-expressing transgenic lines based on molecular analysis were evaluated for resistance to the infection of the two fungal pathogens in comparison to control plants that did not contain Pen4-1. The grasses were mowed prior to inoculation. The plants in each pot were then inoculated with pathogens by applying, in the center of the pot, approximately 0.5 g of rye seeds colonized by S. homoeocarpa or 3 and 6 g of rye seeds colonized by R. solani.

Plants inoculated with S. homoeocarpa (0.5 g of colonized inoculum) were placed in plastic trays containing 4 cm of distilled water, lightly misted with distilled water at 48 h intervals to maintain relative 100% humidity. The plastic trays were placed inside a greenhouse set to maintain a diurnal cycle of 14 h light and 10 h dark. Three to four replicates of each transgenic line or wild type control were used for evaluation. Disease
severity was visually estimated at 3, 5, 7 and 9 days post-inoculation using the Horsfall Barrett scale (Horsfall and Barratt, 1945). Nine days later, the plants were moved to a growth room from the greenhouse to recover for three weeks. Temperatures in the growth room were maintained at 22°C in the light and 17°C in the dark. The inoculation experiment was repeated three times.

Plants inoculated with *R. solani* (3g rye grass seeds colonized by the pathogen) were placed in plastic trays containing 4 cm of distilled water, lightly misted with distilled water at 48 h intervals to maintain humidity. The trays were placed inside a growth chamber to maintain a diurnal cycle of 14 h light and 10 h dark. The temperature and RH were 30°C and 70% during day time, and 24°C and 95% at night. After 14 days, disease severity was either rated by measuring the total distance from the point of inoculation to the farthest point of the lesions extended, or visually estimated using the Horsfall Barrett scale (Horsfall and Barratt, 1945). The inoculation experiment was repeated twice.

**Statistical analysis**

Both *in vitro* plant leaf inoculation and *in vivo* direct plant inoculation tests were conducted using a randomized complete block design. Data were analyzed using JMP® 9.0.0 (2010 SAS Institute Inc). For the data generated using the Horsfall-Barratt scale, a nonparametric test, Wilcoxon test at *P*=0.01 and *P*=0.05, was used to compare the medians. In the case of the data generated on a continuous scale such as lesion length, Tukey’s HSD at *P*=0.01 and *P*=0.05 was used to test for differences in mean disease
severity.

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CHAPTER 3 - CONSTITUTIVE EXPRESSION OF A MIR319 GENE ALTERS PLANT DEVELOPMENT AND INCREASES SALT AND DROUGHT TOLERANCE IN TRANSGENIC CREEPING BENTGRASS (AGROSTIS STOLONIFERA L.)

Keywords: abiotic stress, microRNAs, perennial grasses, transgenic creeping bentgrass, turfgrass,

Abstract

The miR319 family is one of the first characterized and conserved miRNA families in plants and it has been demonstrated to target TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) genes encoding plant-specific transcription factors known to be largely involved in leaf development. We have investigated the role miR319 plays in plant development and plant response to abiotic stress in perennial grass species. A full length cDNA of the rice miR319 gene, Osa-miR319a was introduced into creeping bentgrass (Agrostis stolonifera L.). Transgenic plants overexpressing the Osa-miR319a exhibited morphological changes, including significantly decreased tiller numbers, wider and thicker leaves, coarser stems, increased weight:area ratio and more total wax coverage. Overexpression of miR319 also led to enhanced drought and salt tolerance in transgenics, which might be attributed to the increased weight:area ratio and total wax coverage as well as less sodium uptake. Gene expression analysis in both wild-type and transgenic plants indicated that at least four putative miR319 target genes in turfgrass AsPCF5, AsPCF6, AsPCF8 and AsTCP14 were down-regulated in transgenic plants. And a homologue of rice NAC domain gene
AsNAC60 was also shown to be down-regulated in transgenics. Our results reveal the importance of miR319 in regulating plant resistance to environmental adversities and indicate that the enhanced abiotic stress tolerance in transgenic plants might be related to the significant down-regulation of the putative target genes of miR319. This may lead to development of novel molecular strategies to genetically engineer crop species for enhanced performance under unfavorable environmental conditions, contributing to agriculture production.

Introduction

Plant responses to drought and salt stresses have been studied extensively in understanding the physiological and molecular mechanisms underlying plant adaptation to these drought and salt stress (reviewed by Munns, 2002; Wang et al., 2003a; Chaves et al., 2009). At the physiological level, plant responses to salinity stress are generally divided into two phases: a rapid, osmotic phase inhibiting shoot growth and a slower, ionic phase accelerating senescence of mature leaves (Munns and Tester, 2008). Plant responses to drought share a lot of similarities, especially in the first phase. Plants subjected to drought and salinity both experience a physiological water deficit which alters photosynthesis, cell growth and induces osmotic adjustment to maintain current water uptake and cell turgor (Chaves et al., 2009). However, under salinity stress, plants endure salt-specific effects with very high Na⁺ or Cl⁻ concentrations within cells leading
to toxicity. To tolerate salt-specific effects, plants either minimize the uptake of salt or compartmentalize salt in the vacuoles (Munns, 2005). The current advances is plant biotechnologies for enhanced plant tolerance to drought (Garg et al., 2002; Capell et al., 2004; Chandra Babu et al., 2004; Lian et al., 2004; Oh et al., 2005; Wang et al., 2005a; Hu et al., 2006; Fu et al., 2007a) and salinity (Gaxiola et al., 2001; Rus et al., 2001; White and Broadley, 2001; Zhang and Blumwald, 2001; Zhang et al., 2001; Laurie et al., 2002; Shi et al., 2002; Flowers, 2004; Rus et al., 2004; Wu et al., 2004; Møller et al., 2009; Li et al. 2010) mainly concentrate on manipulating downstream genes which function in the physiological responses discussed above (e.g. osmotic or ionic adjustment).

In recent years, the upstream regulatory networks of plant drought and salt stress response including transcription factors, hormones, protein kinases, protein phosphatases, and other signaling molecules such as calmodulin binding proteins have gradually been uncovered (Zhang et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima et al., 2009). However, the fine details of these regulatory networks are still largely unknown and questions about how the different regulatory elements function together and how they are coordinated by master regulators posttranscriptionally or posttranslationally remain to be addressed. The discovery of plant miRNAs largely involved in abiotic stress response (Phillips et al., 2007; Sunkar et al., 2007; Lu and Huang, 2008; Mazzucotelli et al., 2008; Shukla et al., 2008; Lewis et al., 2009; Nakashima et al., 2009) shed light on these questions. It has been revealed that miRNAs mediate plant abiotic stress response through regulating their target genes, the majority of which are transcription factors.
constituting complicated regulatory network (65% of the confirmed or confidently predicted targets of miRNAs are transcription factors), serving as key players in the gene regulation networks (Jones-Rhoades et al., 2006).

Plant miRNAs are ~20 to 24 nt non-coding RNAs that specifically base pair to and induce the slicing of target mRNAs or cause translational repression (Zhang et al., 2006; Shukla et al., 2008). They have diverse roles in plant development such as phase transition (e.g. from vegetative growth to reproductive phase), leaf morphogenesis, floral organ identity, developmental timing and other aspects of plant development (Lu and Huang, 2008; Rubio-Somoza and Weigel, 2011). In recent years, a large amount of data have been generated by genome-wide high-throughput sequencing and microarray profiling to identify stress-responsive miRNAs (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Zhao et al., 2007; Ding et al., 2009; Yang et al., 2010; Wang et al., 2011), of which miR319 was identified to respond multiple stresses. For example, they are up-regulated by dehydration, salt and cold stress in Arabidopsis (Sunkar and Zhu, 2004; Liu et al., 2008), and by cold stress in sugar cane (Thiebaut et al., 2011), whereas a complicated behavior in expression was observed in rice depending on developmental states (e.g. Osa-miR319 was only identified as up-regulation on tillering stage 12 days after water withholding) (Zhou et al., 2010a). As one of the first experimentally characterized and most conserved miRNA families (Axtell and Bowman, 2008), the miR319 targets, TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) genes encode plant-specific transcription factors sharing a conserved TCP domain region with a basic helix-loop-helix structure (bHLH). The TCP family is known to be largely involved in plant
development such as control of cell proliferation in leaf morphogenesis (Palatnik et al., 2003; Ori et al., 2007; Nag et al., 2009).

Although miR319-mediated changes in plant morphology have been well studied in dicots, there have been no published reports studying monocots especially perennial grass species. As is known, the developmental regulation between monocots and dicots is different (Nelson and Langdale, 1989; Tsiantis et al., 1999; Rudall and Buzgo, 2002; Scarpella and Meijer, 2004). It would be interesting to know how miR319 functions to impact plant development in monocot species. Moreover, although the involvement of miR319 in plant response to drought and salinity stress has been suggested based on microarray data, experimental proof in support of the possible contribution of the miR319 family and the underlying molecular mechanisms are still lacking. In this study, transgenic plants of creeping bentgrass (Agrostis stolonifera L.) overexpressing a rice miR319 gene were generated to investigate the roles miR319 play in controlling plant development and plant response to environmental stress, i.e., (1) what impact the miR319 gene family may have in plant development in perennial grasses? (2) Is miR319 involved in plant abiotic stress response in perennial grasses? (3) What is the molecular mechanism of miR319-mediated plant tolerance to abiotic stress in perennial grasses?

Results

Production and molecular characterization of transgenic creeping bentgrass plants expressing the rice miR319 gene, Osa-miR319
To study the potential of manipulating miR319 expression in perennial species for enhancing plant resistance to environmental stresses, we prepared a chimeric DNA construct containing one of the rice miR319 genes, *Osa-miR319a* under the control of the 35S promoter (Figure 3.1a). Using *Agrobacterium*-mediated transformation of embryogenic callus derived from mature seeds, we introduced the chimeric gene construct, p35S-hyg/p35S-*Osa-miR319a* into the creeping bentgrass cultivar, Penn A-4 to produce a total of 15 independent transgenic (TG) lines ectopically expressing *Osa-miR319a*. PCR amplification of foreign genes using genomic DNA from all transformants confirmed the presence of transgenes (Figure 3.1b). RT-PCR analysis revealed the presence of the rice primary miR319a transcripts in all transgenic lines (Figure 3.1c). Under the higher stringency of PCR conditions (30 cycles with the annealing temperature of 65°C), the high level of mature transcripts of miR319 could only be detected by stem-loop RT-PCR analysis in the transgenics which accumulated in high quantities, suggesting that the rice miR319 was successfully expressed in turfgrass plants and properly processed into mature microRNAs (Figure 3.1d). However, the detection of mature miR319 transcripts in wild type control plants under a lower stringency of PCR conditions suggested that the transcripts of the turf endogenous miR319 was also amplified and shared conservation of mature sequence with rice miR319 (Figure 3.1d).
Figure 3.1  Generation and molecular analysis of the transgenic lines expressing Osa-miR319a. (a) Schematic diagram of the Osa-miR319a overexpression gene construct, p35S-hyg/p35S-Osa-miR319a, in which the Osa-miR319a gene is under the control of CaMV35S promoter. The CaMV35S promoter-driven hyg gene is included for hygromycin resistance. (b) Example of PCR analysis of hyg gene in wild-type (WT) and transgenic (TG) plants to detect transgene insertion into the host genome. (c) Example of RT-PCR analysis of the primary Osa-miR319a transcripts in transgenics. Primary Osa-miR319a transcripts were detected in transgenic plants. (d) Example of stem-loop RT analysis of the mature Osa-miR319a in WT and TG plants.

Overexpression of miR319 causes pleiotropic phenotypes in transgenic creeping bentgrass plants

Analysis of transgenic creeping bentgrass plants constitutively expressing Osa-miR319a showed that compared to control plants without Osa-miR319a, Osa-miR319a plants displayed wider leaves and larger stems (Figure 3.2a, b). Microscopic analysis of
the plant leaf samples indicated that leaf expansion (blade width and vein number) in Osa-miR319a plants was significantly greater than that in control plants (Figure 3.2c, d, e). Moreover, compared to control plants without Osa-miR319a, Osa-miR319a plants also exhibited thicker leaves (Figure 3.2f, g), which might be associated with increased mesophyll cell layers (Figure 3.2f). In stems, overexpression of miR319 led to increased stem diameter in transgenic plants as shown in the Figure 3.2h and 3.2i. Leaf and stem size is dependent on both the number and the size of cells in the organ. To determine whether enlargement of Osa-miR319a plant leaves and stems was associated with increased cell number, cell size or a combination of these two parameters, we compared control and Osa-miR319a plants when they were fully developed after mowing. The size of the cells in both leaves and stems was not significantly different between control and Osa-miR319a plants (data not shown, Figure 3.2c, f, h) indicating that the wide-leaf and large-stem phenotypes of the Osa-miR319a transgenic plants are most likely due to an
Figure 3.2  Morphology change in transgenic plants overexpressing *Osa-miR319a*. (a) The TG plants exhibited wider leaves than WT controls. (b) A closer look at the *Osa-miR319a* TG plants and WT controls. The image shows that the representative TG plant leaf is wider (left) and the stem is bigger (right). (c) Leaf sectioning images of WT and TG plants. (d) Statistic analysis of leaf blade width in WT and TG plants. (e) Statistical analysis of total vein number in WT and TG plants. (f) Examples of representative sectioning images of WT and TG plant leaves. (g) Statistical analysis of leaf thickness in WT and TG plants. (h) Examples of representative sectioning images of WT and TG plant stems. (i) Statistical analysis of stem diameter in WT and TG plants. Statistical analysis of leaf blade width, total vein number, leaf thickness and stem diameter was conducted on WT control plants and various transgenic lines. Data are presented as means ± SE, and error bars represent standard error. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s *t*-test using JMP 9.0.0

increase in cell proliferation, but not the expansion of individual cells. This morphology change in turfgrass quite different from that was observed in dicot species in which overexpression of *miR319* lead to crinkled and more serrated leaves resulting from excessive cell proliferation in the absence of TCP activity (Palatnik et al., 2003; Ori et al., 2007).

To elucidate any potential impact of constitutively expressed miR319 on other aspects of plant development, we grew plants starting from a single tiller and studied plant growth and development by monitoring changes in plant tiller numbers at different stages and measuring shoot biomass as represented by dry weight in control and *Osa-miR319a* plants. As shown in Figure 3.3a and 3.3b, *Osa-miR319a* transgenics exhibited dramatically reduced tillers compared to control plants. However, no significant difference in shoot biomass between control and *Osa-miR319a* plants was observed (Figure 3.3c) suggesting that increased leaf expansion in *Osa-miR319a* plants might compensate for the loss in biomass caused by decreased tillering. In contrast, the root
biomass of the Osa-miR319a plants was significantly lower than that of the controls (Figure 3.3a, d).

Figure 3.3  Tillering and plant development under normal growth conditions. Both WT and TG plants initiated from a single tiller. (a) Tillering, shoot and root development in WT and TG plants 90 days after initiation from a single tiller of the same size. The image shows that TG plants have fewer tillers and less root growth than WT controls. (b) Comparison of tiller numbers in WT and TG plants 30 days, 60 days and 90 days after initiation from a single of the same size. (c) Biomass of WT and TG shoots 90 days after initiation from a single tiller of the same size. (d) Biomass of WT and TG roots 90 days after initiation from a single of the same size. Statistical analysis of tiller number, shoot and root biomass was conducted on WT control plants and various transgenic lines. Data are presented as means ± SE, and error bars represent standard errors. Asterisks * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.
Overexpression of miR319 results in enhanced salt tolerance in transgenic creeping bentgrass plants

Involvement of many microRNAs in plant resistance to abiotic stress, such as drought and salinity has been suggested by their up- or down-regulation in the presence of various environmental stimuli (Sunkar and Zhu, 2004; Liu et al., 2008; Zhou et al., 2010a; Thiebaut et al., 2011). However, direct evidence about the role miR319 plays in plant response to environmental stress is still lacking. To investigate this, we first examined control and transgenic plants constitutively expressing Osa-miR319a under salinity stress. We treated both the control and Osa-miR319a plants with NaCl daily by applying nutrient solution supplemented with 200 mM NaCl. As shown in Figure 3.4a and 3.4b, the growth of the control plants without Osa-miR319a was severely hampered and serious tissue damage was observed 16 days after salt treatment whereas the Osa-miR319a plants were much less affected and grew better. To reveal how plants recover from the release of salinity stress, the salt-treated control and Osa-miR319a plants were both subjected to regular maintenance. The Osa-miR319a plants survived the treatment and the majority of the tillers recovered from salt-elicited damage, whereas only a few tillers in control plants stayed alive (Figure 3.4b).

Osa-miR319a transgenics retain more water and exhibited less cell membrane damage than control plants without Osa-miR319a under salt stress

To further elucidate physiological mechanism of enhanced salt tolerance in Osa-miR319a plants, we investigated water status of these transgenic lines in comparison to
control plants without Osa-miR319a. As exemplified in Figure 3.4c showing data from two of the representative transgenic lines, there was no significant difference in leaf relative water content between control and Osa-miR319a plants under normal growth conditions. When exposed to various concentrations of NaCl, the leaf relative water content in both control and Osa-miR319a plants declined. However, this decline was more significant in control plants than in Osa-miR319a transgenics, especially when high salinity stress (300 mM of NaCl) was applied. Consequently, the leaf relative water content in Osa-miR319a transgenics was significantly higher than that in control plants under salinity stress, indicating greater water retention capacity the Osa-miR319a transgenics than that in control plants without Osa-miR319a gene.

Next, we examined cell membrane integrity in both the Osa-miR319a transgenic and control plants. To do this, we measured the leaf cell electrolyte leakage (EL) in plants grown under normal and salinity conditions. Both control and Osa-miR319a plants exhibited low levels of cell EL, which were not significantly different from each other under normal growth conditions (Figure 3.4d). In contrast, the leaf EL significantly increased in both control and the Osa-miR319a plants upon exposure to various concentrations of NaCl. The higher the concentration of the salt used for plant treatment was, the more the leaf cell EL was (Figure 3.4d). However, the increase of leaf cell EL was significantly more pronounced in control plants than in the Osa-miR319a transgenics (Figure 3.4d), indicating that the control plants without Osa-miR319a are more prone to salt-elicited cell membrane damage than the Osa-miR319a plants.
Figure 3.4  Response of WT and TG plants to salt stress. (a) The fully developed WT and TG plants clonally propagated from individual tillers were grown in sand and subjected to 200 mM NaCl treatment. The images show differences in damages elicited by salinity in WT and TG plants 16 days after treatment. (b) Performance of WT and TG plants 12 days after recovering from a 16-day salt treatment. (c) Relative water content (RWC) of WT and TG plants under salt stress. Fully developed WT and TG plants were watered daily with 200 ppm 20-10-20 (N-P-K) fertilizer supplemented with 0, 200 and 300 mM NaCl, as indicated. Leaf tissues were carefully excised after 12 days of NaCl treatment, and used for measuring RWC. (d) Electrolyte leakage (EL) of leaf cells of WT and TG plants under normal and various salinity conditions. Leaf tissues from WT and TG plants were carefully excised after 12 days of NaCl treatment, and used for measuring EL. Data are presented as means ± SE, and error bars represent standard error. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.
**Osa-miR319a transgenics accumulate less Na\(^+\) than control plants under salinity condition**

A strong correlation between salt exclusion and salt tolerance has been reported in many plant species including rice, durum wheat, bread wheat, barley, pearl millet, *Hordeum* species, tall wheatgrass and *Triticum tauschii* (Yeo and Flowers, 1983; Lauchli, 1984; Tardieu et al., 1992; Bolaños and Edmeades, 1993; Bruce et al., 2002; Munns and James, 2003; Munns et al., 2003; Tester and Davenport, 2003; Campos et al., 2004; Manschadi et al., 2006; Manschadi et al., 2008; Tardieu, 2011). Salt sensitive cultivars tend to have higher accumulation of Na\(^+\). Therefore, lower accumulation of Na\(^+\) in plants as a positive trait has been used to select superior genotypes in different breeding programs (Munns and James, 2003; Munns et al., 2003).

To investigate how Osa-miR319a transgenic plants perform in Na\(^+\) uptake compared to control plants without Osa-miR319a, we measured shoot Na\(^+\) content in plants grown under normal and salt stress conditions. As shown in Figure 3.5a, no significant difference in shoot Na\(^+\) content was observed between Osa-miR319a transgenics and control plants under normal growth conditions (no NaCl application). Both Osa-miR319a transgenic and control plants accumulated low levels of Na\(^+\) (Figure 3.5a). Sodium accumulations increased drastically in both control and Osa-miR319a plants when treated with 200 mM of NaCl. However, the increased Na\(^+\) accumulation was significantly more pronounced in control plants than in Osa-miR319a transgenics (P = 0.01) (Figure 3.5a, c), suggesting that the enhanced salt tolerance of Osa-miR319a plants might be the result of less Na\(^+\) accumulation in the cytoplasm of the cell,
Figure 3.5  Mineral contents in WT and TG plants under normal and salinity conditions. Shoot tissues from WT and TG plants were carefully excised after 12 days of NaCl treatment, and used for measuring mineral contents. (a) The measurement of shoot sodium contents in WT and TG plants propagated and grown in sand under normal conditions and 200 mM NaCl treatment. (b) The K⁺/Na⁺ ratio measured from the WT and TG shoots propagated and grown in sand under normal conditions and 200 mM NaCl treatment. (c) The measurement of shoot sodium contents in WT and TG plants propagated and grown in soil under normal conditions and 200 mM NaCl treatment. (d) The K⁺/Na⁺ ratio measured from the WT and TG shoots propagated and grown in soil under 200 mM NaCl treatment. Statistical analysis of shoot sodium content and the K⁺/Na⁺ ratio was conducted on WT control plants and various transgenic lines. Data are presented as means ± SE, and error bars represent standard error. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.

and consequently reduced Na⁺ toxicity to plant cells. Interestingly, Osa-miR319a plants appeared to accumulate more K⁺ than wild-type controls in both normal and salinity conditions, especially when plants were grown in soil (presented as K⁺:Na⁺ ratio in
Figure 3.5b, d). The discrepancy in significance level for K⁺ uptake in plants grown in sand and soil might be attributed to the characteristics of potting mixture soil used for plant maintenance. The soil itself might be rich in nutrients, facilitating plant uptake of ions. Transgenic plants overexpressing Osa-miR319a might have improved ion transport, therefore accumulating more potassium. It should be noted that the accumulation of phosphate, calcium and magnesium was also higher in Osa-miR319a plants than in the controls without Osa-miR319a gene when plants were propagated in the soil and applied with 200 mM salinity stress (data not shown). The enhanced K⁺:Na⁺ discrimination has been reported to be strongly associated with greater salt tolerance (Asch et al., 2000; Munns et al., 2000; Colmer et al., 2006; Møller et al., 2009). The higher K⁺:Na⁺ ratio observed in OsmiR319a plants (Figure 3.5d) provides additional evidence supporting improved salt tolerance in transgenic plants overexpressing OsmiR319a.

Overexpression of miR319 results in enhanced drought tolerance in transgenic creeping bentgrass plants associated with enhanced water retention, less cell membrane damage and well-maintained photosynthesis

To study whether overexpression of miR319 impacts plant response to water stress, we examined both control and Osa-miR319a transgenic plants subjected to drought stress. As demonstrated in Figure 3.6, after 15 days of water withholding, control plants without Osa-miR319a started to display obvious dehydration symptoms, such as loss of turgor and wilting, whereas Osa-miR319a transgenic plants remained largely turgid without obvious damage. After 20 days of water withholding, control plants had a large area of yellow and wilted leaves, However, more percentage of leaves in
transgenics remained green and turgid (Figure 3.6). Osa-miR319a and control plants were also examined when subjected to limited water supply treatment. As exemplified in Figure 3.7a, the Osa-miR319a plants exhibited greater growth under stressed condition, whereas the control plants were completely arrested, displayed severely disturbed morphology with shortened and deformed leaves and stems (Figure 3.7a, c and Figure A1). Although the tiller number of control plants was still higher than that of Osa-miR319a transgenics (Figure 3.7b), the shoot biomass of control plants was significantly lower than that of Osa-miR319a transgenics (Figure 3.7d) in contrast with the results obtained under normal growth conditions (Figure 3.3c). In addition, contrary to the observation under normal growth conditions that Osa-miR319a plants had less root growth than controls (Figure 3.3a, d), no significant difference in root biomass was observed between them under water deficit, indicating that root development in Osa-miR319a plants was less impacted by the stress than that in controls (Figure 3.7a, e).
Figure 3.6  Response of WT and TG plants to drought stress. The fully developed WT and TG plants clonally propagated from individual tiller were grown in big pots (33 × 44.7 cm) and subjected to complete water withholding. The image showed the performance of WT and two independent transgenic lines 15 days, 16 days and 20 days after water stress.
Figure 3.7  Tillering and plant development of WT and TG plants under the drought stress for 60 days after 30 days normal development starting from a single tiller. (a) Tillering and plant growth in WT and TG plants developed from a single tiller of the same size for 30 days, then subjected to 60 days of water stress (limited water supply) (b) The statistical analysis of tiller numbers in WT and TG plants counted 60 days after the application of limited water supply. (c) Length of the longest stem of WT and TG plants. (d) Biomass of WT and TG shoots weighed 60 days after the the initiation of drought stress. (e) Biomass of WT and TG roots weighed 60 days after the initiation of drought stress. Statistical analysis of tiller number, shoot and root biomass was conducted on WT control plants and various transgenic lines. Data are presented as means ± SE, and error bars represent standard errors. Asterisks *** indicates a significant difference between transgenic and control plants at P < 0.001 by Student’s t-test using JMP 9.0.0.

Further investigation of plant water status and cell membrane integrity revealed that both control and Osa-miR319a transgenics displayed similar relative water content under normal growth condition, whereas under dehydration stress, water loss in Osa-miR319a plants was significantly less than that in control plants without Osa-miR319a (Figure 3.8a) and the cell membrane damage elicited by drought stress was significantly less severe in Osa-miR319a plants than that in controls without Osa-miR319a (Figure 3.8b), suggesting an enhanced water retention capacity and cell membrane integrity in
transgenic plants expressing Osa-miR319a.

Figure 3.8 Leaf relative water content (RWC) and leaf electrolyte leakage (EL) of WT and TG plants under normal and drought stress conditions. (a) Leaf RWC of WT and TG plants 20 days after water withholding. (b) Leaf EL of WT and TG plants 20 days after water withholding. Statistical analysis of RWC and EL was conducted on WT control plants and the two transgenic lines. Data are presented as means ± SE, and error bars represent standard error. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.

Photosynthesis is one of the primary processes affected when plants are subjected to environmental stress (Munns et al., 2006; Chaves et al., 2009). An increase in stomatal
conductance and the maintenance of photosynthesis have been demonstrated to be positively correlated to plant performance under stress (Nelson et al., 2007). Our study on Osa-miR319a transgenic and control plants revealed that under normal growth conditions, there were no significant differences between Osa-miR319a and control plants in photosynthesis rate and stomatal conductance (Figure 3.9a, b). However, when subjected to drought stress (5 days of water withholding), Osa-miR319a transgenic plants displayed higher stomatal conductance and higher photosynthesis rate than control plants without Osa-miR319a gene (Figure 3.9a, b).

![Figure 3.9](image)

**Figure 3.9** Photosynthetic characteristics of WT and a representative Osa-miR319a TG line (TG-1) measured under normal growth conditions and 5 days after dehydration stress. (a). Net photosynthetic rate (b) Stomatal conductance. Data are presented as means ± SE, and error bars represent standard errors. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.
Overexpression of *miR319* results in increased leaf weight:area ratio and higher accumulation of total wax coverage in transgenic creeping bentgrass plants

Plants adapted to dry and saline soil possess a common feature, i.e., their leaves have a higher weight:area ratio (Munns, 2005). Although osa-miR319a plants were not significantly different from controls without osa-miR319a in shoot biomass (Figure 3.3a, c, d), they exhibited drastically reduced tillering (Figure 3.3a, b), increased leaf expansion (Figures 3.2a-d) and leaf thickness (Figure 3.2f, g). This may lead to change in plant weight to surface area ratio. Indeed, compared to control plants without osa-miR319a, osa-miR319a transgenic plants displayed an increased weight:area ratio (Figure 3.10a).

Wax coverage on the leaf cuticle is positively correlated with plant performance under dehydration condition. When subjected to drought stress, plants may be triggered for increased wax production as one of the avoidance mechanisms to keep water from loss (Kosma et al., 2009). To investigate whether miR319 overexpression impacts leaf epicuticle wax content in transgenic plants, we conducted gas chromatography analysis of leaf cuticles from both transgenic and control plants grown under normal conditions. Although wax accumulation per unit weight in the Osa-miR319a transgenic plants (2083 μg/g) was not significantly higher than that in controls (1919 μg/g), the total leaf wax coverage per unit surface area in Osa-miR319a transgenics was significantly higher than that in control plants without osa-miR319a (Figure 3.10b). It should be noted that wax composition is highly species-specific. There has been no report on the composition of wax in creeping bentgrass so far. The identity of a couple of components detected
remains to be determined.

The changes in physical parameters, such as weight:area ratio and leaf wax content resulting from the overexpression of miR319 in transgenic plants might contribute to better plant adaptation to physiological water deficit elicited by drought or salinity (Kosma et al., 2009; Zhou et al., 2009).

![Figure 3.10](image)

**Figure 3.10** Measurement of leaf weight/area ratio and total leaf cuticle wax coverage in WT and a representative Osa-miR319a TG line (TG-1). (a) The leaf weight/area ratio (mg/cm²) of WT and TG plants grown under normal conditions. (b) The total leaf cuticle wax coverage of WT and TG plants. Data are presented as means ± SE, and error bars represent standard error. Asterisk * indicates a significant difference between transgenic and control plants at $P < 0.05$ by Student’s t-test using JMP 9.0.0.

Expression of at least four putative miR319 target genes were down-regulated in the Osa-miR319a transgenic creeping bentgrass plants

MiRNAs exert their effects through regulating the expression of target genes. To investigate molecular mechanism of miR319-mediated alterations in plant development and plant response to environmental stress, we sought to search for putative miR319
target genes in plants for further characterization. Bioinformatics analysis of the rice genome sequence allowed identification of 5 putative miR319 target genes, OsPCF5, OsPCF6, OsPCF7, OsPCF8 and OsTCP14 (Li et al., unpublished data) all belonging to TCP gene family of plant-specific transcription factors (Palatnik et al., 2003; Schommer et al., 2008). Based on sequence information of these five putative miR319 target genes in rice, we designed primers and partially amplified and cloned the corresponding creeping bentgrass homologues, AsPCF5, AsPCF6, AsPCF7, AsPCF8 and AsTCP14 and identified the highly complementary putative target sites (Figure 3.11 and Figure A.2). Semi-quantitative and real-time RT-PCR analyses demonstrated that the expression of the four putative miR319 target genes AsPCF5, AsPCF6, AsPCF8 and AsPCF14 were all down-regulated in both leaves and roots of the Osa-miR319a transgenic creeping bentgrass plants (Figure 3.12a, b). Although the semi-quantitative might indicate that AsPCF7 was also down-regulated (Figure 3.12a), we failed to amplify this gene by real-time RT-PCR and further experimental proof is still required for confirmation.

These results indicated negative regulation of the target genes by miR319 and they suggested the potential direct involvement of these target genes in altering plant development and plant response to environmental stresses.
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**Figure 3.11**  Comparison of target sites in the 5 putative miR319 target genes in rice and creeping bentgrass with the mature sequence of Osa-miR319. The complementary sequence of the mature Osa-miR319 was used to facilitate comparison.
Figure 3.12  Expression levels of the 5 putative miR319 target genes in WT and TG plants. (a) Semi-quantitative RT-PCR analysis of the expression levels of the 5 putative miR319 target genes in WT and TG leaves. (b) Real-time RT-PCR analysis of the expression levels of the 5 putative miR319 target genes in WT leaves and TG leaves. (c) Real-time RT-PCR analysis of the expression levels of the 5 putative miR319 target genes in WT and TG roots. ΔΔCt method was used for real-time RT-PCR analysis. Three biological replicates and three technical replicates for each biological replicate were used for data analysis. ACTIN was used as the endogenous control. Error bars indicate ±SE (n=9)

The impact of miR319 on the expression of other stress-related genes

To examine how other stress-related genes are affected in Osa-miR319a transgenic plants, we studied the transcription factor gene AsNAC60, a homolog of the rice NAC-like gene ONAC60 whose expression has been shown to be dramatically impacted by overexpression of Osa-miR319 (Li et al., unpublished data). Moreover, ONAC60 is the target gene of miR164 in rice (Wu et al., 2009), and in Arabidopsis, TCPs positively regulate miR164 (Rubio-Somoza and Weigel, 2010). Partial sequences of
AsNAC60 were cloned from creeping bentgrass and found to be highly homologous to ONAC60 (Figure A.6). Semi-quantitative and real-time RT-PCR analyses demonstrated that AsNAC60 expression was down-regulated in Osa-miR319a transgenic creeping bentgrass plants in both leaves and roots (Figure 3.13a and b).

**Figure 3.13** Expression levels of AsNAC60 in WT and TG plant leaves and roots. (a) Semi-quantitative RT-PCR analysis of AsNAC60 expression in WT and TG leaves. (b) Real-time RT-PCR analysis of AsNAC60 expression in WT and TG roots. ΔΔCt method was used for real-time RT-PCR analysis. Three biological replicates and three technical replicates for each biological replicate were used for data analysis. ACTIN was used as the endogenous control. Error bars indicate ±SE (n=9).
Expression of miR319 target genes alters in response to salt and drought

To investigate whether the five putative miR319 target genes are directly involved in the miR319-mediated plant response to abiotic stress, we first examined expression levels of these genes in wild-type creeping bentgrass plants treated with 200 mM NaCl. Semi-quantitative analyses suggested a significant increase in the accumulation of the As-PCF5 transcript upon salinity stress (Figure 3.14a). Real-time RT-PCR results agreed with the trend but only had two-fold difference at 0.5 h after exposure to NaCl (Figure 3.14b). At 6h time point, the expression level of As-PCF5 decreased demonstrated by both semi-quantitative RT-PCR and real-time RT-PCR (Figure 3.14a, b). Although the expression of other putative target genes was not significantly changed in response to salinity stress demonstrated by end-point PCR and gel-based assay (Figure 3.14a), real-time RT-PCR analysis suggested a trend of upregulation of three putative target genes AsPCF6 (1.16 fold), AsPCF8 (1.56 fold) and AsTCP14 (3.79 fold) at 6h after exposure to salt stress but not statistically significant (Figure 3.14b).
Figure 3.14  Expression pattern of putative miR319 target genes in WT plants under 200 mM NaCl treatment. (a) Semi-quantitative RT-PCR analysis of 5 putative miR319 targets in WT plant leaves 0h, 0.5h and 6h after salt stress. (b) Real-time RT-PCR analysis of AsPCF5 gene expression after exposure to salt stress. (c) Real-time RT-PCR analysis of AsPCF6 gene expression after exposure to salt stress. (d) Real-time RT-PCR analysis of AsPCF8 gene expression level after exposure to salt stress. (e) Real-time RT-PCR analysis of AsTCP14 gene expression level on three time points after exposure to dehydration stress. \(\Delta\Delta C_t\) method was used for real-time RT-PCR analysis. ACTIN was used as the endogenous control. Error bars indicate ±SE (n=3 technical replicates).

To further explore the turf TCP gene activities in dehydration stress response, the wild-type plants were taken out from pots and put on filter paper for desiccation treatment. Samples were collected 2.5h and 6h later. Semi-quantitative RT-PCR results suggested that AsPCF6, AsPCF8 and AsTCP14 were all upregulated after 2.5h exposure to desiccation stress. Real-time RT-PCR results agreed with the trend at 2.5h but not significantly different from expression level at 0h. However, after 6-hour desiccation, the expressions of these three genes were all upregulated (Figure 3.15 c-d).
Figure 3.15  Time course expression pattern of putative miR319 targets under dehydration treatment. (a) Semi-quantitative RT-PCR analysis of 5 putative miR319 targets in WT plant leaves on 3 time points 0h, 0.5h and 6h after applying dehydration stress. (b) Real-time RT-PCR analysis of AsPCF5 gene expression level on three time points after exposure to dehydration stress. (b) Real-time RT-PCR analysis of AsPCF6 gene expression level on three time points after exposure to dehydration stress. (c) Real-time RT-PCR analysis of AsPCF8 gene expression level on three time points after exposure to dehydration stress. (b) Real-time RT-PCR analysis of AsTCP14 gene expression level on three time points after exposure to dehydration stress. ΔΔCt method was used for real-time RT-PCR analysis. ACTIN was used as the endogenous control. Error bars indicate ±SE (n=3 technical replicates).

The expression level of AsNAC60 change upon 200 mM NaCl was also analyzed. Semi-quantitative RT-PCR demonstrated a significant increase of AsNAC60 expression level at time point 0.5h and also a significant decrease at time point 6h (Figure 3.16a). Real-time RT-PCR data agreed with the trend and have 2.5 fold change at 0.5h point and 1.67 fold change at 6h point (Figure 3.16b).
Figure 3.16  Time course expression pattern of \textit{AsNAC60} under 200 mM NaCl treatment. (a) Semi-quantitative RT-PCR analysis of \textit{AsNAC60} in WT plant leaves on three time points 0h, 0.5h and 6h after treatment of 200 mM NaCl. (b) Real-time RT-PCR analysis of \textit{AsNAC60} in WT plant leaves on three time points 0h, 0.5h and 6h after treatment of 200 mM NaCl. \(\Delta\Delta C_t\) method was used for real-time RT-PCR analysis. \textit{ACTIN} was used as the endogenous control. Error bars indicate ±SE (n=3 technical replicates).

Discussion

Leaf shape and plant abiotic stress tolerance

Leaves are very important plant organs responsible for trapping solar energy through photosynthesis for plant growth and development (Tsukaya, 2005). The transgenic turfgrass plants overexpressing Osa-miR319a exhibit wider and thicker leaves, bigger stems, less tillering, increased weight:area ratio and increased total leaf cuticle wax coverage, of which thicker leaves, increased weight:area ratio and increased total wax coverage are thought to be positively correlated with plant abiotic stress tolerance (Bondada et al., 1996; Zhang et al., 2005; Zhou et al., 2009), whereas leaf width is thought to be negatively correlated with plant abiotic stress tolerance (Deák et al., 2011)
due to the increased transpiration area. Despite the observation that leaf width frequently correlates with plant abiotic stress response, the underlying physiological mechanisms remain largely unknown.

A recent study showed that a rice mutant with a defected zinc finger transcription factor, DST (Drought and Salt Tolerance), had remarkably wider leaves than wild-type control plants. However, the dst mutant plants exhibited significantly enhanced tolerance to drought and salinity stress than the controls. The dst mutant plants also had lower stomata density and less stomata aperture than controls, thus reducing water loss and maintaining higher water content. Moreover, sodium uptake of the dst mutants was significantly less than that in the wild-type control plants when subjected to 100 mM NaCl treatment. The introduction of a wild-type genomic DNA fragment of DST into the dst mutant restores the wild-type phenotype, i.e. narrow leaves and sensitivity to drought and salt stress (Huang et al., 2009).

In the present study, although the transgenic turfgrass plants overexpressing Osa-miR319a exhibited wider leaves than wild-type controls, no significant difference in stomata opening (Figure A3), stomata conductance (Figure 3.9) and stomata density (Figure A3), was observed between the Osa-miR319a transgenic and wild-type control plants. However, the Osa-miR319a transgenic plants had thicker leaves, increased weight:area ratio, increased wax contents, which most likely helped reduce water loss, thus contributing to the enhanced plant resistance to drought stress. Although wider leaves are more likely to be beneficial for plant photosynthesis and thinner leaves would be more efficient in gas exchange (O\(_2\), CO\(_2\) and H\(_2\)O) (Tsukaya, 2005), thus desirable for
photosynthesis too, these two features are less desirable for plant response to water stress. However, other characteristics of leaves such as, stomatal density, stomatal aperture, increased weight:area ratio and increased wax contents may override the cost of increased leaf width and thickness. Thus, like the dst mutant plants, the better adaption of the Osa-miR319a transgenic plants to abiotic stress might be a result of good balance of the costs, benefits and associated trade-offs for each morphological trait.

As reported in the dst mutant (Huang et al., 2009), sodium uptake of the Osa-miR319a transgenic plants was also significantly less than that in the wild-type controls when subjected to salinity stress, indicating the important role salt exclusion mechanism may play in plant salinity resistance. Although our data suggest the possible involvement of some of the putative miR319 target genes in plant response to both drought and salt stress (see more discuss later), further study investigating molecular mechanisms underlying miR319-mediated plant resistance to abiotic stress will shed light on how plants cope with adverse environmental conditions through coordinated function of various regulatory networks.

It was also observed that the morphology of creeping bentgrass is highly associated with environmental conditions and has developmental and morphology plasticity. Thus, normalizing the environmental conditions for control and transgenic plants are very important. The data we presented (Figure 3.2) were all obtained from measuring plants propagated in sand and subjected to regular trimming. If plants were grown in soil and subjected to less trimming, the leaf width of fully developed control leaves could achieve an average of 4 mm and that of transgenics could reach an average
of 4.7 mm. Although the absolute value could vary, transgenic leaves overexpressing rice miR319 were wider than control plants as long as their environmental conditions were same.

The root architecture and plant abiotic stress tolerance

As one of the very first plant organs that sense many environmental changes, root system plays an important role in plant response to abiotic stress. In many circumstances, enhancing soil exploitation through more robust root system with increased root length or root biomass have been considered as a positive feature under drought conditions (de Dorlodot et al., 2007; Tardieu, 2011). For example, in fields with deep soil, increased root biomass and length would make plants reach to deeper soil layer and explore wider soil area thus more water and nutrient uptake (Javaux et al., 2008; Schroder et al., 2008; Tardieu, 2011). However, new plant cultivars with enhanced drought tolerance generated in several breeding programs exhibited decreased root biomass (Bolaños and Edmeades, 1993; Bruce et al., 2002; Campos et al., 2004; Tardieu, 2011). When grown in relatively poor conditions with limited soil depth it makes sense for plants to invest photosynthetic products to other sinks rather than roots. Under such circumstances, enhanced root biomass or length may not be the key factors impacting plant water uptake. Data from the current research show that the Osa-miR319a TG plants display less robust root system, but normal shoot growth compared to controls under normal conditions (Figure 3.3). However, when subjected to water stress, TG plants maintain their growth in both shoot and root, displaying less stress-elicited impact (Figure 3.7). This suggests that under
normal conditions, Osa-miR319a TG plants mainly invest photosynthetic products to the above-ground shoot development, but distribute more for root development while still keep enough ensuring leaf growth under stress conditions. To some extent, it is an effective strategy for perennial grasses to keep enough photosynthesizing leaves gaining opportunity to enter a state akin to dormancy hence surviving the stress (Munns, 2005).

It has also been argued that besides other factors that contribute to plant response to water stress, plant root capacity in water uptake is determined by root spatial distribution rather than root biomass or length even when plants are grown in better field conditions with deep soil layers (Tardieu et al., 1992; Manschadi et al., 2006; Manschadi et al., 2008; Tardieu, 2011). Thus, it is not surprising to observe that with a less robust root system than wild-type controls, the Osa-miR319a TG creeping bentgrass plants still perform better under drought stress. Further study analyzing both the Osa-miR319a TG and control plants grown in the field under normal and stress conditions for their root spatial distribution would provide information for a better understanding of the physiological mechanisms of miR319-mediated morphological change in roots and its impact on plant response to environmental stress.

**Leaf senescence and abiotic stress tolerance**

In some cases, early leaf senescence is thought to serve as one of the stress avoidance mechanisms induced to avoid cellular stresses by decreasing water demand (Tardieu, 1996, 2011). Younger leaves are conserved under stress by sacrificing older leaves (Munné-Bosch and Alegre, 2004; Munns, 2005). On the other hand, senescence is
also assumed to serve as a type of cell death program that could be induced during drought and salinity which impose negative effects to plants. Suppression of this kind of induced leaf senescence would make plants maintain higher water contents and better photosynthetic activity during the stress (Lutts et al., 1996; Rivero et al., 2007).

In the present study, when WT plants started to display the symptoms elicited by sodium toxicity 5 days after 200 mM NaCl treatment, TG plants did not show any typical salinity stress-elicited symptoms (wilting and loss of cell turgor) as observed in WT controls, but appeared to show slight senescence symptom earlier than WT controls (Figure A4). However, the progression of leaf senescence in TG plants was much slower than that in WT plants (Figure 3.4). Considering that miR319 has been reported to positively regulate plant leaf senescence through jasmonic acid (JA) biosynthesis pathway (Schommer et al., 2008), it is hypothesized that overexpression of miR319 in transgenic creeping bentgrass impaired the function of the miR319 target, TCP, leading to less accumulation of JA and consequently, the delay of leaf senescence in general. However, this hypothesis cannot explain the earlier leaf senescence that appeared to take place in the TG plants. As JA is not the only pathway which regulates senescence (Schommer et al., 2008), it is likely that some other factors triggered by miR319 overexpression may be involved, contributing to this particular response in transgenic plants. Another possibility would be that better sodium exclusion in the TG plants (Figure 5) may contribute to its delayed leaf senescence in the long and slower ionic phase minimizing the sodium toxicity.
**Extreme stress response by WT and TG turfgrass plants**

In response to adverse environmental conditions, turfgrass also develops escape mechanism fighting against drought and salt stress. When subjected to extremely harsh conditions, turfgrass leaves become desiccated and the whole plant growth is arrested. The above-ground parts suffer the most and usually all die. However, the crowns, stolons, or rhizomes are mostly likely to stay alive. When released from the stressed condition, the “dormant” turfgrass can regenerate new shoots and roots and revive again (McCann, 2008). The difference in plant recovery from this “dormancy” between WT and the Osa-miR319a TG creeping bentgrass plants are significant and can be demonstrated by Figure A5. After exposure to 300 mM NaCl for 12 days, both WT and TG plants exhibited growth arrest and the shoots were all dead. However, when released from the salt treatment and maintained by regular irrigation, the Osa-miR319a TG plants recovered much more quickly than WT controls (Figure A5a). Similarly, when subjected to water deficiency, both WT and the Osa-miR319a TG plants lost cell turgor and exhibited plant damages 6 days after total desiccation stress. However, after 9-day recovery, the Osa-miR319a TG plants quickly recovered and started growing, whereas the WT controls barely survived the damages (Figure A5b).

**Molecular basis of salt exclusion mechanisms in the Osa-miR319a TG plants**

Our results demonstrated that the Osa-miR319a TG plants accumulated less Na⁺ than the controls (Figure 3.5). The low Na⁺ accumulation in transgenic plants should lead to less cell damage and might contribute to the enhanced salt tolerance observed in
transgenic plants. However, the salt exclusion mechanism in creeping bentgrass remains unknown. In wheat, it has been demonstrated that low and high Na⁺ accumulation is controlled by two major genes \textit{Nax1} and \textit{Nax2}. \textit{Nax1} was located in chromosome 2A (Lindsay et al., 2004) and was identified as a Na⁺ transporter of \textit{HKT} family, \textit{HKT7} (orthologue \textit{HKT1;4} in \textit{Triticum monococcum}) (Huang et al., 2006). \textit{Nax2} was located in chromosome 5A and was identified as \textit{HKT8} (orthologue \textit{HKT1;5} in rice) (Byrt et al., 2007). It is deduced that \textit{Nax1} correlated with the loading of Na⁺ in the xylem in the roots, while \textit{Nax2} correlates with the relocation of Na⁺ in the upper parts of plants (Munns and James, 2003; Munns, 2005). Genetic engineering of salt exclusion mechanism by tissue-specific overexpression of a Na⁺ transporter \textit{HKT1;1} in \textit{Arabidopsis} reduced 37% to 64% Na⁺ accumulation in the shoots (Møller et al., 2009). However, the detailed mechanism of how HKT transporters mediate the sodium management is still unknown. In transgenic rice plants overexpression miR319, the Na⁺ transporter gene \textit{OsHKT2} was also found to be highly up-regulated (Li et al, unpublished). In the current study, we observed that some of the putative miR319 target genes were up-regulated upon salt stress (Figure 3.14). It is likely that these target genes are involved in plant response to salt stress via participation in controlling various downstream biological pathways, including Na⁺ transporter production. This can be further supported by the salt-induced up-regulation of \textit{AsNAC60}, a stress-responsive rice homolog gene that is not a miR319 target (Figure 3.13). Modification of miR319 expression interferes this regulatory network, leading to enhanced salt resistance phenotype in transgenic plants. Further characterization of the miR319 target genes will provide information for a better understanding of the molecular
mechanism underlying miR319-mediated plant resistance to salt stress. Additionally, our data also demonstrated the possible involvement of the putative miR319 target genes in plant response to drought stress (Figure 3.15). Studies of the stress-responsive miR319 targets would also elucidate molecular mechanism determining plant response to drought stress.
Experimental Procedures

Cloning of Osa-miR319a gene and construction of the plant expression vectors

The pre-miR319a sequence obtained from miRBase (version 13.0c, http://microrna.sanger.ac.uk/sequences/) was used to search against the rice full length cDNA database (Kome, http://cdna01.dna.affrc.go.jp/cDNA/) and a full-length cDNA clone AK064418 corresponding to the pri-miR319a sequence was identified. In rice genome, the AK064418 corresponds to Os01g0659400, which encodes rice miR319a gene (Osa-miR319a). Gene specific primers Os-miR319aFL_XbaIF (5’-TCTAGAAGAGCCATGGCATTGCT-3’) and Osa-miR319aFL_SalIR (5’-GTCGACGCAAAAGAAAAAATACTACATGATTG-3’) were used to clone the fragment containing the stem-loop structure from the full length cDNA of Osa-mir319a. Upon double digestion by XbaI and SalI, the PCR product was cloned to the binary vector pZH01 (Xiao et al., 2003), producing the chimera construct p35S-Osa-miR319aFL/p35S-hyg. The construct contains the CaMV35S promoter driving the Osa-miR319aFL and the 35S promoter driving the hyg gene for hygromycin resistance as a selectable marker. The construct was transferred into Agrobacterium tumefaciens strain LBA4404 by electroporation for subsequent plant transformation.

Plant materials and transformation

A commercial genotype of creeping bentgrass (A. stoloniferal L.) Penn A-4 was used for transformation in this study. Transgenic (TG) creeping bentgrass overexpressing
*Osa-miR319a* were produced using *Agrobacterium*-mediated transformation of embryogenic callus initiated from mature seeds as previously described (Luo et al., 2004). The regenerated plants were transferred to commercial potting mixture soil (Fafard 3-B Mix, Fafard Inc., Anderson, SC, USA) in the greenhouse under a 16h photoperiod with supplemented lighting at 27°C in the light and 25°C in the dark.

**Plant propagation, maintenance, and abiotic stress treatments**

General procedures of conducting plant propagation, maintenance, and abiotic stress treatments were developed based on a previous protocol (Li et al. 2010). The *Osa-miR319a* TG plants as well as control plants (untransformed plants either derived from seeds or regenerated from tissue culture and transgenic lines harboring other expression vectors without *Os-miR319a*) maintained in greenhouse were transferred to a growth room at a 14h photoperiod for propagation. Both TG and control plants were clonally propagated from tillers and grown in small cone-tainers (4.0 × 20.3 cm, Dillen Products, Middlefield, OH, USA), middle pots (15 × 10.5 cm, Dillen Products, Middlefield, OH, USA) and big pots (33 × 44.7 cm Dillen Products, Middlefield, OH, USA) using pure silica sand. In order to achieve uniform plant growth, the grass shoots were clipped weekly. Illumination in growth room was 350-450μmol m⁻² s⁻¹ provided by AgroSun Gold 1000 W sodium/halide lamps (Maryland Hydroponics, Laurel, MD, USA). Temperatures and humidity were maintained at 25°C and 30% in the day time and 17°C and 60% at night. Plants were irrigated every other day with 200 ppm of water-soluble fertilizer [20-10-20 (N-P-K), Peat-Lite Special; The Scotts Company, Marysville, OH,
USA].

For plants grown in cone-tainers, the salinity treatment was conducted by injecting 10 ml 200 mM NaCl supplemented with 200 ppm 20-10-20 (N-P-K) fertilizer twice every day. For plants grown in the middle pots, the salinity treatment were conducted by immersing the whole pots in 1L of 0mM or 200 mM NaCl supplemented with 200 ppm 20-10-20 (N-P-K) fertilizer and changing solution every day. The grass shoots were harvested 12 days later, and used for measuring mineral contents and other analyses. To investigate plant recovery from salt stress, the plants were watered with 200 ppm fertilizer described above. The progress of recovery was documented by photograph.

For drought treatments, we either completely withheld watering or provided limited water supply to plants (10ml water for each plant every two days).

For measuring leaf net photosynthesis rate and stomatal conductance, plants were propagated and maintained in nutrient rich soil Fafard 3-B Mix without trimming to achieve maximum leaf growth to facilitate measurements.

**Plant DNA isolation**

Plant genomic DNA was extracted as previously described using the cetyltrimethyl ammonium bromide (CTAB) method (Luo et al., 2005).

**RNA isolation and cDNA synthesis**

Total RNA was isolated from the leaves and roots of the TG and control plants using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s
instructions. DNase-treated total RNA was reverse transcribed using Super Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions.

**Stem-loop reverse transcription and real-time RT-PCR analysis**

Stem-loop reverse transcription was preformed following the protocol of Varkonyi-Gasic et al. (Varkonyi-Gasic et al., 2007). The sequences of the *Osa-miR319a* stem-loop RT primer and the *Osa-miR319* forward primer were GTCGTATCCAGTGAGGGTGTTCCGAGGTATTCCGACTGGAT ACGACGGGAGC and CGGCGGTTGGACTGAAGGGT. Based on DNA sequences of the PCR products we cloned, real-time RT-PCR primers were designed using Primer3 software. Each PCR reaction contained 12.5 μl iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). The reaction was performed using the Bio-Rad iQ5 Real-time detection system (Bio-Rad Laboratories, Hercules, CA). The iQ5 Optical System Software V.2.0 (Bio-Rad Laboratories, Hercules, CA) was used to collect the fluorescence data. Two reference genes *AsACT1* and *AsUBQ5* were used as endogenous controls in analyzing the gene expression level in WT and TG plants (Figure 3.12 and Figure 3.13). *AsACT1* was used as endogenous control in analyzing the *TCP* gene expression level change upon stress (Figure 3.14, Figure 3.15 and Figure 3.16). The ΔΔCt method was used for real-time PCR analysis. ΔCt values were calculated by first normalizing Ct values to the endogenous control, and subsequently calculating ΔΔCt values using the ΔCt value of 0 hour as a reference. Relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ formula.
Measurement of mineral contents

After salt treatment, plant leaf samples were collected to determine the sodium content. All the above ground shoots of the creeping bentgrass plants were rinsed in Millipore (Billerica, MA, USA) water for 30 s. The shoots were dried for 48h at 80°C, and the dry weights (DWs) were measured. The sodium contents were determined using Spectro ARCOS ICP (Spectro, Mahwah, NJ, USA) in Clemson University Agricultural Service Laboratory following protocols by Haynes (1980) and Plank (1992).

Measurement of leaf relative water content (RWC)

The leaves from both the WT and TG plants were harvested and immediately weighed (Fresh Weight, abbreviated as FW). They were then immersed in Millipore water at 4°C for 16h, and then weighed (Turgid Weight, abbreviated as TW). After measuring the TW, the leaves were dried at 80°C for 24h, and weighed (Dry Weight, abbreviated as DW). Leaf RWC was calculated using the following formula: RWC=[(FW-DW)/(TW-DW)] × 100% (Li et al. 2010).

Measurement of leaf electrolyte leakage (EL)

Leaf EL was measured to evaluate cell membrane integrity followed by a previous protocol (Li et al. 2010). Fresh leaf samples (0.2-0.5g) were incubated in 20 ml Millipore water at 4°C for 16h. The initial conductance (Ci) was measured using a conductance meter (AB30, Fisher Scientific, Suwanee, GA, USA). Then the leaf tissue
with the incubation solution was autoclaved for 30 min. After cooling down on a shaker with 24h incubation, the conductance of the incubation solution was determined again (Cmax). Relative %EL was calculated as \((\frac{C_i}{C_{max}}) \times 100\%\). This analysis reflects the percentage ions released from the plant cells, thus measuring the difference of the cell membrane stability of TG and control plants under different conditions.

**Leaf sections and microscopic analysis**

Top third fully expanded leaves were collected and fixed in FAA, followed by dehydration through graded ethanol, and infiltration in catalyzed resin (1.25g of benzoyl peroxide/100 ml of immunobed monomer A). The samples were then embedded, polymerized at room temperature and put in desiccator under vacuum till ready to block. The pictures were taken using microscope (MEIJI EM-5) connected with a 35mm SLR camera body (Canon).

**Measurement of photosynthesis parameters**

Single-leaf net photosynthesis rate was measured on at least 2 leaves from 3 pots of plants respectively using a PLC-6 narrow leaf chamber connected to a CIRAS-2 (PP Systems, Amesbury, MA, USA). Leaf chamber CO₂ concentration was maintained constantly at 375 µmol mol⁻¹. Light intensity was controlled at 400 µmol m⁻²s⁻¹ with the LED light sources installed in the leaf chamber of the CIRAS-2 system. Leaf chamber temperature was maintained equilibrium to the ambient temperature.
**Cuticular wax analysis**

Leaf wax composition measurement was conducted essentially after Kosma et al. and Bethea et al. (Kosma et al., 2009; Bethea et al. unpublished). Leaves were submerged in hexane for 45 s. Wax extracts were evaporated under N₂ gas and derivatized by heating at 100°C for 15 min in N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA; Supelco). Silylated samples were analyzed by gas chromatography (GC). Quantification was based on flame ionization detector (FID) peak areas relative to the internal standard tetrococcine.

The total amount of cuticular wax was expressed as unit weight per unit of leaf surface area (ug cm⁻²). Leaf areas were determined by ImageJ software (http://rsb.info.nih.gov/ij/) using digital images of flattened leaves.

**Statistical analysis**

Student’s t-test was used to analyze all the data to compare the obtained parameters from TG and control plants under normal and stressed conditions. A p value <0.05 was considered to be statistically significant.

**Acknowledgements**

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CHAPTER 4 - CONCLUSIONS AND FUTURE DIRECTIONS

Plant performance under adverse environmental conditions is one of the key factors determining crop production. Genetic improvement of crop plants for enhanced tolerance to biotic and abiotic stresses play a pivotal role in agriculture economy. Using recombinant DNA and transgenic technologies, I have explored different molecular strategies to genetically engineer creeping bentgrass, one of the economically and environmentally important perennial turfgrass species for enhanced resistance to disease, water stress and salt stress.

In an effort to improve plant biotic resistance in creeping bentgrass, I have explored the use of a novel antimicrobial peptide Pen4-1 from shrimp to engineer enhanced fungal disease resistance into creeping bentgrass plants. In order to make it efficiently expressed in plants, the coding sequence of Pen4-1 was modified for monocot plant-preferred codon usage. Northern blot analysis demonstrated that Pen4-1 was successfully expressed at transcriptional level. Transgenic plants overexpressing Pen4-1 exhibited enhanced fungal disease resistance, but did not show any comprised phenotypic changes. I also found that the addition of a signal peptide to the Pen4-1 did not significantly impact its antifungal activity.

In another project, I examined what impact of rice miRNA 319 would have on plant development and response to abiotic stress in transgenic turfgrass. Of particular significance, we are the first to present what impact of miR319 would have on monocot
species and experimentally prove the involvement of miR319 in plant abiotic stress tolerance. We demonstrated that transgenic plants overexpressing Osa-miR319a had dramatic morphological changes including wider and thicker leaves, bigger stems, less tillering, decreased root biomass, increased weight:area ratio and increased total wax coverage. Some of these morphological changes can in turn contribute to enhanced plant abiotic stress tolerance. We also found that the Osa-miR319a plants had enhanced salt and drought tolerance. Further characterization of the underlying physiological mechanisms showed that when subjected to water deficit, the Osa-miR319a transgenic plants could maintain higher water content, higher cell membrane integrity and better photosynthesis. We also found that the Osa-miR319a transgenic plants adopted salt exclusion mechanism to minimize sodium toxicity to cells. To further explore molecular mechanisms of miR319-mediated plant abiotic stress tolerance, we investigated gene expression change of 5 potential miR319 targets in both control and the Osa-miR319a transgenic plants. We found that at least four of the targets AsPCF5, AsPCF6, AsPCF8 and AsTCP14 were all significantly down-regulated in transgenics. To examine whether these four targets were directly involved in plant abiotic stress response, we studied expression of these target genes in response to salt and drought stress by semi-quantitative RT-PCR and real-time PCR analyses. Except AsPCF5, the other three putative target genes were all up-regulated upon salt and dehydration stress. These results suggest that the enhanced abiotic stress tolerance in transgenics might be attributed to significant down-regulation of the miR319 target genes.
Our data clearly demonstrate the impact of miR319 in various aspects of plant development and plant response to environmental stress and the possible involvement of its target genes in these processes. This provides basic information for further study to unravel the respective underlying molecular mechanisms of each phenotype change, contributing to a better understanding of the basic biology of plant development. Our data also indicate that not all miR319-induced changes in plant characteristics are desirable for all plant species from agricultural production perspectives, for example, wider leaves and less tillering displayed in the Osa-miR319a transgenic plants are not necessarily the desirable traits for turfgrass. Next step would be to functionally characterize miR319 target genes and related gene regulatory network to better understand what specific role each target gene plays. We expect to engineer specific target genes, which are probably responsible for plant response to abiotic stress to avoid the pleiotropic effect miR319 has on plant morphology and development.

Collectively, this current study with creeping bentgrass as a target species provides the very first example of using Pen4-1 and rice miR319 for genetic engineering of enhanced environmental stress tolerance in perennial monocot grasses, pointing out the potential of applying similar strategies in other agriculturally important crop species. In the future, it would be interesting to utilize gene stacking technology to combine both Pen4-1 and rice miR319 into one transgenic line to achieve a broader range of tolerance to environmental stress. Besides, considering the possibility of transgene escape and the potential ecological impacts of releasing transgenic plants of perennial species for commercialization, it is also important to incorporate gene containment strategy, such as
male sterility or total sterility in the final product. Genetic engineering of environmentally safe plants with enhanced disease resistance and abiotic stress tolerance will lead to materials that can be directly used for commercialization, benefiting the environment and the agriculture production.
Figure A1  A close look of the leaves and stems of WT and TG plants subjected to 60-day limited water supply. (a) WT plant leaves and stems were shortened and deformed. (b) TG plant stems elongated normally and the leaves maintain the normal shape.
Figure A.2  Alignments between 5 putative miR319 targets in turfgrass and their corresponding homologues in rice (a) Alignment between $AsPCF5$ and $OsPCF5$. (b) Alignment between $AsPCF6$ and $OsPCF6$. (c) Alignment between $AsPCF7$ and $OsPCF7$. (d) Alignment between $AsPCF8$ and $OsPCF8$. (e) Alignment between $AsTCP14$ and $OsTCP14$. 

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Target site
Figure A.3  Stomata study in WT and TG turfgrass plants. (a) A picture capturing guard cell shape and stomata opening. (b) The epidermal cell layer of WT and TG plants. (c) stomata density in WT and TG plants.
**Figure A.4** The performance of WT and TG plants 5 days after 200 mM NaCl treatment.
Figure A.5  The performance of WT and TG plants under the extreme salinity and drought stress. (a) TG plants revived much better than WT plants 6d (left panel) and 15d after recovery (right panel). (b) WT plants showed severe wilting after 6-day desiccation (left panel). TG plants regained the vitality 9 days after the recovery from 6-day desiccation, but WT plants almost died (right panel).
Figure A.6  Alignment between AsNAC60 and ONAC60.