CREATION OF A FRAMEWORK GENETIC LINKAGE MAP OF COLONIAL BENTGRASS AND THE IDENTIFICATION OF GENOMIC REGIONS ASSOCIATED WITH DOLLAR SPOT RESISTANCE

by

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Plant Biology

written under the direction of

Prof. Faith C. Belanger

and approved by

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New Brunswick, New Jersey

January, 2009

ABSTRACT OF THE DISSERTATION

Creation of a framework genetic linkage map of colonial bentgrass and the identification of genomic regions associated with dollar spot resistance.

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Creeping bentgrass (*Agrostis stolonifera* L. 2n=4x=28, $A_2A_2A_3A_3$) is a cool season turfgrass known for its lateral growth habit, fine texture and ability to tolerate low mowing heights. One of the biggest maintenance problems for creeping bentgrass is the fungal disease dollar spot caused by *Sclerotinia homoeocarpa*. Currently the disease is controlled through heavy fungicide use but a more practical long term approach lies through the development of resistant cultivars. Colonial bentgrass (*A. capillaris* L. 2n=4x=28, $A_1A_1A_2A_2$) is a species related to creeping bentgrass which is resistant to dollar spot. We have generated fertile creeping x colonial bentgrass interspecific hybrids. One plant, designated TH15, was backcrossed with a different creeping plant to generate a mapping population which segregated for dollar spot resistance. The objective of this thesis was to determine which areas of the colonial bentgrass genome are associated with dollar spot resistance. To do this we used the hybrid backcross population to generate a framework genetic linkage map of colonial bentgrass. We created and annotated EST libraries for both creeping and colonial bentgrass and used these libraries as a resource for mapping genes. A new approach to marker development, termed dideoxy

polymorphism scanning, was developed to efficiently map genes on the colonial bentgrass map. The colonial bentgrass linkage map contains 212 AFLPs and 110 gene based markers and totals 1157 cM.

By comparing phenotypic data obtained from field trails we identified regions potentially associated with dollar spot resistance in colonial bentgrass. The segregation of resistance in the backcross population suggests a three gene recessive epistasis model for dollar spot resistance in colonial bentgrass. By comparing genotypes we identified colonial bentgrass loci on groups 2A1 and 3A1 that are common in all resistant individuals. We hypothesize that genes related to dollar spot resistance may lie on these groups. Other resistant backcross individuals not included in the mapping population also shared these loci proportions significantly higher then expected by random chance ($p < 0.05$). This lends additional support to our model of dollar spot resistance in colonial bentgrass.

ACKNOWLEDGEMENT

My sincere appreciation goes to my advisor, Dr. Faith Belanger for her never ending support and patience. I would like to thank my committee members, Dr. Stacy Bonos, Dr. Joseph Goffreda, Dr. Barbara Zilinskas, and Dr. Gerben Zylstra.

My special thanks to Dr. Scott Warnke and Keenan Amundsen for their assistance. I would like to thank former lab members Dr. Huaijun Mike Li and Ruth Dorn for all the help and aid that they have given me. I would also like to thank Pierre Tannous for his advice and friendship. I am thankful for support from the United States Golf Association, as well as the Rutgers Turfgrass Center.

Finally I would like to thank my parents, my brother, Jonathan, and my fiancée, Joanna, for their care, understanding and encouragement.

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Introduction

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Introduction

Turfgrasses are found on home lawns where they are used as an area for play and home recreation. They provide economical, year round soil stabilization along roadsides and airfields without inhibiting the sight line of drivers (Beard & Green, 1994). Turfgrass is found in our parks and around schools where it provides an area to play and gather. Industrial parks, large businesses, and hospitals use turfgrass to create large lawns and natural areas that have been shown to relax and relieve stress, and aid in recovery of patients (Ulrich, 1984). Churches and cemeteries use turfgrass to allow for movement around the grounds, minimizing the presence of mud and water puddles (Beard, 1973). It is also used to stabilize soils on nature and fitness trails minimizing the impact of recreational use of our natural areas. Additionally, turfgrass is used as athletic playing surfaces in many sports including golf, field hockey, football, soccer, and even horse racing. The use of turfgrass in such sports was the driving force to the development of modern, high quality turfgrass cultivars of many species (Beard, 1973).

The bentgrasses

Bentgrasses, *Agrostsis* spp, are probably most symbolic of what selective breeding with turfgrasses can accomplish. Originally native to Western Europe, *Agrostis* is a complicated genus comprising over 200 species (Watson, 1990). Of these 200, four are commercially relevant as turfgrasses in North America. They are creeping

bentgrass (*A. stolonifera* L.), velvet bentgrass (*A. canina* L.), colonial bentgrass (*A. capillaris*), and redtop bentgrass (*A. gigantean* L.) (Warnke, 2003). Brown bentgrass (*A. vinealis* L.) and dryland bentgrass (*A. castellana* L.) are other species also used in turf or forage applications (Brilman, 2001). The genus is characterized by having small spikelets with a single floret. Inflorescences are open panicles. Glumes are acute or acuminate with the first usually 1-nerved and the second 1- or 3-nerved; awnless; often as long as, or longer than, the floret. Lemmas may be awnless or awned from below the middle. Paleas usually are absent or very small, rarely well-developed (Watson, 1990).

More specifically, creeping bentgrass is a cool season turfgrass known for its lateral growth habit, fine texture, and its ability to tolerate low mowing heights . These qualities make it ideal for use on golf courses, primarily on greens and tees, where it can be maintained at mowing heights as low as 3 mm. Its name comes from its highly stoloniferous growth habit. Ideally, creeping bentgrass is suited to cool regions where it is known to tolerate extremely cold winters. In many areas of the Unites States, maintaining creeping bentgrass on golf courses requires concentrated maintenance efforts (Warnke, 2003). One of the biggest maintenance problems for creeping bentgrass is the fungal disease dollar spot caused by *Sclerotinia homoeocarpa* (Walsh et al., 1999).

Colonial bentgrass is a related *Agrostis* sp. that is also used on golf courses. Like creeping bentgrass, colonial bentgrass has rolled vernation and narrow pointed

leaves. However, it lacks the distinctive aggressive stoloniferous growth habit of creeping bentgrass. Because of this its use on golf courses is limited to fairways and driving tees. However, it is known for having strong natural resistance to dollar spot (Brilman, 2001).

Extensive cytological work was done by Jones (1956 a,b,c) who examined hybrids of *Agrostis* spp. The conclusions of Jones are summarized in Table 1.1, but recent data from out lab suggests some revisions should be made. Colonial and creeping bentgrass are both allotetraploids and share common A2 genomes but have divergent A1 and A3 genomes, respectively. Creeping and colonial bentgrass both exhibited strict bivalent pairing. However, due to the substantial homology between the A1 and A2 genomes, Jones concluded that colonial bentgrass was a segmental allotetraploid. The A2 and A3 genomes present in creeping bentgrass are sufficiently divergent, and therefore, creeping was considered a strict allotetraploid. Since 1956 a number of other studies have attempted to confirm this relationship originally outlined by Jones. Isozyme analysis conducted by Warnke et al. (1998) confirmed Jones' original hypothesis of the allotetraploid nature of creeping bentgrass. Bonos et al. (2002) used flow cytomerty to positively correlate the chromosome numbers and ploidy levels that Jones originally reported with nuclear DNA content. Vergara et al. (2004) used AFLPs to differentiate bentgrass genotypes and determine the genetic relationships among genotypes.

Dollar spot

Dollar spot is caused by the fungal pathogen *Sclerotinia homoeocarpa* F. T. Bennett. The disease primarily blights leaf tissue but does not affect turfgrass roots or crowns (Hsiang & Mahuku, 1999). Recently, taxonomists are in dispute as to whether this fungus belongs in the genus *Sclerotinia* (Carbone & Kohn, 1993). They argue that members of this genus typically produce a compact mass of mycelium known as a sclerotium, which *S. homoeocarpa* apparently does not. Biochemical and immunological studies conducted on infertile apothecia from isolates grown in pure culture indicate that this fungus may belong to either the genus *Rutstroemia, Pocolum, Lanzia* or *Moellerodiscus* (Carbone & Kohn, 1993; Holst-Jensen et al., 1997). However, the correct classification cannot be made until the teleomorphic state of the fungus is discovered, so in practice and in the literature the causal agent of dollar spot is generally referred to as *S. homoeocarpa* (Powell & Vargas, 2007).

Of all the turf diseases, dollar spot might be the most readily identifiable with characteristic round (2.5 cm diameter) necrotic patches appearing in clusters. Initially, on individual leaves these lesions appear as small and circular brown spots that eventually turn necrotic and extend across the blade (Jo et al., 2006). The fungus survives as mycelium and becomes most active typically in early spring when there are extended periods of dew accumulation on leaves (Nelson & Craft, 1991). Occasionally during early morning hours, the mycelium can be visualized as feathery, web-like, structures affixed to the individual blades of grasses. It is generally accepted that transfer of mycelium is the primary method that the fungus

is dispersed, though Hsiang and Mahuku (1999) reported that sexually produced spores may also play a role.

The environment seems to play a large role in the occurrence and severity of infection (Koh et al., 2003). The dollar spot fungus will start growing at 15°C, with optimal growth between 21° and 27°C, combined with relative humidity greater than 85% and heavy dew at night (Lee et al., 2003). Many of the cultural practices used to manage the disease focus on limiting the amount and duration of leaf wetness. Additionally a fertility program that ensures adequate nitrogen balance can prevent infection. Turf grown with minimal fertilization is generally more susceptible to disease and is slower to recover. The "greening" effect that nitrogen applications have can also aid in masking some of the initial symptoms as well (Markland et al., 1969).

All of the popular creeping bentgrass cultivars are susceptible to dollar spot (Bonos et al., 2006). Vincelli (1997) examined recovery rates in 15 different cultivars of creeping bentgrass and determined that though some cultivars exhibited partial resistance none were completely resistance to infection. Though the aforementioned cultural management practices have been shown to improve the quality of turgrasses infected with dollar spot, much of the control of dollar spot is done through the use of fungicides. In fact, a large portion of the fungicides used on turfgrasses is directed toward controlling dollar spot (Bonos et al., 2006). However, in the long term, repeated fungicide use may not be the most effective

approach. Resistance to various fungicide chemistries by *S. homoeocarpa* has been reported as far back as 1968 (Taylor & Duich, 1968). More recently, one study showed that prolonged fungicide use on creeping bentgrass led to the evolution of resistant fungal strains (Burpee, 1997). Bishop et al. (2008) saw resistance in *S. homoeocarpa* isolates to three different classes of fungicides in Tennessee and northern Mississippi. There have been reports of decreased sensitivity of isolates to fungicides in Canada as well (Hsiang et al., 2007). This casts doubt on the long term viability for control of dollar spot via chemical fungicides.

A more practical, long term, approach to manage dollar spot may lie in the development of resistant cultivars. Though creeping bentgrass is susceptible to dollar spot, colonial bentgrass is known for being resistant to infection (Belanger et al., 2004). Interspecific hybridization between creeping and colonial bentgrass may hold potential for the development of cultivars that have much of the aesthetic turf-type qualities that creeping bentgrass is known to have while maintaining the dollar spot resistance seen in colonial bentgrass.

Interspecifc hybridization as a tool for introgression of new traits

Interspecific hybridization has been used by breeders of many crops to introduce beneficial traits from related species into crop species. It is especially useful for introducing new phenotypes that do not exist in the species of interest. Many times the intensive selection of particular traits of interest creates a bottleneck that limits the genetic variability within a crop species. When researchers are faced with the

prospect of having to select for a new phenotype (such as resistance to a new disease) they become limited by the lack of variation contained in their germplasm. Interspecific hybridization with related species allows for the introduction of new variation that has not been subjected to the same selection pressure. It has been successfully utilized in many important agronomic crops such as coffee (*Coffea arabica* L.) (Lashermes et al., 2000), grapes (*Vitis* spp.) (Lodhi et al., 1995), garlic (*Allium sativum* L.) (Yanagino et al., 2003), and tomato (*Lycopersicon esculentum* L.) (Bernatzky & Tanksley, 1986).

For example, desirable traits have been introgressed into allotetraploid coffee (*Coffea arabica* L.) via interspecific hybridization with the related wild species *Coffea canephora* L. RFLP analysis was used to track *C. canephora* alleles within the backcross population (Lashermes et al., 2000).

Interspecific hybridization has not yet been widely utilized by turfgrass breeders (Brilman, 2001) and may offer new opportunities for cultivar improvement. Bradshaw (1958) initially examined the potential for interspecific hybridization within wild *Agrostis* stands and determined that natural interspecific hybrids did exist between creeping and colonial bentgrass and the hybrids had increased ecological fitness relative to their parental species (Bradshaw, 1958). Bradshaw concluded that the hybrids he observed were sterile, though other researchers later on successfully obtained creeping x colonial bentgrass hybrids that were found to be fertile, both through the pollen and the egg (Belanger et al., 2003b).

In 1998 colonial x creeping bentgrass hybrids were generated in projects investigating the potential outcrossing of transgenic creeping bentgrass (Belanger et al., 2004; Belanger et al., 2003b). A transgenic creeping bentgrass plant expressing the *bar* gene, which confers resistance to the herbicide glufosinate, was used as the pollen parent. Herbicide resistance was used as a marker to screen the progeny of nontransgenetic plants of related *Agrostis* species to identify the hybrids. From controlled crosses, it was determined that the frequency of interspecific hybridizations was low, lower than the frequency of selfing which was estimated to be about 0.5% (Belanger et al., 2003b).

Since the hybrids were fertile and since colonial bentgrass in general has good dollar spot resistance, the colonial x creeping bentgrass interspecific hybrids were evaluated for dollar spot resistance in 2001 and 2002. Some of the hybrids exhibited excellent resistance (Belanger et al., 2004).

Of these hybrids one hybrid in particular (designated TH15) consistently exhibited excellent dollar spot resistance in addition to many desirable aesthetic turf-type qualities and growth habits. This plant was selected and backcrossed with a new creeping plant (designated 9188). The resulting population was evaluated in a field test in 2003 and 2004. The population exhibited large variation with respect to resistance, growth habit, and leaf morphology. Figure 1.2 illustrates the structure of the mapping population.

Genetic mapping to determine loci that are associated with disease resistance

Genetic mapping is a powerful tool for uncovering the genes associated with specific traits. The information garnered from these maps can lead to improved cultivars as well as further understanding of the molecular mechanisms that lead to agronomically important phenotypes such as disease resistance and yield (Tanksley et al., 1992). These data can ultimately lead to QTL (quantitative trait loci) mapping of specific traits and the implementation of marker assisted breeding strategies (Lynch & Waksh, 1998). Gene mapping can also yield powerful data regarding the evolution of a species and the nature of its genome organization (Kellogg, 1998). Bentgrasses have undergone a number of polyploidization events (Jones, 1956 a,b,c). By comparing genetic maps of various bentgrass species, information can be gathered regarding their genome organization within and among species. Genetic maps with thousands of mapped loci are now available for a number of model organisms such as arabidopsis (*Arabidopsis thaliana* L.), rice (*Oryza sativa* L.), and maize (*Zea mays* L.) as well as other important agronomic crops (Ahn & Tanksley, 1993; Chang et al., 1988). The information has allowed for the creation of comparative maps that have been made for a number of related species such as rice, maize, sorghum (*Sorghum bicolor* L.) and wheat (*Triticum aestivum* L.) (Devos, 2005). Comparison maps have also been made for tomato (*Lycopersicon esculentum* L.), potato (*Solanum tubersum* L.), and pepper (*Capsicum annum* L.) (Tanksley et al., 1992). These studies revealed synteny over large regions of DNA, as well as the conservation of alleles and QTL information.

The development of fertile colonial bentgass x creeping bentgrass hybrids, as well as the resulting backcross progeny, affords a unique opportunity to study the genetic nature of disease resistance in colonial bentgrass. By mapping markers that appear in the hybrid parent (TH15) and not in the creeping parent (9188) and grandparent (5061), one can, in effect, map the colonial portion of the hybrid genome. This is an approach taken by Wu and colleagues (2003) in constructing framework genetic linkage maps of two related allotetraploid *Leymus* wildryes (Wu et al., 2003).

Making a genetic map of colonial bentgrass using these hybrids would allow for the ability to determine loci on the colonial bentgrass genome that may be associated with dollar spot resistance. Previous observations have shown that the phenotype that is seen in colonial bentgrass does segregate within the hybrid backcross population.

Researchers use careful consideration in determining what type of markers to use in creating a linkage map. Each marker system is a compromise between how informative they are versus how costly (time and labor included) they are to find and evaluate. The compromise tends to be a combination of strategies that are used to efficiently create an informative map. A genetic linkage map of creeping bentgrass using RAPD, AFLP, and RFLP markers has already been developed by one group (Chakraborty et al., 2005), and work is currently being done using primarily SSRs by another group (Bonos et al., 2008). To take full advantage of the information gained from maps created with these populations, it is important to

create markers based on specific genes rather then other random based approaches. Markers derived from genes have the benefit of referring to specific loci that can be compared on other maps. Expressed sequence tag (EST) libraries are a resource for the development of gene based markers. These libraries are sequences from cloned mRNA fragments isolated from the species of interest. EST libraries have been made of many plant species and have resulted in the production of comprehensive catalogues which can be used for the discovery of both molecular markers and candidate genes.

In summary, this thesis will report the creation of the first framework genetic linkage map for colonial bentgrass. To accomplish this, two EST libraries of creeping and colonial bentgrass were created from mRNA taken from field samples that were inoculated with dollar spot (and exhibiting symptoms in the controls). A new marker strategy, termed dideoxy polymorphism scanning was developed to take advantage of the sequence polymorphism data obtained by comparing the creeping and colonial bentgrass EST libraries.

Goals of this thesis

This thesis reports the following:

1. the creation and annotation of EST libraries created for creeping and colonial bentgrass,

2. the development of markers for genetic linkage mapping of colonial bentgrass using information gained from the creeping and colonial EST resources, and
3. the creation of a framework genetic linkage map of colonial bentgrass and the identification of loci that are associated with dollar spot resistance.

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Table 1.1. *Genetic relationships between commercially relevant bentgrass species, as proposed by Jones (1956 a,b,c).*

Species	***Common Name***	***Chromosome Number***	***2C DNA, pg (Bonos et al, 2002)***	***Genome Composition (Jones, 1956abc)***
A. canina	Velvet bentgrass	14	3.42	$A_1 A_1$
A. vinealis	Brown bentgrass	28	6.31	$A_1 A_1 A_1 A_1$
A. capillaris	Colonial bentgrass	28	5.87	$A_1 A_1 A_2 A_2$
A. stolonifera	Creeping bentgrass	28	5.27	$A_2 A_2 A_3 A_3$
A. gigantea	Redtop bentgrass	42	8.18	$A_1 A_1 A_2 A_2 A_3 A_3$

Figure 1.1. *Photographs comparing the extent of disease in creeping bentgrass parental line 5061 with the resistant interspecific hybrid #15.*

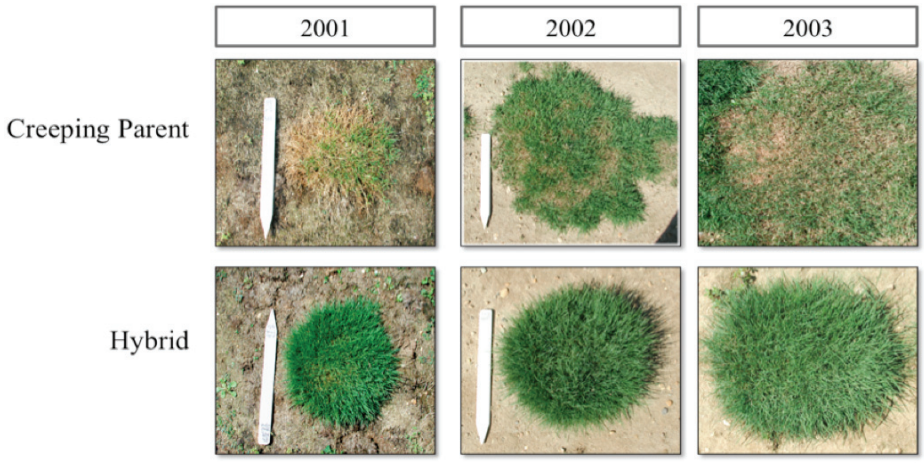
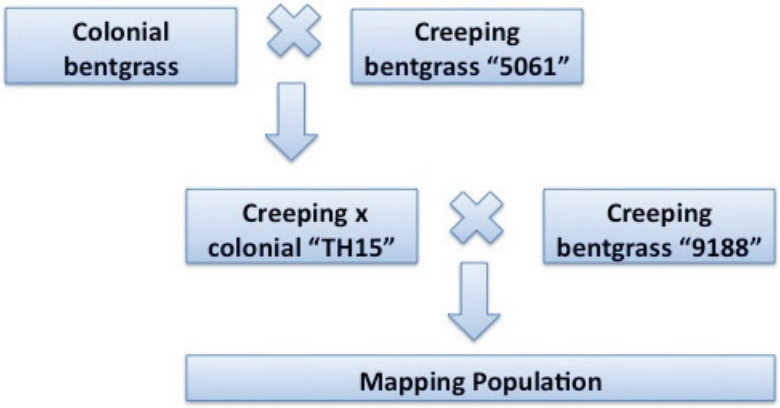


Figure 1.2. *Lineage of the colonial x creeping backcross population*.



Chapter 2

Construction and analysis of expressed sequence tag libraries of creeping and colonial bentgrass created under biotic stress

Chapter 2

Construction and analysis of expressed sequence tag libraries of creeping and colonial bentgrass created under biotic stress

Introduction

Creeping bentgrass (*Agrostis stolonifera* L. 2n=4x=28, $A_2A_2A_3A_3$) and colonial bentgrass (*A. capillaris* L. 2n=4x=28, $A_1A_1A_2A_2$) are two related commercially relevant turfgrass species in the genus *Agrostis*. They are known to tolerate a low mowing height and form a dense turf and as such are commonly used on the greens, tees, and fairways of golf courses (Warnke, 2003). Dollar spot is one of the biggest management problems facing creeping bentgrass (Walsh et al., 1999). Currently the disease is controlled though fungicide use (Lee et al., 2003), but a more practical long-term approach lies in the development of resistant cultivars. Colonial bentgrass is known for having good resistance to dollar spot and can be a potential source for novel genes that can be used for the genetic improvement of creeping bentgrass (Belanger et al., 2004; Belanger et al., 2003). The previous chapter described our approach for using colonial x creeping bentgrass interspecific hybrids to determine the genetic basis for dollar spot resistance in colonial bentgrass. Our goal was to use molecular tools to determine loci in colonial bentgrass that are associated with disease resistance.

Work done in model species such as tomato, rice, and corn show the potential for molecular approaches such as quantitative trait loci (QTL) analysis and marker assisted selection (MAS) for crop improvement (Ahn & Tanksley, 1993; Messing & Llaca, 1998; Qi et al., 2004; Ribaut & Ragot, 2007). Researchers are now using this type of analysis to aid in the genetic improvement of bentgrasses. We have contributed to this effort by creating a genetic linkage map of colonial bentgrass using the hybrid backcross mapping population. To fully take advantage of the information from these maps and others created in model species such as rice and wheat it is important to use markers based on genes. Gene based markers can serve as anchor points that can align maps for comparative genomic analysis (Messing & Llaca, 1998).

In an effort to enhance the development of molecular markers for linkage mapping of genes, we have generated a collection of expressed sequence tags (ESTs) from both creeping and colonial bentgrass. EST libraries are comprised of sequences obtained from cloned mRNA fragments contained within cDNA libraries. They are a repository of sequences that provide a window toward understanding the expression of genes under specific conditions. To ensure that genes related to disease pressure were included, the RNA for these libraries was isolated from field samples that were inoculated with the dollar spot fungus. Samples were taken at points where the creeping bentgrass control plants were exhibiting the strongest disease symptoms. Because the primary focus of these libraries was for marker creation, the ESTs were sequenced from the 3' end. The 3' non coding regions of

genes are known for being more variable than coding regions, presumably because they are not subjected to the same selection pressure as the coding regions (Bhattramakki et al., 2002).

We generated 8,470 creeping bentgrass EST sequences and 7,528 colonial bentgrass EST sequences. Analysis of these sequences revealed some differences in gene expression between the two species that may relate to the dollar spot resistant or susceptible phenotypes of the colonial and creeping bentgrass plants used for library construction. Numerous ESTs for genes involved in plant stress responses were found in both libraries and will be candidates for further research on the responses of the two species to dollar spot infection.

Here we report the creation and annotation of EST libraries for colonial and creeping bentgrass. To date, they represent the largest sequence resource for the bentgrasses.

Materials and methods

EST sequencing

The RNA isolation and cDNA library construction was done by H. M. Li. For preparation of cDNA clones for sequence analysis the pBK-CMV phagemids were mass excised from the λ ZAP-Express vector, transformed into bacterial strain XLOLR, and plated onto Luria-Bertani (LB) plus kanamycin medium as described by the manufacturer. Approximately 2000 phagemids per plate were grown in 243 x 243 mm BioAssay Dishes (Nalge Nunc International Corp., Rochester, NY). To allow discrimination between recombinant and nonrecombinant phagemids, a solution containing 1 mL LB, 1 mL of 100 mM isopropylthio-β-D-galactoside (IPTG), and 200 mL of 100 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGAL) was spread on the agar surface of each plate prior to plating the bacterial cultures. White colonies were picked on a 'Q' Pix2 automated colony picker (Genetix USA Inc., Boston, MA) into 96 well plates containing 200 mL per well of LB plus kanamycin freezing medium (Zimmer and Gibbins, 1997). The cultures were incubated at 37 °C for 16 hours, duplicated, and stored at – 80 °C.

The cDNA clones were sequenced in the laboratory of J. Messing as described below. The glycerol stocks were used to inoculate deep well plates with 1.2 mL of LB plus kanamycin using a 'Q' Bot robot (Genetix USA Inc.). Plasmid DNA was isolated from these overnight cultures using the alkaline lysis method in 96-well format using Whatman filters (Whatman Inc., Clifton, NJ). The DNA was pelleted using isopropanol and dissolved in 1 mM Tris-Cl, pH 8.0. The cDNA inserts were

sequenced from the 3' end with the T7 primer using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied BioSystems, Foster City, CA). The unincorporated dyes were removed by solid phase reversible immobilization (SPRI) using magnetic beads (Agencourt Biosciences Corp., Beverly, MA). The reactions were then suspended in 0.1 mM EDTA and run on an ABI 3730xl automated capillary sequencer with 50 cm capillary arrays. Base-calling was done using the ABI KB basecaller with lower quality sequences being trimmed using the "Lucy" software (TIGR) for quality control (.Q16). Sequences were also screened for vector contamination. All sequences > 100 bp were processed for GenBank submission. A total of 15,998 successful sequence reads were obtained and deposited to GenBank.

Computational analysis

ABI trace files were converted to fasta format using the AutoEditor program (TIGR). Cleaned sequences were assembled into contigs using the CAP3 program (Huang & Madan, 1999). The ESTs were imported into the openSputnik EST database and clustered into unigenes for functional annotation (Rudd et al., 2005). Functional assignments were performed using BLASTX (threshold value of 1e-10) against the MIPS catalog of functionally assigned proteins (FunCat) (Ruepp et al., 2008).

To identify the most abundant transcripts, the creeping and colonial EST sequences were searched against the NCBI database using the BLASTX function. The resulting data were imported into an Access database for further manipulation. The data

were sorted by e value (with a 10^{-10} cutoff) and by matching accession number. The top 15 most abundant accessions were then confirmed by searching against the formatted creeping and colonial EST libraries using the BLASTN function.

Phylogenetic analysis

The CLUSTAL-X (Thompson et al., 1997) program was used to align DNA sequences. Phylogenetic analysis was performed with the PAUP program (Swofford, 2002). Phylogenetic trees were generated using the maximum parsimony (1000 bootstrap replications) and the neighbor joining methods.

Results

EST generation

Leaf tissue from creeping and colonial bentgrass plants from the 2002 field test was used as the source of RNA for construction of the cDNA libraries (Belanger et al., 2004). The field had been inoculated with the dollar spot fungus and the tissue samples were taken at a time when the creeping bentgrass plant was exhibiting symptoms of the disease. The colonial bentgrass plant had no disease symptoms.

The creeping and colonial bentgrass cDNAs were cloned unidirectionally into the λ ZAP Express vector. The cDNA clones were sequenced from the 3' ends to generate sequence data from the untranslated region of the transcripts. The 3' untranslated regions of genes are generally more polymorphic than the coding sequences, and therefore more useful for molecular marker development (Bhattramakki et al., 2002; Brady et al., 1997). The characteristics of the colonial and creeping bentgrass ESTs are summarized in Table 2.1. A total of 10,944 randomly chosen cDNA clones from each library were single pass sequenced from the 3' end. From the creeping bentgrass cDNAs, 8,470 usable sequences were obtained with an average read length of 567 bases after vector trimming. From the colonial bentgrass cDNAs, 7,528 usable sequences were obtained with an average read length of 745 bases after vector trimming. These sequences have been deposited in the NCBI dbEST (colonial bentgrass accession nos: DV852741-DV860268; creeping bentgrass accession nos: DV860269-DV868738).

Chloroplast and microbial sequences

To determine the level of chloroplast derived cDNA contamination among the ESTs, the colonial and creeping bentgrass sequences were compared with the wheat chloroplast genome sequence by a BLASTN search (Ogihara et al., 2002). Some sequences nearly identical to the wheat chloroplast genome were present among the colonial and creeping bentgrass ESTs (Table 2.1). The contamination level was 0.5% and 1.4% for the colonial and creeping bentgrass ESTs, respectively.

Since the cDNA libraries were constructed from field grown plants that were inoculated with the dollar spot fungus, some microbial sequences would be expected among the ESTs. To estimate the level of microbial ESTs in the libraries, the colonial and creeping bentgrass ESTs were compared to the 14,522 protein sequences of the plant pathogen *Sclerotinia sclerotiorum* using a BLASTX search. Although the dollar spot fungus should not be classified in the genus *Sclerotinia*, *S. sclerotiorum* is the most closely related species for which there is a whole genome sequence available (Holst-Jensen et al., 1997). For the bentgrass ESTs that had a match to a *S. sclerotiorum* protein sequence, the expected value was compared with to the best match in a BLASTX search of the NCBI database. Those bentgrass ESTs for which the expected value was lower with the *S. sclerotiorum* match than with the NCBI match were considered to be likely of microbial origin. This comparison was done since most of the *S. sclerotiorum* protein sequences are not included in the NCBI database. For the colonial and creeping bentgrass ESTs, 184 and 99 sequences, respectively, met these criteria. Sequences of microbial origin in the

libraries are therefore estimated to be 2.4% for colonial bentgrass and 1.2% for creeping bentgrass.

Unigene analysis

The sequences from each library were imported into the openSputnik EST database and clustered into unigenes (Rudd et al., 2005). Analysis of the colonial bentgrass sequences resulted in 1,095 multimember unigenes and 3,579 singletons. Analysis of the creeping bentgrass sequences resulted in 980 multimember unigenes and 3,901 singletons. The unigene sequences were assigned to functional categories based on the MIPS catalog of functionally assigned proteins (Table 2.2) (Ruepp et al., 2008). Of the 4,425 colonial bentgrass unigenes, 1,843 could be classified. Of the 4,747 creeping bentgrass unigenes, 1,048 could be classified. There were more creeping bentgrass unigenes with no match. This may be a reflection of the shorter average length of the creeping bentgrass sequences. Since the sequences were from the 3' ends, there may be inadequate coding sequence information in some of the creeping bentgrass ESTs on which to base a match for functional classification. Of those unigenes that could be assigned a functional classification, the percentage distribution between colonial and creeping bentgrass was similar, with one exception. There was a 10-fold higher representation of creeping bentgrass unigenes in the category of transposable elements, relative to that of colonial bentgrass. The difference in transposable elements between the creeping and colonial bentgrass ESTs is discussed more below.

Differential EST abundance between the colonial and creeping sequences

In addition to unigene classification, it was also of interest to examine the relative abundance of different sequences among the ESTs. The 15 most abundant sequences from both libraries were compared. Among the most abundant sequences from the colonial bentgrass library were several proteins involved in photosynthesis, chlorophyll a/b binding proteins, rubisco small subunit, rubisco activase, FBP aldolase, and PSII proteins (Table 2.3). cDNAs from photosynthesis genes are typically abundant in healthy leaf EST libraries (Brandle et al., 2002; Fernandes et al., 2002; Jantasuriyarat et al., 2005; Pratt et al., 2005). Other abundant cDNAs in the colonial bentgrass library were carbonic anhydrase and metallothionein, which are also often found to be highly expressed genes (Brandle et al., 2002; Matsumura et al., 1999). With one exception, the cDNAs that were the most abundant in the colonial bentgrass library were also recovered from the creeping bentgrass library, but at considerably reduced levels. In rice leaves inoculated with the rice blast fungus, photosynthesis genes were found to be suppressed relative to their abundance in libraries prepared from control tissue (Jantasuriyarat et al., 2005). One of the most abundant colonial bentgrass cDNAs with similarity to group 5 late embryogenesis abundant (*Lea5*) proteins was not found among the creeping bentgrass ESTs.

Most of the abundant ESTs in the creeping bentgrass library were the same as the abundant ESTs from the colonial bentgrass library, but were recovered at lower relative levels than in the colonial library. There were a few interesting exceptions

that may relate to the diseased state of the creeping bentgrass tissue used as the source of RNA for library construction. Three of the abundant creeping bentgrass ESTs were for proteins known to be involved in plant stress responses and they were recovered in considerably higher levels in the creeping library relative to the colonial library. Cyp709C1 is a long chain fatty acid hydroxylase induced in wheat by jasmonic acid and by infection with the fungal pathogen *Fusarium graminearum* (Kandel, 2005). The enzymatic activity of Cyp709C1 results in the production of hydroxlated fatty acids, which are considered to play a role in plant defense (Kandel et al., 2005). Other stress related abundant ESTs in the creeping bentgrass library were a 12-oxo-phytodienoic acid reductase involved in the synthesis of jasmonic acid and an ABA-inducible protein (Agrawal et al., 2003; Moons et al., 1997). Another abundant EST from the creeping library was nearly identical to an unknown rice protein, and there were no similar clones recovered among the colonial bentgrass ESTs.

The cDNA sequences that were unique to either the colonial bentgrass or creeping bentgrass libraries, and represented by at least 5 ESTs, are reported in Tables 2.5 and 2.6, respectively. Sequences unique to either of the libraries are candidates for further examination as genes important to the dollar spot resistant or susceptible phenotypes of the two species. As mentioned above, one of the sequences unique to the colonial bentgrass cDNAs was for a *Lea5* protein. Most *Lea* proteins are highly hydrophilic, but the *Lea5* group is atypical in having less hydrophilic character (Galau et al., 1993). Similar *Lea5* proteins were expressed in leaves of cotton,

barley, and citrus, and in some cases were induced by gibberellic acid, drought, heat, or salt stress (Galau et al., 1993; Naot et al., 1995; Speulman & Salamini, 1995). Some of the other unique colonial bentgrass ESTs have roles in plant responses to pathogens. The iron storage protein ferritin is induced in response to pathogen attack and may protect plants from iron-mediated oxidative damage (Deak, 1999; Dellagi et al., 2005; Mata et al., 2001). Glutamate decarboxylase catalyzes the decarboxylation of glutamate producing γ-aminobutyric acid (GABA), which is involved in stress responses (Bouche & Fromm, 2004; Kinnersley & Turano, 2000). The wheat LRR14 gene is a nucleotide-binding site leucine rich repeat gene that has not yet been functionally characterized but this type of gene typically functions as a disease resistance gene (R gene) (Feuillet et al., 2001; Pan et al., 2000). LEM2 is a jacalin-like lectin that is induced by salicylic acid and is involved in systemic acquired resistance (Abebe et al., 2005).

The most abundant creeping bentgrass unique ESTs were good matches to uncharacterized proteins from other grasses, with the exception of a COX VIIa-like protein and a calcium ion binding protein (Table 2.6).

Expression of disease response genes

While ESTs from both species can be used to develop genetic maps, the pathogen-induced gene expression can also aid in the identification of the underlying basis of dollar spot resistance in colonial bentgrass and QTLs for enhanced dollar spot tolerance in creeping bentgrass. In addition to the genes discussed above that may

be involved in pathogen response, the EST sequences were systematically searched for disease resistance genes and pathogenesis-related genes. Resistance could also be based on allelic differences rather than the absence or presence of gene products.

Many plant disease resistance genes are nucleotide-binding site leucine rich repeat proteins (NBS-LRR proteins) or receptor-like kinases (Belkhadir et al., 2004; Morris & Walker, 2003). Sequences similar to both types of proteins were present among the colonial and creeping ESTs (Table 2.7). Interestingly, resistance gene-like sequences represented a five-fold higher percentage of the colonial bentgrass ESTs than the creeping bentgrass ESTs, and could contribute to a general response to resistance to dollar spot disease.

Pathogenesis-related proteins (PR-proteins) are proteins of several different classes that are induced in response to pathogens and are considered to function in plant defense (Muthukrishnan et al., 2001). PR-protein induction is associated with systemic acquired resistance in incompatible plant-pathogen interactions (Ryals et al., 1996). Overall, PR-protein ESTs represented similar proportions of the colonial and creeping bentgrass sequences (Table 2.7). PR-proteins are often induced in pathogen susceptible plant genotypes, but expression is delayed relative to resistant genotypes (Muthukrishnan et al., 2001). This could be further investigated by comparison of the induction and expression levels of PR-proteins in colonial and creeping bentgrass in response to inoculation with the dollar spot pathogen.

Transposable element sequence representation in the colonial and creeping bentgrass ESTs

In the FunCat analysis of the unigenes, transposable element sequences represented a 10-fold higher percentage of the creeping bentgrass unigenes, relative to the colonial bentgrass unigenes. The distribution of transposable element ESTs between the two species was, therefore, further investigated. Transposable elements are found in all plant genomes and are classified into two groups, the Class I retroelements (retrotransposons with LTRs and retrotransposons without LTRs) and the Class II DNA transposons (*h*AT, CACTA, *Mutator*, MITEs, Helitrons, etc.). Many grass genomes are largely comprised of retrotransposon sequences (Bennetzen, 2000; Feschotte et al., 2002; Haberer et al., 2005; Messing et al., 2004). An analysis of 7.8×10^5 EST sequences from numerous plant species revealed that retrotransposons were active and represented 0.12% of the ESTs (Vicient et al., 2001). Some of the colonial and creeping bentgrass EST sequences were most similar to retrotransposons described from other species, particularly rice. The colonial and creeping bentgrass ESTs were systematically searched with the coding sequences of numerous rice retrotransposons of different classes and the best matches were tabulated. The results are summarized in Table 2.8. The expected values of the matches ranged from $8e^{-5}$ to e^{-128}. For all the ESTs included in the data summarized in Table 2.8, a retrotransposon sequence was the best match. There is a striking difference in the representation of retrotransposons among the colonial bentgrass ESTs, relative to that among the creeping bentgrass ESTs. In the colonial bentgrass library, retrotransposon ESTs were 0.18% of the total, similar to the

0.12% reported from the survey of ESTs from many plant species (Vicient et al., 2001). In contrast, in the creeping bentgrass EST library retrotransposon sequences represented 1.4% of the total, an 8-fold higher representation than in the colonial library.

The Class II DNA elements are found in lower copy numbers than the retrotransposons. The bentgrass ESTs were searched with several Class II DNA element coding sequences and some significant matches were found (Table 2.8). Similar to the case with the retrotransposons, there was a 16-fold higher representation of the Class II DNA elements among the creeping bentgrass ESTs, relative to the colonial bentgrass ESTs.

This difference in the transposable element representation between the creeping bentgrass and colonial bentgrass ESTs may be related to the metabolic state of the tissues used for cDNA library construction. The creeping bentgrass sample was exhibiting symptoms of dollar spot disease, whereas the colonial bentgrass sample was not. Transposable elements can be activated by various abiotic and biotic stresses, including pathogen attack (Casacuberta & Santiago, 2003; Grandbastien, 1998; Kimura et al., 2001; McClintock, 1984). The difference in transposable element representation in the two EST libraries is suggestive of an activation of the transposable elements in the creeping bentgrass plant due to the stress of infection by the dollar spot fungus.

Conserved ortholog set cDNAs

An important feature of colonial and creeping bentgrasses is that they arose by polyploidization. The evidence for this comes from extensive cytological investigation into some *Agrostis* spp. and their interspecific hybrids by (Jones, 1956a,b,c). Based on the cytological results, Jones proposed a model for genome organization of creeping and colonial bentgrass in which both species were considered to be allotetraploids, having one ancestral genome in common. Both species were found to have 14 chromosome pairs. The genome organization of colonial bentgrass was designated as $A_1A_1A_2A_2$ and that of creeping bentgrass $A_2A_2A_3A_3$ (Jones, 1956a,b,c). The A_1 subgenome of colonial bentgrass was considered to be related to the diploid species *A. canina* L. (velvet bentgrass). The diploid origins of the A_2 and A_3 genomes were unknown. Analysis of marker segregation in creeping bentgrass demonstrated that inheritance is strictly disomic so distinct subgenomes are expected (Chakraborty et al., 2005).

Conserved ortholog set (COS) genes are single copy genes that have been conserved throughout evolution (Fulton et al., 2002). Similar COS sequences among species have, therefore, been considered to be orthologous. As allotetraploids, both creeping and colonial bentgrass would be expected to have homoeologous COS genes. Sequence comparisons and mapping of orthologous creeping and colonial bentgrass COS genes will be helpful in the identification of the subgenomes of both species. However, such an assessment should be considered preliminary because it ultimately requires positional information of the two related sequences within their

genomes (Swigonova et al., 2004). COS genes have been identified in rice (1,290) in the Michelmore lab (http://cgpdb.ucdavis.edu/COS Arabidopsis/). We therefore compared the creeping and colonial bentgrass EST sequences to the rice COS genes. Orthologs of 177 and 161 rice COS genes were found among the colonial and creeping ESTs, respectively. For some of the COS genes represented in the bentgrass ESTs, multiple sequences for each species were obtained. Phylogenetic analyses of some of these homoeologous sequence sets revealed that some creeping and colonial sequences were more similar to each other than they were to the other similar sequences from within each species. These results are consistent with expectations based on the previous cytological work. For cases where multiple colonial and creeping bentgrass ESTs were obtained for a COS gene and for which two distinct sequence types were found among the colonial and the creeping ESTs, predictions regarding the subgenome assignment could be made from phylogenetic analyses of the sequences. Since the A_2 genome is shared between colonial and creeping bentgrass (Jones, 1956a,b,c), presumably the A_2 genome sequences will be more similar to each other than to the A_1 or A_3 genome sequences.

Phylogenetic analysis of two COS gene sequences is shown in Fig. 2.1. Representative colonial and creeping bentgrass EST sequences were chosen for the analysis based on a preliminary analysis of all the similar sequences obtained. The similar oat (*Avena sativa* L.) sequence was used to root the trees. The genera *Agrostis* and *Avena* are taxonomically grouped in sister clades in the tribe Aveneae (Soreng & Davis, 1998). The phylogenetic tree in Fig. 1A is from sequences closely

related to the rice COS gene encoding the photosystem II 10K protein (rice COS 29683030). This analysis included 384 bases of coding sequence, beginning with the ATG start codon, and 136 bases of 3' untranslated sequence. The colonial and creeping sequences that are most similar to each other were assigned to the A_2 subgenome. The other colonial and creeping bentgrass sequences were therefore assigned to the A_1 and A_3 subgenomes, respectively.

The phylogenetic tree in Fig. 1b is based on sequences similar to the coding sequence of an uncharacterized protein (rice COS 8954978). Full-length ESTs were not recovered for all the representative bentgrass sequences. The region of shared sequence was therefore used for the phylogenetic analysis, which was based on 402 bases of coding sequence and 73 bases of 3' untranslated sequence. Again, the colonial and creeping bentgrass sequences most closely related to each other were assigned to the A_2 genome. This phylogenetic analysis was used to make subgenome assignments of the colonial bentgrass linkage groups (described in chapter 4).

Novel gene discovery

Most of the colonial (87%) and creeping (73.1%) bentgrass ESTs had BLASTX matches (cutoff $1e^{-10}$) when compared individually to the NCBI nonredundant database. However, a significant fraction, 13% of the colonial and 26.9% of the creeping, had no BLASTX match. These numbers differ from those regarding the FunCat distribution of unigenes (Table 2.2), because the catalog used by the FunCat

program is based on the *Arabidopsis* genome annotation (Ruepp et al., 2008). Numerous genes have been reported from other species that are not found in *Arabidopsis* and are not included in the FunCat annotations.

The colonial and creeping bentgrass ESTs with no BLASTX match to the NCBI nonredundant database were compared to the EST-others database using a BLASTN search to determine if similar sequences from other species have been recovered in ESTs but are not yet annotated regarding protein coding sequences. To minimize the number of ESTs whose sequence was only of a 3' noncoding region, only ESTs with a sequence greater than 400 nucleotides were considered for this analysis. Of the 686 and 668 colonial and creeping ESTs, respectively, that met this criterion, 238 colonial bentgrass and 345 creeping bentgrass ESTs did have a match to the EST-others database. These were mainly to sequences from other grasses in the genera *Avena, Festuca, Hordeum, Lolium*, and *Triticum*. These sequences are presumably for genes common to other grasses but not present in the fully annotated rice genome (The International Rice Genome Sequencing Project, 2005).

There were 448 and 323 colonial bentgrass and creeping bentgrass ESTs, respectively, that did not have any match to the EST-others database. These sequences may thus represent novel genes not reported from any other plant species. The bentgrasses are not food crops and have not been subjected to thousands of years of human selection, as have the cereal grasses, which represent most of the monocot sequence data currently available. The bentgrasses have

evolved in response to nonhuman selection pressures and it is, therefore, not surprising that some novel genes, relative to the cereals, would be found in their genomes.

Potential EST simple sequence repeats

As the next step in developing genetic maps for both species, the ESTs were analyzed for the presence of simple sequence repeats (SSRs), which are direct tandem repeats of a short nucleotide sequence. SSRs have been found to be frequent and widely distributed in many plant species (Morgante, 1993). Markers based on SSRs are commonly used in plant genome mapping since they are easy to use and tend to be highly polymorphic (Powell et al., 1996). SSRs within ESTs are particularly useful for mapping since they represent genes (Varshney et al., 2005). Repeat Masker was used to screen the creeping and colonial bentgrass ESTs for the presence of simple sequence repeats. Di-, tri-, and tetranucleotide repeats were identified. One hundred and forty-two creeping bentgrass potential SSRs were found for a frequency of about 1.7%. Two hundred and sixty-six colonial bentgrass potential SSRs were identified for a frequency of 3.5%. The number of each repeat type and the most common repeats are included in Table 2.9.

Discussion

These two EST resources represent the largest sequence repository for the bentgrasses to date. As such, they provide a valuable resource for the discovery of polymorphisms for marker development. Analysis of these libraries led to the development of the gene based markers used in the construction of the colonial bentgrass linkage map. This opens the door for other investigators working on related species to perform comparative mapping studies utilizing information gained from these sequences. Since the RNA for these libraries was taken from field samples under disease stress, these libraries have increased potential for aiding in the discovery of novel genes that might be expressed in response to disease pressure.

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Table 2.1. *Characteristics of the colonial and creeping bentgrass EST sequences.*

	Colonial ESTs		**Creeping ESTs**	
EST count	7,528		8,470	
Total nucleotides	5,611,738		4,809,144	
Average EST length (nt)	745		567	
Unigenes	4,425		4,747	
Multimember	1,095		980	
Singleton	3,330		3,767	
Average unigene length (nt)	795		555	
Apparent coding potential	70.48%		54.53%	
Colonial/Creeping shared ESTs	5,754	(76.4%)	6,911	(81.6%)
Colonial/Creeping unique ESTs	1,774	(23.6%)	1,559	(18.4%)
BlastX match	6,548	(87.0%)	6,190	(73.1%)
No BlastX match	980	(13.0%)	2,280	(26.9%)
Chloroplast sequences	38	(0.5%)	123	(1.4%)
Microbial sequences	184	(2.4%)	99	(1.2%)

Table 2.2*. Distribution of the Functional Classes of Colonial and Creeping Bentgrass Unigenes.*

Category	Colonial ESTs[a]		Creeping ESTs[b]	
Number of unigenes represented	1843	42%	1048	28%
Number of unigenes with no match	2582	58%	3699	78%
Total unigenes	4425	100%	4747	100%
Metabolism	500	19.40%	244	18.30%
Energy	256	9.90%	134	10.10%
Cell growth, cell division, DNA synthesis	62	2.40%	33	2.50%
Transcription	172	6.70%	63	4.70%
Protein synthesis	122	4.70%	120	9.00%
Protein destination	151	5.90%	102	7.70%
Transport facilitation	159	6.20%	65	4.90%
Cellular transport and transport mechanisms	86	3.30%	40	3.00%
Cellular biogenesis	119	4.60%	48	3.60%
Cellular communication/signal transduction	264	10.20%	129	9.70%
Cell rescue, defense, cell death and ageing	262	10.20%	119	8.90%
Ionic homeostasis	2	0.10%	1	0.10%
Cellular organization	358	13.90%	155	11.70%
Motility	1	0.04%	0	0%
Tissue specificity	0	0%	0	0%
Development	47	1.80%	19	1.40%
Transposable elements, viral and plasmid proteins	10	0.40%	57	4.20%
Organism specific proteins	3	0.10%	0	0%
Number of matches[c]	2574		1329	
Classification not yet clear-cut	409		155	
Unclassified proteins	845		384	
Total number of matches	3828		1868	

[a]Out of 4425 colonial bentgrass unigenes, 1843 (42%) could be assigned a putative function at 1e-10, which includes unclassified proteins and those whose classification is not yet clear-cut.
[b]Out of 4747 creeping bentgrass unigenes, 1048 (28%) could be assigned a putative function, which includes unclassified proteins and those whose classification is not yet clear-cut.
[c]Number of matches exceeds the number of unigenes represented because the same unigene may have more than one match from different role categories

Table 2.3. *Most abundant coding sequences among the colonial bentgrass ESTs and comparison with the number of similar creeping bentgrass ESTs. In this analysis cDNA clones that may originate from different members of a gene family but that encode the same protein were combined.*

Accession [a]	Gene Identification	# Colonial ESTs (%)	# Creeping ESTs (%)
DV856307	Chlorophyll a/b binding, XP_464478	205 (2.72)	41 (0.48)
DV859324	Rubisco small subunit, AAF07942	145 (1.92)	112 (1.32)
DV859428	Carbonic anhydrase, P40880	101 (1.34)	55 (0.65)
DV859387	Rubisco activase, AAP83927	87 (1.15)	41 (0.48)
DV857642	Metallothionein, type 3, CAD88266	82 (1.09)	6 (0.07)
DV855046	FBP aldolase, AAF74220	69 (0.92)	6 (0.07)
DV855915	Glyceraldehyde-3-phosphate dehydrogenase XP_472744	61 (0.81)	19 (0.22)
DV859702	Photosystem II 10 kD, XP_480562	59 (0.78)	46 (0.54)
DV858645	Lipid transfer protein, CAA65680	41 (0.54)	11 (0.13)
DV856328	Glycine decarboxylase P subunit, AAB82711	40 (0.53)	9 (0.11)
DV854079	Lea5 protein, CAA55482	38 (0.50)	0
DV856704	Tetraubiquitin, CAA49200	37 (0.49)	14 (0.16)
DV854202	ACC oxidase, AAU44031	28 (0.37)	4 (0.05)
DV855859	Photosystem II 23 kD, CAA40669	25 (0.33)	13 (0.15)
DV856488	Alanine aminotransferase, AAO84040	25 (0.33)	14 (0.16)

[a] Accession number of a representative colonial bentgrass EST.

Table 2.4*. Most abundant coding sequences among the creeping bentgrass ESTs and comparison with the number of similar colonial bentgrass ESTs. In this analysis cDNA clones that may originate from different members of a gene family but that encode the same protein were combined.*

Accession [a]	Gene Identification	# Creeping ESTs (%)	# Colonial ESTs (%)
DV862209	Rubisco small subunit, AAF07942	112 (1.32)	145 (1.92)
DV862120	Carbonic anhydrase, P40880	55 (0.65)	101 (1.34)
DV867848	Photosystem II 10 kD, XP_480562	46 (0.54)	59 (0.78)
DV862831	Rubisco activase, AAP83927	41 (0.48)	87 (1.15)
DV866945	Chlorophyll a/b binding, XP_464478	41 (0.48)	205 (2.72)
DV863394	Unknown rice protein, XP_550450	26 (0.31)	0
DV867236	Cyp709C1 fatty acid hydroxylase, AY641449	21 (0.25)	10 (0.13)
DV861467	12-oxo-phytodienoic acid reductase, BAD35825	20 (0.24)	9 (0.12)
DV861272	Glyceraldehyde-3-phosphate dehydrogenase, XP_472744	19 (0.22)	61 (0.81)
DV866058	Phosphoribulokinase, CAB56544	17 (0.20)	7 (0.09)
DV861115	Alanine aminotransferase, AAO84040	14 (0.16)	25 (0.33)
DV867434	Tetraubiquitin, CAA49200	14 (0.16)	37 (0.49)
DV864646	Photosystem II 23 kD, CAA40669	13 (0.15)	25 (0.33)
DV861049	ABA-inducible protein, XP_479573	12 (0.14)	1 (0.01)
DV867996	Thioredoxin h isoform 1, AAP72290	11 (0.12)	1 (0.01)

[a] Accession number of a representative creeping bentgrass EST

Table 2.5. *Most abundant unique colonial bentgrass ESTs.*

Accession [a]	Gene Identification, Accession	# ESTs
DV857428	Lea5, CAA55482	38
DV855748	Ferritin, AAT67051	11
DV857725	Glutamate decarboxylase, AAM47304	9
DV860028	Wheat Lrr14, AAK20742	9
DV855253	Jacalin-like protein LEM2, AAM18206	8
DV855054	Reversibly glycosylated protein, CAA77237	7
DV858431	Sorbitol dehydrogenase, XP_483619	6
DV856443	Unknown rice protein, BAD38379	6
DV852804	1,3; 1,4-Endoglucanase, AAU10802	5

[a] Accession number of a representative colonial bentgrass EST

Table 2.6. *Most abundant unique creeping bentgrass ESTs.*

Accession [a]	Gene Identification, Accession	# ESTs
DV863394	Unknown rice protein, XP_550450	26
DV861141	Unknown rice protein, NP_917975	7
DV863838	Unknown barley protein, AAM22814	6
DV860902	Unknown rice protein, AAT85231	5
DV865227	COX VIIa-like protein, AAT36216	5
DV868724	Calcium ion binding, AAP68277	5

[a] Accession number of a representative creeping bentgrass EST

Table 2.7. *Disease response colonial and creeping bentgrass ESTs.*

Accession[a]	Gene Identification	# Colonial ESTs		# Creeping ESTs	
DV859732	Disease resistance gene-like	38	(0.5%)	11	(0.1%)
Pathogenesis-related proteins					
DV858600	PR-1 protein	2		0	
DV855135	PR-2, Beta-1,3-glucanases	13		7	
DV856397	PR-3, Class I, III, IV chitinases	20		8	
DV856896	PR-4, Endochitinase	3		2	
DV856236	PR-5, Thaumatin-like proteins	3		7	
DV856911	PR-6, Protease inhibitors	3		16	
DV854233	PR-9, Peroxidases	14		3	
DV857430	PR-10, Ribonucleases	2		1	
DV864619	PR-12, Defensins	0		2	
DV855553	PR-15, Germin/oxalate oxidase	1		3	
	Total	61	(0.8%)	49	(0.6%)

[a] Representative accession. For most of these protein categories multiple distinct sequences are known in other species. The representative accession therefore may not be highly similar to all the EST sequences identified for that category.

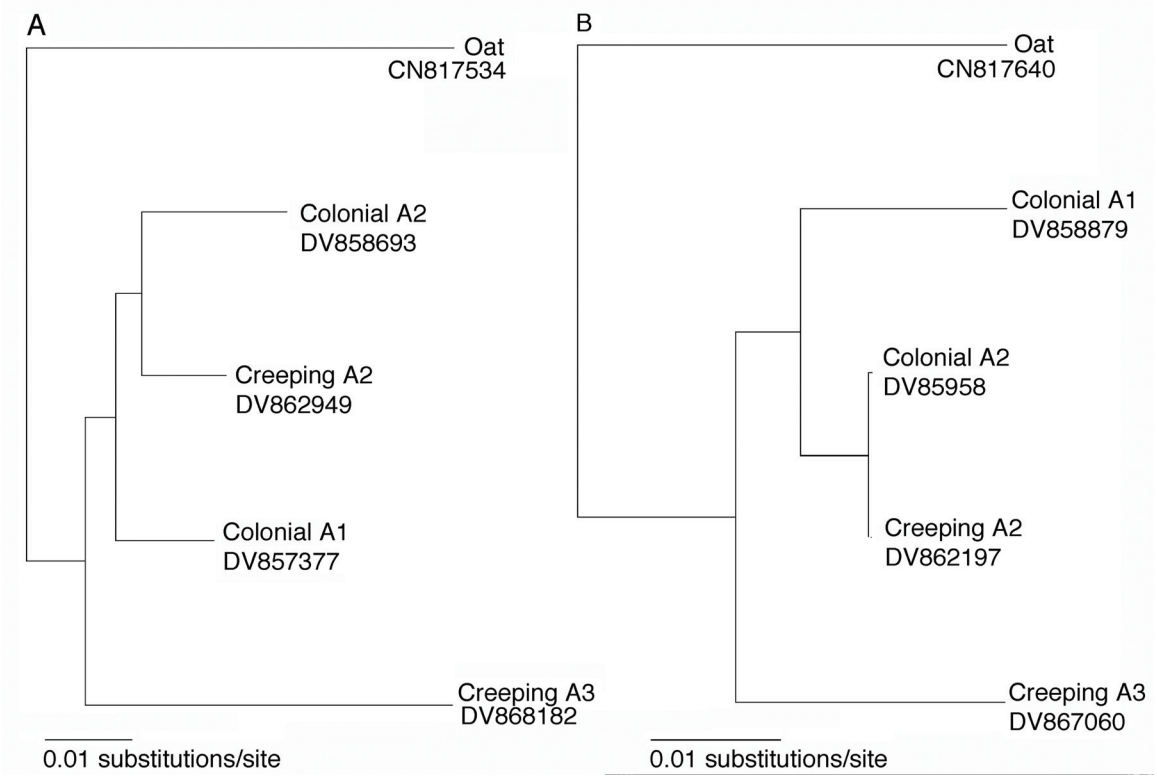
***Table 2.8**. Colonial and creeping bentgrass transposable element ESTs.*

	# Colonial ESTs		# Creeping ESTs	
Class I – Retroelements				
Ty1-copia-like elements	7		16	
Ty3-gypsy-like elements	3		88	
Unclassified retrotransposon	4		16	
Total	14	(0.18%)	120	(1.4%)
Class II – DNA Transposons				
hAT Superfamily	0		2	
CACTA En/Spm	1		12	
Total	1	(0.01%)	14	(0.16%)

***Table 2.9**. Characteristics of the creeping and colonial bentgrass potential EST-SSRs.*

Species	Repeat type	Number	Most Common Type
Creeping	Dinucleotide	34	TA (20)
	Trinucleotide	87	CTG and TTC (16)
	Tetranucleotide	21	CATA (11)
Total		142	
Colonial	Dinucleotide	24	CA (12)
	Trinucleotide	195	CCG and CTG (42)
	Tetranucleotide	47	CATA (25)
Total		266	

Figure 2.1. *Rooted neighbor-joining phylogenetic trees comparing representative colonial and creeping bentgrass COS genes for subgenome assignment. The similar oat sequences were designated as the outgroup for rooting. A. Photosystem II 10K protein. B. Uncharacterized COS gene.*



Chapter 3

Three methods to exploit sequence based polymorphisms for construction of a genetic linkage map of colonial bentgrass

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Three methods to exploit sequence based polymorphisms for construction of a genetic linkage map of colonial bentgrass

Introduction

In constructing a genetic map, researchers take great care in determining what type of markers to incorporate. The term "marker" basically refers to any polymorphism that is observed to segregate within the mapping population. Each marker brings with it an array of positives and negatives that must be weighed. Many times there are constraints imposed by the type of mapping population being used and the information available. When Morgan and his students embarked on the first genetic map of *Drosophila* he counted the segregation of visual morphological traits (Jenkins, 2003). The success of this work led to maps made of other species and the search for segregating traits. Current genetic linkage mapping uses almost exclusively molecular markers and is based on DNA sequence polymorphisms between parents whose segregation is followed in their progeny.

The previous chapter highlighted the value of EST libraries for gene based marker development. With the rapidly increasing availability of EST sequences, numerous approaches to developing markers based on these sequences have been reported (Gupta et al., 2005). Single nucleotide polymorphisms (SNPs) and small insertion/deletions (indels) are the most widespread types of polymorphisms in

both plant and animal genomes (Berger et al., 2001; Gut, 2001; Rafalski, 2002a; Rafalski, 2002b; Sachidanandam et al., 2001). Although SNPs and indels are relatively common, development of gene-based markers for mapping based on them can be difficult.

The types of molecular markers can be divided into two broad categories, random and gene based. Of the random markers, the simplest are known as random amplified polymorphic DNA (or RAPDs). They involve the amplification of genomic DNA with short (around 10 bp) random primers. They quickly became popular with researchers because they are simple to modify to different species and relatively inexpensive to use and screen large populations. Maps of a number species have been made using these markers (Giorio et al., 1997; Gore et al., 2002; Liu et al., 2006; Sharma et al., 2008). RAPDs are also a common tool used for genetic diversity studies. RAPDs are easy to implement but they have long been plagued with reproducibility issues (Muralidharan & Wakeland, 1993). Additionally, RAPDs are not co-dominate and it is difficult to determine if identical migrating amplification products from different individuals actually represent the same loci. These issues alone make RAPDs not an option for use as anchor markers between different mapping populations. A related method known as amplified fragment length polymorphism (AFLPs) was developed in 1995 in an effort to maximize the utility of RAPDs while minimizing its reproducibility issues. AFLP uses restriction enzyme-digested genomic DNA as the template for a PCR reaction with primers that contain the restriction enzyme recognition site as well as a number of additional random

nucleotides (Vos et al., 1995). This method allows for more specificity while still being able to identify a large number of random markers distributed throughout the genome. We utilized AFLPs as one marker type for the creation of our colonial genetic linkage map. Since AFLPs can be generated relatively quickly they were used to develop a framework on which to map the gene-based markers. The AFLP reactions were carried out by our collaborator Scott Warnke of the USDA. Analysis of the data was carried out at Rutgers.

Sequence tagged sites as markers

Introduction

One of our motives in linkage mapping of colonial bentgrass was to compare its genome organization with other grass species. Such comparisons require mapping of genes. AFLP's are useful for generating maps with many markers to identify QTLs within a population. However, since genes are not mapped with these methods, the maps cannot be used for comparisons with other populations of the same species or for comparisons of genomic organization among other species. We therefore also concentrated on other methods that map genes.

The standard approach for mapping genes in plants has been RFLPs (Lander & Botstein, 1989). This procedure entails digesting genomic DNA with restriction enzymes and blotting the resulting DNA fragments onto a nitrocellulose membrane. The membrane is probed with a cDNA to reveal the polymorphisms. This method is considered very reliable and has been used to make maps in many plant species. However, it requires large amounts of DNA per individual, which is laborious and expensive to obtain in a large population size. It would be more desirable to use a PCR-based approach to mapping genes.

Exploiting polymorphisims in the 3' non-coding regions of expressed genes may be a good approach to developing a PCR based marker system. The 3' untranslated regions (UTR) of genes can be a source of polymorphisms due to the lower selective

pressures to which they are subjected compared with the coding regions (Bhattramakki et al., 2002). By taking advantage of this characteristic, sequence tagged site (STS) markers can be created which can combine the ease of use of RAPDs with the specificity of SSRs. This method is well suited to our population, since we were looking for polymorphisms between two species within our backcross population.

The method itself consists of using sequence data to determine the 3' noncoding region of the gene of interest. Primers were designed to amplify a region starting roughly 50 bp upstream of the stop codon and ending before the poly-A tail. In the most ideal situation, copies of the gene of interest were found in both the creeping and colonial bentgrass EST sets. This permits the alignment of sequences and the discovery of potential insertion-deletion sites that could be exploited. Whenever possible primers were designed to selectively amplify the colonial version of the allele. Potentially this could increase the chances of discovering a useful polymorphism. Initially, the parents were screened to determine if a useful polymorphism exists. A useful polymorphism is one where one can visualize a difference between the hybrid parent (TH15) and both the creeping bentgrass parent (9188) and grandparent (5061). This ensures that the source of the allele is from the colonial bentgrass portion of TH15 genome. PCR products were then separated on a MetaPhor agarose gel and evaluated for the existence of polymorphisms. MetaPhor (Cambrex Bio Science, Rockland, ME) is a high resolution agarose which can resolve DNA fragments that differ in sizes by 4 bp. When a

potential maker was discovered the procedure was repeated on the mapping population which was scored for segregation of the colonial bentgrass specific marker.

Methods

Selection of amplification primers

Using the BLASTX results, the presumed stop codon and 3' UTR was identified. In each sequence a reverse primer was selected in the 3' UTR and a forward primer was selected 20 to 40 bp upstream of the stop codon. Ideally, the primers selected were 21 bp, G+C contents +50%, with minimal secondary structure and no significant inter-primer complementary. However, due to the limited range of sequence sometimes available, this was not always achieved.

Screening candidate markers for polymorphisms

Primer pairs were screened against the parents (TH15 hybrid, 9188 creeping bentgrass), the creeping grandparent (5061), as well as an unrelated colonial bentgrass plant. The amplified products were evaluated for fragments that were specific for TH15, indicating they originated from its colonial bentgrass parent. One hundred ng of DNA was used per 25ul PCR reaction which contained 10mM Tris-HCl, pH 8.3, 50mM KCl, 2.5mM $MgCl_2$, 0.2mM of each dNTP, 0.5ug of each primer, and 1ul (2 units) Taq polymerase (Life Technologies, Gaithersburg, Maryland). PCR was carried out in a GeneAmp 9600 thermocycler (Perkin Elmer Corp., Foster City, CA). The PCR cycling parameters were 94C for 7 minutes followed by 30 cycles of

94C for 30 s denaturing, 55C for 30 s annealing, 72C for 1 min extension, and finally followed by 72C for 10 min. The resulting PCR amplification products were separated on a 3% Metaphor agarose gel (Cambrex Bio Science, Rockland, ME).

Marker Segregation Analysis

The same PCR conditions were used and the resulting products were run on a 3% Metaphor agarose gel.

Results

Primer design

When evaluating a sequence for use as a potential marker the initial step is the evaluation of the 3' noncoding region through the determination of the stop codon preceding the poly-A tail. Figure 3.1 shows the results of a typical EST sequence (in this case DV859794 mapped to linkage group 4A1) that has been searched against the non redundant (NR) protein database using the BLASTX function at the NCBI website. The E value ($5e^{-45}$) indicates this is a significant match to the database and, additionally, the sequence aligns in the correct frame and direction.

These data are then used to annotate the sequence to determine the stop codon and the downstream 3' UTR. Figure 3.2 depicts the CLUSTAL/W pairwise alignment of the DV859794 deduced protein sequence with its best BLASTX match, phospholipid hydroperoxide glutathione peroxidase from rice (CAC17628.1). The asterisk (*) indicates the predicted stop codon and the shaded region is the 3' untranslated

region that was used to construct a marker. Primers were designed to amplify the designated region using the Primer3 program under conditions highlighted in the methods.

Screening of markers for polymorphisms.

Ten percent of all the primer pairs screened generated some type of polymorphism which was useful for linkage analysis (Figure 3.3). Markers that generated null amplification alleles were the most common (Figure 3.4a). Thirty five percent of the markers screened generated co-dominate alleles (Figure 3.4b). Successful markers segregated in a Mendelian fashion within our population. A marker is judged as successful if one of the amplification products is of the predicted size and there is a band present in TH15 (hybrid parent) and absent from 9188 (creeping bentgrass parent) and 5061 (creeping bentgrass grandparent). Such amplification products originated from the original colonial bentgrass parent of the hybrid. An unrelated colonial bentgrass sample was included for interest only. Figure 3.4 shows the segregation of a marker across the mapping population using the STS method.

Discussion

The variability in the 3' untranslated region of expressed genes allows for its use as a target region for efficient marker development. Though this technique is limited in its ability to exploit all the polymorphisms that may be present, it does have great value for its ease of use. One can maximize results by utilizing preexisting sequence information and taking a proactive approach in designing primers to selectively amplify certain alleles of interest. There are two different types of polymorphisms

that were observed using this method. The most common type was plus/minus polymorphisms which are illustrated in Figure 3.4A. A plus/minus polymorphism results from primers that are specific to the colonial bentgrass allele. There was not successful amplification of the creeping bentgrass allele because of insertion/deletions or sequence polymorphisms that existed at the primer annealing site (leading to no band being detected). A second type of polymorphism that was observed was a co-dominate marker, as illustrated in Figure 3.4B. Here polymorphisms result from insertion/deletions present within the amplified region which generate different size fragments. Lane 3 of Figure 3.4B shows the hybrid containing both the creeping and colonial bentgrass versions of this allele. Lane 3 and 4 are the creeping bentgrass parent (9188) and grandparent (5061) respectively. Lane 5 is an unrelated colonial bentgrass plant. As can be seen the upper band, migrating at roughly 180bp, is the creeping bentgrass allele. The lower band, migrating at roughly 160bp, is the colonial bentgrass allele. In this case the marker is co-dominate because both alleles can be detected and followed for segregation. However, since we were mapping the colonial portion of the hybrid genome, co-dominate markers were only scored for segregation of the colonial bentgrass allele across the mapping population.

Minisequencing

Introduction

The 3' untranslated region in genes is a valuable resource to exploit for marker development. However, it does not represent all the variability contained within the genome. The most common type of polymorphisms are in fact variations of single base pairs that differ between alleles. These are known as single nucleotide polymorphisms (SNPs) (Rafalski, 2002b). Often these single base differences are silient mutations and have no effect on the resulting amino acid sequence (and therefore the final protein). Because of this they are not subjected to selection pressure and persist within populations (Arbiza et al., 2006; Nephawe & Spurlock, 2007). Minisequencing is a method which allows for the exploitation of known SNPs as markers (Carvalho & Pena, 2005). The technique consists of an initial PCR reaction which amplifies a region containing the SNP of interest. Unincorporated primers and dNTPs are then inactivated. A primer extension reaction is then performed starting from a specific primer designed to anneal one base upstream from the target SNP site. In this reaction a single, fluorescently labeled dideoxy nucleotide is incorporated in the target oligo in individuals which have the SNP corresponding to the allele of interest. In a modified version of this technique the now labeled oligo is then separated on a polyacrylamide gel and evaluated.

Methods

Selection of initial amplification primers

Unigenes contained within the creeping and colonial bentgrass libraries were determined using the CAP3 program as described in Chapter 2 (Huang & Madan,

1999). Unigenes which contained both creeping and colonial bentgrass sequences were initially selected for sequence analysis. Using the MegaAlign program (DNA*) alignments were made from creeping and colonial sequences and evaluated for potential SNPs. Oligos were designed to amplify a 200 base pair region surrounding the site of interest.

DNA extraction and PCR analysis

Genomic DNA was isolated by using a commercial kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO). One hundred nanograms of genomic DNA was used per 20 ul PCR reaction which contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.14 ug of each primer, and 0.2 ul (1 unit) Taq polymerase (Applied Biosystems Inc, Foster City, CA). PCR was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems Inc.). The initial denaturation was conducted at 94°C for 4 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 1 min extension at 72°C, followed by a final extension at 72°C for 10 min. When screening the parental samples for polymorphisms, 15 ul of the PCR product was checked on a 3% MetaPhor agarose gel (Cambrex BioScience Rockland, Inc., Rock- land, ME) to confirm that a single DNA fragment was amplified.

Minisequencing reaction

Five microlitre of the PCR product was transferred to a new tube and treated with 2 ul (1 unit) ExoSAP-IT (USB, Cleveland, Ohio) to remove unincorporated primers and

excess dNTPs that would interfere with the dideoxy termination reactions. The ExoSAP-IT reaction was carried out at 35°C for 15 min followed by 80°C for 15 min to inactivate the enzymes.

The entire 7 ul cleaned PCR product was used for a new 20 ul PCR reaction which contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of the Cy-5 fluorescently labeled dideoxy nucleotide being used, 0.14 ug of the sequencing primer, and 0.2 ul (1 unit) Taq polymerase (Applied Biosystems Inc, Foster City, CA). PCR was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems Inc.). The initial denaturation was conducted at 94°C for 4 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 1 min extension at 72°C, followed by a final extension at 72°C for 10 min.

The reaction was stopped by adding 15 ul of stop solution (95% formamide, 0.2 M NaOH, 0.05% bromophenol blue) to each tube. The reaction products were denatured by heating the mixture at 94°C for 5 min and immediately cooling on ice. The Cy-5 labeled dideoxy terminated olgios were separated on a 6% polyacrylamide gel containing 8 M urea and visualized on a Storm imaging system (GE Healthcare, Piscataway, NJ).

Results

Figure 3.6 is a diagram depicting the steps necessary for the minisequencing method. Before you can begin this step, intensive sequence analysis must be performed to identify sites that potentially contain nucleotides which are

polymorphic. Figure 3.6A illustrates the initial amplification of the target region. This step serves to ensure an adequate amount of the target region is present for the subsequent steps. The principle of minisequencing is based on an oligo which binds one base pair upstream of the potential polymorphic site. Figure 3.6B shows the SNP and the location of the minisequencing primer in relation to it. If the particular nucleotide in question is present in the sample, the fluorescently labeled didoxy nucleotide will be incorporated into the oligo, labeling it for detection (Figure 3.6C). Figure 3.7 illustrates the minisequencing method performed on 5 selected ESTs with dideoxycytidine triphosphate (ddC). The primer sequences used for PCR amplification of the ESTs and the minisequecing oligo are given in Table 3.2. The labeled oligo is visible as a single band present in each lane (if the sample contains the appropriate SNP). Fragments unique to the interspecific hybrid were detected in sample 4. This is indicated by the unique band in lane a. Although for our purpose we are not following the polymorphisms between the two creeping bentgrass plants in the pedigree, polymorphisms between them can be observed in Fig. 3.7 in EST samples 3 and 5. Samples 1 and 2 revealed no polymorphisms at the particular site being investigated. In those cases, all the samples contained a cytosine at the site in question revealing no polymorphisms. The CLUSTAL/W alignment of sequences that lead to the selection of primers for sample 4 are shown in Figure 3.8. Here the expected SNP is indicated with the black box. The corresponding minisequencing sequence can be seen aligning one bp upstream of the SNP. This marker was screened against the mapping population and the polymorphism segregated in the expected Mendelian fashion.

Discussion

With the appropriate sequence data available, the minisequencing method can be effective in identifying single nucleotide polymorphisms for linkage mapping in colonial bentgrass. The upper range of the efficiency for discovering polymorphims is always the inherent variability of the parents selected for the mapping population. In using the minisequencing method, the amount of informative sequence data may be just as important. Only one particular site is being examined for the presence of polymorphisms. Contrast that with the STS tagging method where any insertion/deletion contained within the amplified region may generate a useable size variation. Care must be taken that the appropriate number of sequences are being compared to determine which site may contain a polymorphism. Much of the variation present across genotypes is contained as SNPs. Other PCR based methods such as the aforementioned STS tagging may not have the adequate sensitivity to take advantage of this. The minisequencing method can be an effective way to take advantage of single nucleotide polymorphisms and convert sequence information (when it is adequately available) to gene based markers.

Dideoxy polymorphism scanning

Introduction

Single nucleotide polymorphisms (SNPs) and small indels are the most widespread types of polymorphisms in both plant and animal genomes and SNPs are becoming the marker type of choice (Berger et al., 2001; Gut, 2001; Rafalski, 2002a; Rafalski, 2002b; Sachidanandam et al., 2001). Although SNPs and indels are relatively common, development of gene-based markers for mapping based on them can be difficult. Methods previously mentioned, allele-specific PCR (STS tagging) and minisequencing, rely on sequence availability from multiple individuals and target known SNPs for marker development (Drenkard et al., 2000; Syvanen, 2001). Other methods such as single-strand conformation, denaturing high-performance liquid chromatography, and heteroduplex cleavage are methods that can detect SNPs and indels with the advantage that they do not require prior knowledge of particular polymorphisms (Orita et al., 1989; Rungis et al., 2005). However, these methods can be technically difficult since they depend on precise experimental conditions or require specialized expensive equipment.

Regardless of marker type, the biggest problem in genetic linkage mapping using gene-based markers is the identification of polymorphisms between the parents of the population. Considerable effort is often expended in screening potential markers, many, and sometimes most, of which prove not to be useful.

To improve the efficiency of marker generation, we have developed a simple and relatively inexpensive method of polymorphism detection adapted from the dideoxy fingerprinting technique (Sarkar et al., 1992). Dideoxy fingerprinting has found widespread utility in mutant identification but has not been used previously for marker development and genetic linkage mapping (Felmlee et al., 1995; Martincic, 1999; Puck et al., 1997). The dideoxy fingerprinting method combines dideoxy chain termination DNA sequencing with a single-strand conformational polymorphism (SSCP) component by electrophoresis of single dideoxy chain-terminated fragments through a nondenaturing polyacrylamide gel. In our adaptation of this approach to EST marker development we have eliminated the SSCP component and the single dideoxy-terminated sequencing fragments are separated on a denaturing polyacrylamide gel.

Methods

Primer design

Primers were designed using the Primer3 program (http://www.frodo.wi.mit.edu/cgi-bin/primer3/pri- mer3_www.cgi) with default settings or by visual inspection of the DNA sequence (Untergasser et al., 2007). All primers were designed to have a Tm of 60°C. A product size of about 200 bp was used. The M13 (–21) sequence (5'- TGTAAAACGACGGCCAGT-3') was added to the 5' position of each forward primer.

DNA extraction and PCR analysis

Genomic DNA was isolated by using a commercial kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO). One hundred nanograms of genomic DNA was used per 20 ul PCR reaction which contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.14 ug of each primer, and 0.2 ul (1 unit) Taq polymerase (Applied Biosystems Inc, Foster City, CA). PCR was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems Inc.). The initial denaturation was conducted at 94°C for 4 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 1 min extension at 72°C, followed by a final extension at 72°C for 10 min. When screening the parental samples for polymorphisms, 15 ul of the PCR product was checked on a 3% MetaPhor agarose gel (Cambrex BioScience Rockland, Inc., Rock- land, ME) to confirm that a single DNA fragment was amplfied.

Dideoxy termination reaction

Five microlitre of the PCR product was transferred to a new tube and treated with 2 ul (1 unit) ExoSAP-IT (USB, Cleveland, Ohio) to remove unincorporated primers and excess dNTPs that would interfere with the dideoxy termination reactions. The ExoSAP-IT reaction was carried out at 35°C for 15 min followed by 80°C for 15 min to inactivate the enzymes. Reagents from the Amplicycle Sequence Kit (Applied Biosystems Inc.) were used to perform the dideoxy termination reactions. The reaction mix containing 5 ul water, 1 ul 10x cycling mix, and 0.02 ug Cy-5 5'-labeled M13 (-21) primer was added to new PCR tubes containing 2 ul termination mix of the specific dideoxy nucleotide being screened. To this mix, 2 ul of cleaned PCR

product was added. The PCR cycling parameters for the dideoxy termination reactions were, 94°C for 2 min followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. The reaction was stopped by adding 5 ul of stop solution (95% formamide, 0.2 M NaOH, 0.05% bromophenol blue) to each tube. The reaction products were denatured by heating the mixture at 94°C for 5 min and immediately cooling on ice. The Cy-5 labeled dideoxy terminated products were separated on a 6% polyacrylamide gel containing 8 M urea and visualized on a Storm imaging system.

Results

The ddPS method is illustated in Figure 3.10. Figure 3.10 shows the incorporation of the M13 primer to the 5' end of the final PCR product with the resulting products shown in Figure 3.9. The possible fragments that result from the dideoxy termination reaction are listed in 3.9. Figure 3.10 illustrates ddPS of PCR products from 8 randomly selected ESTs with dideoxythymidine triphosphate (ddT). The primer sequences used for PCR amplification of the ESTs are given in Table 3.3. Fragments unique to the interspecific hybrid were detected for EST samples 2, 3, 4, and 6. For samples 1 and 5 that did not have hybrid-specific polymorphic bands with the ddT-termination reaction, polymorphic bands were observed when screened with other ddNTPs. Although for our purpose we are not following the polymorphisms between the two creeping bentgrass plants in the pedigree, polymorphisms between them can be observed in Fig. 3.10 in EST samples 3, 5, 7, and 8.

Figure 3.11A depicts 5 positive and 5 negative mapping population individuals that are segregating for the polymorphism identified in sample 2 from Fig. 3.10. For this gene, which encodes a photosystem II 10 kD protein, several polymorphic bands were detectable and were inherited together in the mapping population. To understand the basis for the observed polymorphisms, the PCR products used were cloned and plasmids from several colonies were sequenced. From the hybrid PCR product two sequence types were recovered, whereas from the creeping grandparent and creeping parent samples, a single sequence type was recovered (Fig. 3.11B). The ddPS pattern observed with the hybrid PCR fragment reflects the presence of the two sequence types originating from a colonial bentgrass allele and a creeping bentgrass allele. In the sequence region depicted in Fig. 3.11A there is a T/C SNP and a single base indel between the creeping and colonial sequences. The T/C SNP results in a hybrid specific band originating from the colonial bentgrass allele. The single base indel results in a size shift in the dideoxy terminated fragments, also producing hybrid specific bands. The A/G and G/T SNPs in this sequence region generated hybrid specific bands when the ddPS reactions were terminated with ddA or ddG (data not shown). The ddPS method has also been adapted for capillary sequencing machines, which are available in many research laboratories. Figure 3.12 depicts the results of ddPS using the same primers, and ddT as the terminator, as shown in Fig. 3.11A. As expected, the same hybrid-specific polymorphisms are detectable. The data in Figure 3.12 were generated by Scott Warnke at the USDA.

Discussion

The dideoxy polymorphism scanning method described here has proved highly efficient for identification of gene-based polymorphisms for linkage mapping of colonial bentgrass. This method was used for 53 of the gene based makers on the colonial bentgrass genetic map (see Chapter 4). This method was also highly efficient in detecting polymorphisms between the two creeping bentgrass plants in the pedigree, indicating that it will be useful for pedigrees comprised of intraspecific crosses. For any marker type, the efficiency of marker development will depend on the inherent genetic polymorphisms between the individuals chosen as parents of the population and the sensitivity of the method used to detect the polymorphisms. The incidence of polymorphisms within cultivated plant species is known to vary widely (Shattuckeidens et al., 1990). The resequencing of parental and progeny DNA provides the ultimate in sensitivity for polymorphism detection and linkage (Furman et al., 2004), but will not be possible for most species of agronomic interest. Dideoxy polymorphism scanning provides a practical, yet sensitive and cost effective alternative. With this method one is scanning hundreds of bases at once for polymorphisms, improving the chances of detection. Screening the parental samples with all four dideoxy terminators would systematically scan the entire amplified DNA region for any polymorphisms. We anticipate that this method will be broadly applicable for marker development for mapping of many species. Since most of the time required to develop a gene-based linkage map is spent in identification of useful polymorphisms, this method will significantly shorten the time required for map generation and therefore reduce the overall cost. The biggest

advantage of this method is that no prior knowledge of a particular SNP or other sequence polymorphism is needed. Therefore, extensive sequence data from many individuals are not needed. For many crop plants, EST sequence data are available from a limited number of genotypes and the resources for generation of additional sequences are not available. Another advantage of this method is that it can be effective even with heterogeneous PCR products. This method is based on the Sanger dideoxy sequencing technology, but is not used to generate actual sequence data (Sanger et al, 1977). In systems such as the one described here, the PCR products are often heterogeneous due to the presence of heterozygous alleles and possibly alloalleles, since both colonial and creeping bentgrasses are allotetraploids (Jones, 1956). *Agrostis* species are self-incompatible outcrossing species resulting in individuals that are highly heterozygous. Therefore, even single-band PCR products can be heterogeneous. In the interspecific hybrid here, the PCR products may consist of the creeping bentgrass alleles as well as the colonial bentgrass alleles, as was observed for the example in Fig. 3.11A. Most attempts at direct sequencing of PCR products from the hybrid and the creeping bentgrass plants yielded uninterpretable mixed sequences (data not shown). With standard sequencing, a single base indel between two similar sequences in a mixed PCR product will generate mixed bands at all positions. Even without knowledge of specific SNPs or other sequence variations, and in the presence of heterogeneous PCR products, ddPS revealed segregating polymorphisms originating from the colonial bentgrass alleles. Dideoxy polymorphism scanning will thus be an ideal new approach for efficient identification of markers for mapping complex genomes

where direct sequencing of PCR products is not an option, such as those of polyploids and outcrossing heterozygous species. By focusing on sequence regions more likely to be variable, such as the 3' untranslated regions and introns, markers specific for individual members of multigene families may also be discovered.

This is also a cost-effective method of marker development. Only minimal sequence data are required. To save time and reagents, candidate markers do not need to be screened with all four dideoxy reactions. In general, candidate markers are screened sequentially in groups with a single dideoxy reaction until a clear polymorphism is found. For many candidates, only a single screening of the parents with one dideoxy is required prior to screening the mapping population. Manual sequencing kits provide reagents for 100 sequences but yield 400 reactions for ddPS, since only one dideoxy terminator mix is used for each reaction. This method was originally developed using manual sequencing and denaturing polyacrylamide gel electrophoresis. It has also been adapted for automated sequencing machines, which could improve the throughput of marker identification and population screening. One of the fastest growing areas of biotechnology research today is marker assisted breeding of crops, even minor crop species. The dideoxy polymorphism scanning method described here can provide a new highly efficient approach to marker development for linkage mapping and QTL analysis, especially for situations where resources are limiting.

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Table 3.1. *List of primers used for Figure 3.1.*

Figure 3-1	Accession	Forward Primer	Reverse Primer
A	DV859830	CACCTCCTCATGGGTTGC	GCATTTTATTCTTTAAATTTGCTTCTT
B	DV859826	GCCTTCGCCACCAACTTC	ATCTCGACTCGCCTTCACAC
C	DV859821	CAAGTTCCACTAGGCCGAAA	GACGATCAGAGATGCGATCC
D	DV859820	AGCTGCCTCTGACCAGAAAA	CAATGCCACTGAGGTACACAA
E	DV859812	GGCAAGGCAAAAGATGTAGG	GAACAAGAGTAGCGCAGATGA
F	DV859818	AAGACCAGCGGTTAATCGAA	CCGAAAGCCTGACTCTGAAC
G	DV859804	CAAGTGGCAGAGGAAGCTG	AAGGTGGCATCTGAGGAGAA
H	DV859803	GAGGAGGAGGACGCACACTA	TTTGCTCTAGACATAGCAGCAGT
I	DV859802	GCAAGCACAGGACATCAGTC	TTCACGACAGCTCACTCACC
J	DV859798	GGGCGTTTACCAGTTTGTTG	TGATTCCTTAGTTGGCGAGAG
K	DV859806	GGACCCAGAAGTATGCGATG	TGCTCACTGAAGGGTTGAAG
L	DV859797	CCGTTGGTGTGAACTTTGTG	TGAAGCATCACTTGGAACCT
M	DV859794	CCGTTGGTGTGAACTTTGTG	TGAAGCATCACTTGGAACCT
N	DV859794	CAAATATGCAAACCGAAAGG	AATAACGGCAGCAGCATACA
O	DV859792	GAGTTTGGCTTGCTTTCTGC	GCGTCGCAAATCTGTGTTT
P	DV859791	GCTGGAAGAATATGCCAGAAG	AGAATGAAGAAGCGGTTCCA
Q	DV859788	TGCCAGAAGCCTAAAGGATG	AGAATGAAGAAGCGGTTCCA
R	DV859846	GAGCAAGGAGCCAAAGAAGA	AGGGAAAATTCAGCAGCAAA
S	DV859832	ACGAGGAGTTCGTCAAGGTC	GATACACCAGGGAGGAGCAA
T	DV859837	CCCCGTGCGTATGTATGTCT	GAAGAGGGGTGAACTGGAAC

Table 3.2*. Primer sequences used to amplify PCR products used for minisequencing presented in Figure 3.7.*

Fig. 3.7 sample	Accession	Forward primer Reverse primer Sequencing oligo
1	DV853660	CCATCGAGGACATCAAGCTC CGATCCACGCAACCTTATTC GTGATCGTCGGGTCGCAGTC
2	DV853632	TCCTCTTGAGATTGCCATGA CACGCCAGTTCAGTGCTAGA TGGGGAGTTGATGAGCTCAT
3	DV854208	ATTGCCATTTTTGCTTGGTC TGGAGGATTTTCATTTGGATG CTCTTTTTGCCACTAGTTCA
4	DV856585	TGGCATGTGAATTTGGGTAG CACCCGTTTACATCAAAGCA TAGAGTATTTTGTTTTCTCC
5	DV858390	CGCCTATGTGGTTGGATTCT TGTGAGGGAAGGCAAACTTTA CTTGTGAAAATGTCGAACTA

Table 3.3*. Primer sequences used to amplify PCR products used for dideoxy polymorphism scanning presented in Figure 3.9.*

Fig. 3-9 sample	Accession	Gene ID	Forward primer	Reverse primer
1	DV854092	Oxophytodienoic acid reductase	tgtaaaacgacggccagt CCGGATTTGCCTAAGAGATTC	GGGGGAAAGATACAATGCAC
2	DV855554	Photosystem II 10 kD protein	tgtaaaacgacggccagt TCTCTGGGCAGTCACTCTGGCTG	CGTGCTTGCACCATGGTAC
3	DV853221	LHY transcription factor	tgtaaaacgacggccagt GCAGGTGACGAGGTTGGTA	AGTCTAGAGGGCCTGCTTGG
4	DV853574	Ribosomal protein S1	tgtaaaacgacggccagt TGTTAAGATTCCAGCCAGAGA	CATTTCTTTCCAGCCGTTTT
5	DV854851	Pseudoresponse regulator	tgtaaaacgacggccagt TCCTGGCGTTCAGTATCAGA	TTGGACATCCATGACACCAC
6	DV853371	Cytoplasmic malate dehydrogenase	tgtaaaacgacggccagt AGGAGCTCTCGGAGGAGAAG	AGCCCTCCAGCAAATATTCA
7	DV853359	Unknown protein	tgtaaaacgacggccagt TCGTGAAACCGTAGACACCA	CAGACTTTTCAGCTCTCCGTT T
8	DV855297	Actin depolymerizing factor	tgtaaaacgacggccagt CTAGCGAGATCAGCCTCGAC	TTCGTGGTCTGATGATGGAA

Figure 3.1. *Results of the best match of clone DV859794 to the NR database at NCBI using the BLASTX function.*

```
ID: BLASTX   P all non-redundant genbank cds         01 02 040402 10:59:54
>emb|CAC17628.1| putative phospholipid hydroperoxide glutathione peroxidase [Oryza
sativa]  Length = 169

 Score =  182 bits (462), Expect = 5e-45
 Identities = 86/91 (94%), Positives = 90/91 (98%)
 Frame = -3

Query: 654 QEPGSDQQIKDFACTRFKAEYPVFQKVRVNGPDAAPLYKFLKASKPGLFGSRIKWNFTKF 475
           QEPGSDQQIKDFACTRFKAEYPVFQKVRVNGPDAAPLYKFLKASKPGLFGSRIKWNFTKF
Sbjct: 79  QEPGSDQQIKDFACTRFKAEYPVFQKVRVNGPDAAPLYKFLKASKPGLFGSRIKWNFTKF 138

Query: 474 LVDKNGKVINRYATATTPFSFEKDILKALEE 382
           L+DKNGKVINRY+TAT+P SFEKDILKALE+
Sbjct: 139 LIDKNGKVINRYSTATSPLSFEKDILKALED 169
```

Figure 3.2. *The CLUSTAL/W pairwise alignment of the DV859794 (designated as COLEST 5A9) deduced protein sequence with its best BLASTX match, phospholipid hydroperoxide glutathione peroxidase from rice (CAC17628.1). The asterisk (*) indicates the predicted stop codon and the grey shaded region is the subsequent 3' untranslated region that was used to construct a marker. Primers are indicated by the black region.*

```
COLEST_5A9 : CAA GAG CCA GGC AGT GAC CAG CAG ATA AAA GAC TTT GCT TGC ACA AGA TTC  51
COLEST_5A9 :  Q   E   P   G   S   D   Q   Q   I   K   D   F   A   C   T   R   F
CAC17628   :  Q   E   P   G   S   D   Q   Q   I   K   D   F   A   C   T   R   F

COLEST_5A9 : AAA GCT GAA TAT CCA GTT TTT CAG AAG GTG CGT GTA AAT GGC CCA GAT GCT  102
COLEST_5A9 :  K   A   E   Y   P   V   F   Q   K   V   R   V   N   G   P   D   A
CAC17628   :  K   A   E   Y   P   V   F   Q   K   V   R   V   N   G   P   D   A

COLEST_5A9 : GCG CCG CTT TAC AAG TTT CTG AAA GCT AGC AAA CCT GGT TTG TTT GGG TCA  153
COLEST_5A9 :  A   P   L   Y   K   F   L   K   A   S   K   P   G   L   F   G   S
CAC17628   :  A   P   L   Y   K   F   L   K   A   S   K   P   G   L   F   G   S


COLEST_5A9 : AGA ATC AAG TGG AAC TTT ACC AAG TTT CTT GTT GAT AAG AAT GGA AAA GTC  204
COLEST_5A9 :  R   I   K   W   N   F   T   K   F   L   V   D   K   N   G   K   V
CAC17628   :  R   I   K   W   N   F   T   K   F   L   I   D   K   N   G   K   V

COLEST_5A9 : ATA AAC AGA TAT GCA ACT GCG ACT ACT CCA TTT TCA TTC GAG AAA GAC ATC  255
COLEST_5A9 :  I   N   R   Y   A   T   A   T   T   P   F   S   F   E   K   D   I
CAC17628   :  I   N   R   Y   S   T   A   T   S   P   L   S   F   E   K   D   I

COLEST_5A9 : CTG AAG GCG CTT GAG GAG GAA CAT GTT CCC TCT CTC CCT GAC TCG CAG AAG  306
COLEST_5A9 :  L   K   A   L   E   E   E   H   V   P   S   L   P   D   S   Q   K
CAC17628   :  L   K   A   L   E   D   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : GCG CCT GAG GAG GAA CCA AAG TAG GCG CTT GCT AAA GTA TCC TCG TTG TAA  357
COLEST_5A9 :  A   P   E   E   E   P   K   *   -   -   -   -   -   -   -   -   -.
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : TAT CTA GAC CTC TCC CTG GTA GTA AAT TAA TCT GAT ACT TCC GTT GGT GTG  408
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : AAC TTT GTG  CTT ACT GTA TTT TTG ATT GCC GTT TTG TAT GTA GAA GTT ATG  459
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : TAG ACG CCA CTT TGT TTG TAC CGC TAT CCA TGA CTT GTC CAT ACC GCC AAC  510
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : CCA TGA CTT GTC CTT TTT TTT GAA CTT TGT TGA GGA AGT CGA GAC ATT GAA  561
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : TAA CTG TGA CTA TAC AGT TAA TGT GAG TTT TGT ATT TGT GCT CAT AAG GTT  612
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

COLEST_5A9 : CCA AGT GAT GCT TCA TGA CCA AAA AAA AAA AAA AAA  648
COLEST_5A9 :  -   -   -   -   -   -   -   -   -   -   -   -
CAC17628   :  -   -   -   -   -   -   -   -   -   -   -   -
```

Figure 3.3. *A typical screen of 20 potential markers with the STS tagging method. Primers a-t are listed in Table 3.1. For each primer set the lanes are as follows: lane 1, 50 bp ladder; lane 2, TH15 (hybrid parent); lane 3, 9188 (creeping bentgrass parent); lane 4, 5061 (creeping bentgrass grandparent); lane 5, 8283 (unrelated colonial bentgrass). Marker "m" is a successful marker. The primers for these markers are listed in Table 3-1.*

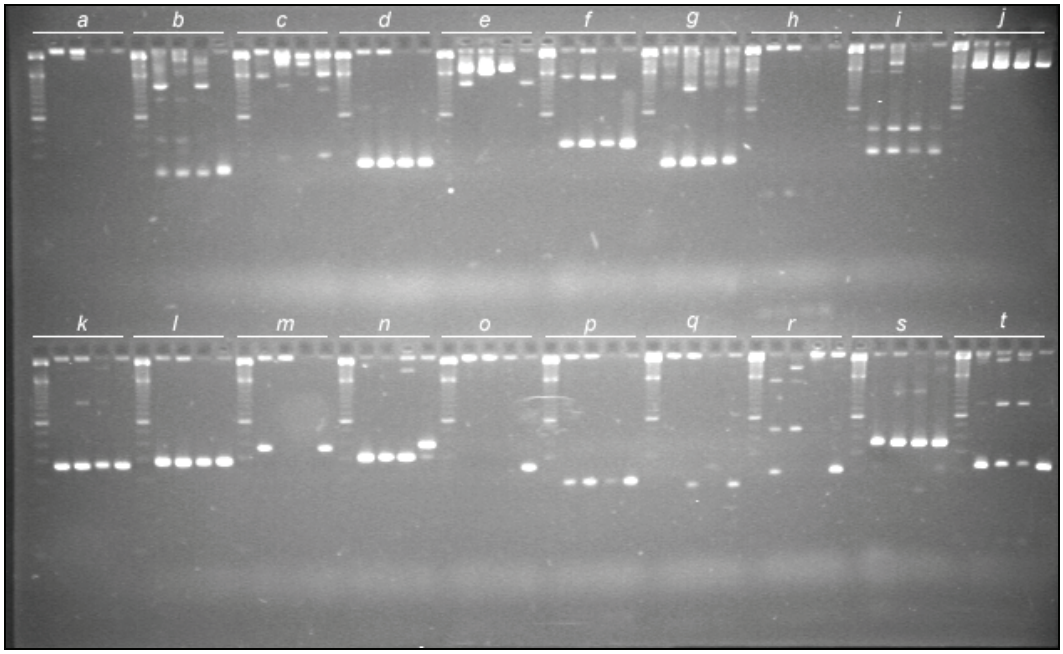


Figure 3.4. *A demonstration of STS markers with (A) null amplification alleles, and (B) codominate amplification alleles. Lanes are as follows: lane 1, 50 bp ladder; lane 2, TH15 (hybrid parent); lane 3, 9188 (creeping bentgrass parent); lane 4, 5061 (creeping bentgrass grandparent); lane 5, 8283 (unrelated colonial bentgrass); lane 6, 50bp ladder.*

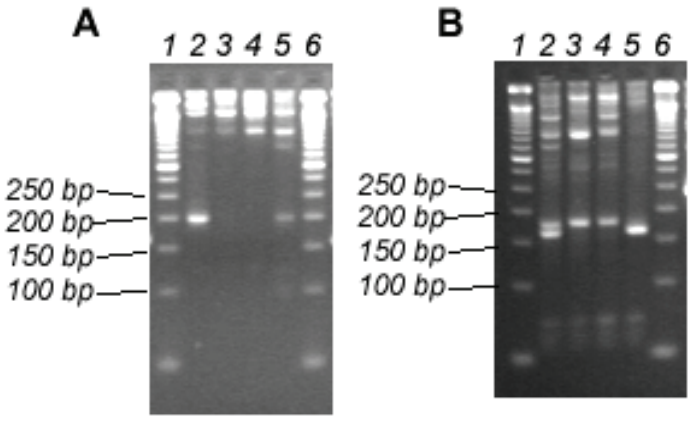


Figure 3.5. *A demonstration of the segregation of a STS marker (DV859794) across the hybrid backcross mapping population with. Lanes are as follows: lane 1, TH15 (hybrid parent); lane 2, 9188 (creeping bentgrass parent); lane 3, 5061 (creeping bentgrass grandparent); lane 4, individuals in the mapping population.*

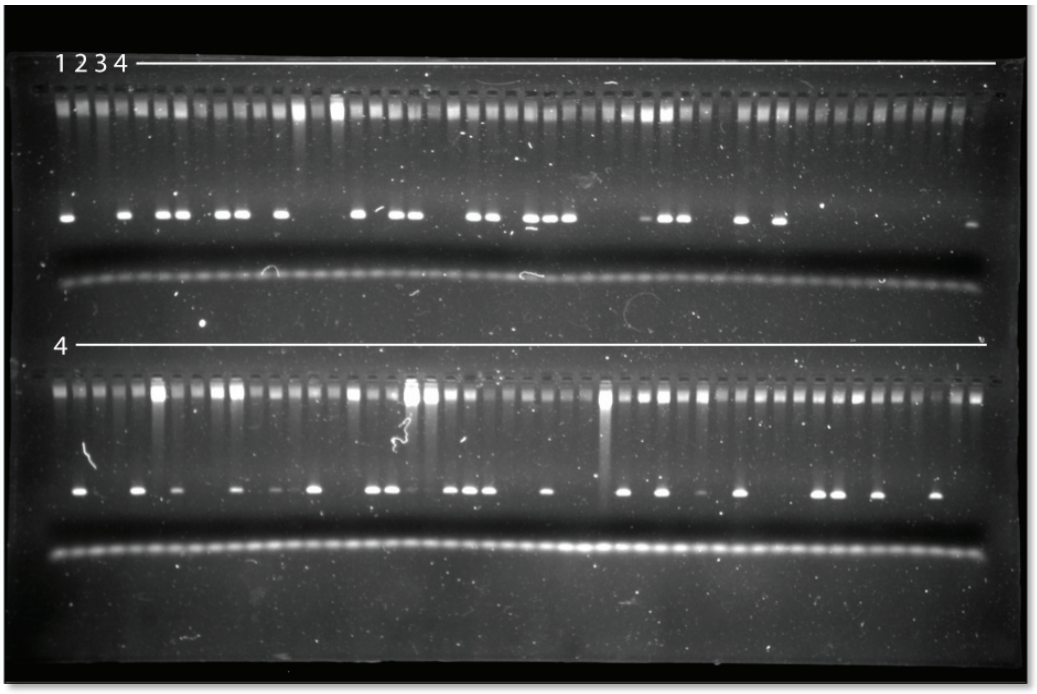


Figure 3.6*. Diagram of the minisequencing method.*

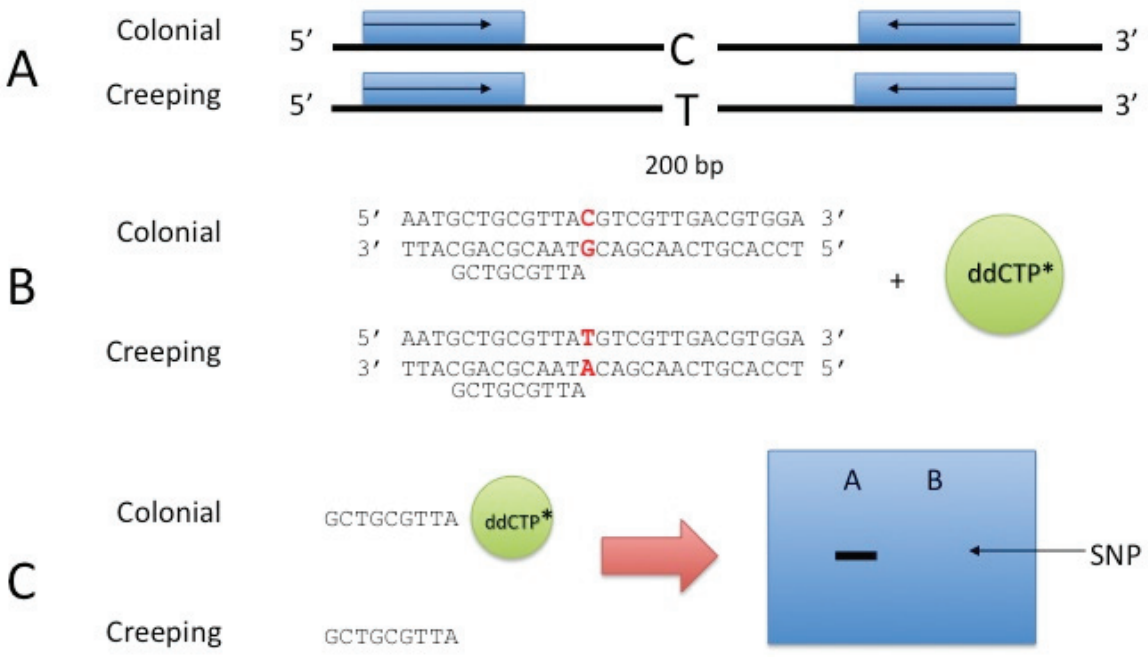


Figure 3.7. *Minisequencing analysis with ddC of 5 colonial bentgrass EST sequences for identification of polymorphisms useful for linkage mapping of colonial bentgrass. The samples within each EST set are as follows: lane a, interspecific hybrid parent of the mapping population; lane b, creeping bentgrass grandparent of the mapping population; lane c, creeping bentgrass parent of the mapping population; lane d, an unrelated colonial bentgrass plant. Bands unique to the interspecific hybrid and therefore useful for mapping colonial bentgrass can be seen in sample 4. Gene identifications of the ESTs and the primer sequences used are presented in Table 3.2.*

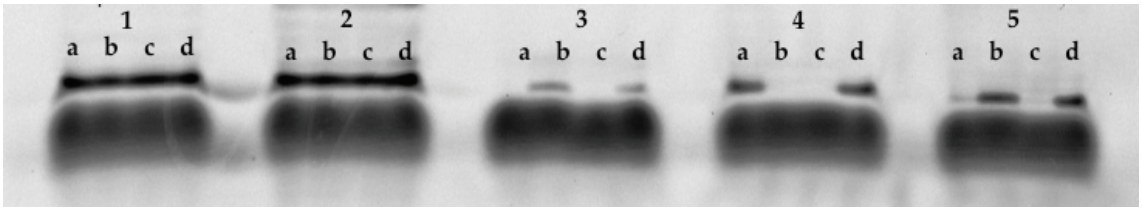


Figure 3.8*. The CLUSTAL/W pairwise alignment of the EST sequences used for Sample 4 (Figure 3.7). The location of the forward and reverse PCR oligos are indicated by the light grey boxes. The minisequencing primer is indicated by the dark grey box and corresponding SNP is indicated by the black box.*

Figure 3.9. *Diagram of the dideoxy polymorphism scanning method.*

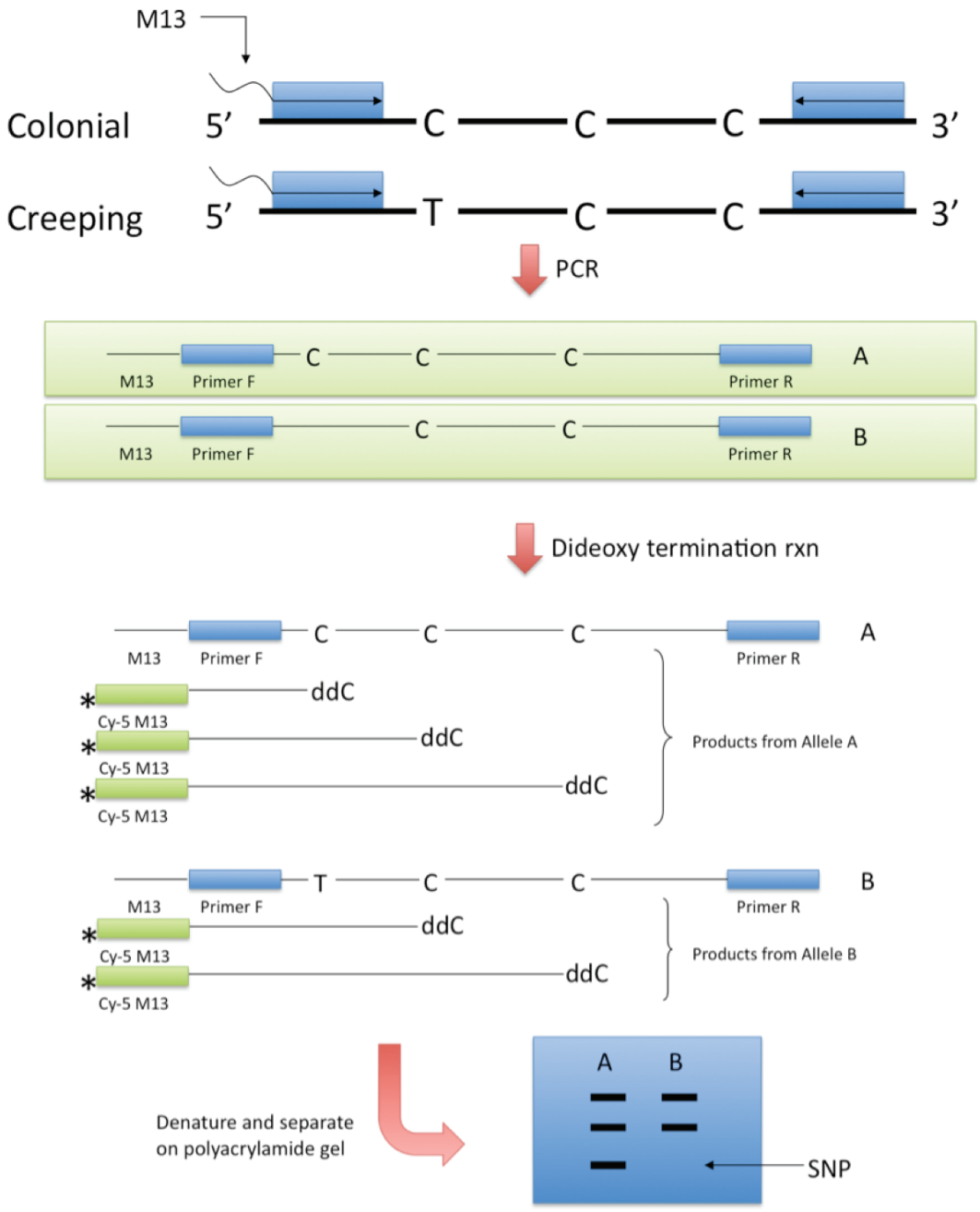


Figure 3.10. *Dideoxy polymorphism scanning with ddT of 8 colonial bentgrass EST sequences for identification of polymorphisms useful for linkage mapping of colonial bentgrass. The samples within each EST set are as follows: lane a, interspecific hybrid parent of the mapping population; lane b, creeping bentgrass grandparent of the mapping population; lane c, creeping bentgrass parent of the mapping population. Bands unique to the interspecific hybrid and therefore useful for mapping colonial bentgrass are enclosed in gray boxes. Polymorphic bands between the two creeping bentgrass plants are enclosed in black boxes. Gene identifications of the ESTs and the primer sequences used are presented in Table 3.3.*

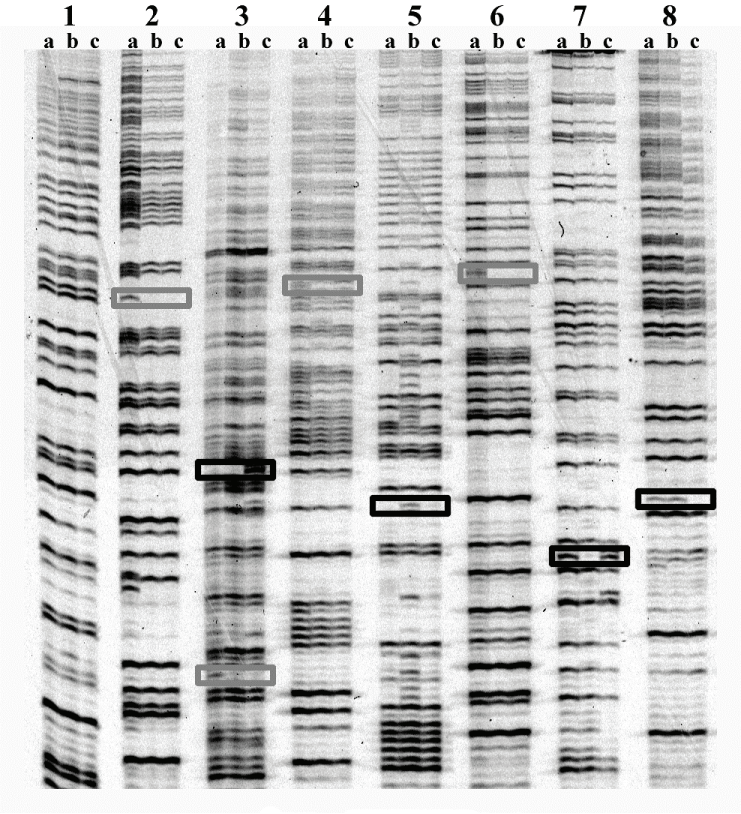
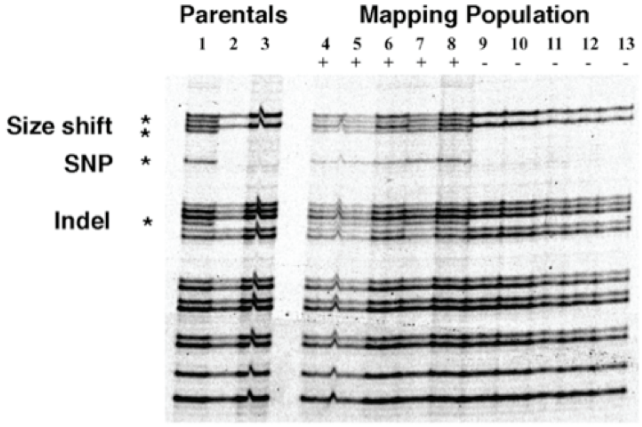


Figure 3.11. *(A) Segregation in the mapping population of the bands unique to the interspecific hybrid from sample 2 in Figure 3.11. Lane 1, interspecific hybrid parent of the mapping population; lane 2, creeping bentgrass grandparent of the mapping population; lane 3, creeping bentgrass parent of the mapping population; lanes 4–8, five individuals of the mapping population that inherited the colonial bentgrass allele; lanes 9–13, five individuals of the mapping population that did not inherit the colonial bentgrass allele. Asterisks mark hybrid specific bands originating from the colonial bentgrass allele. (B) Sequences of cloned PCR products from the parental DNAs corresponding to the region of the gel shown in (A). The sequence regions that result in the hybrid specific bands with ddPS are highlighted by bold larger font sizes.*

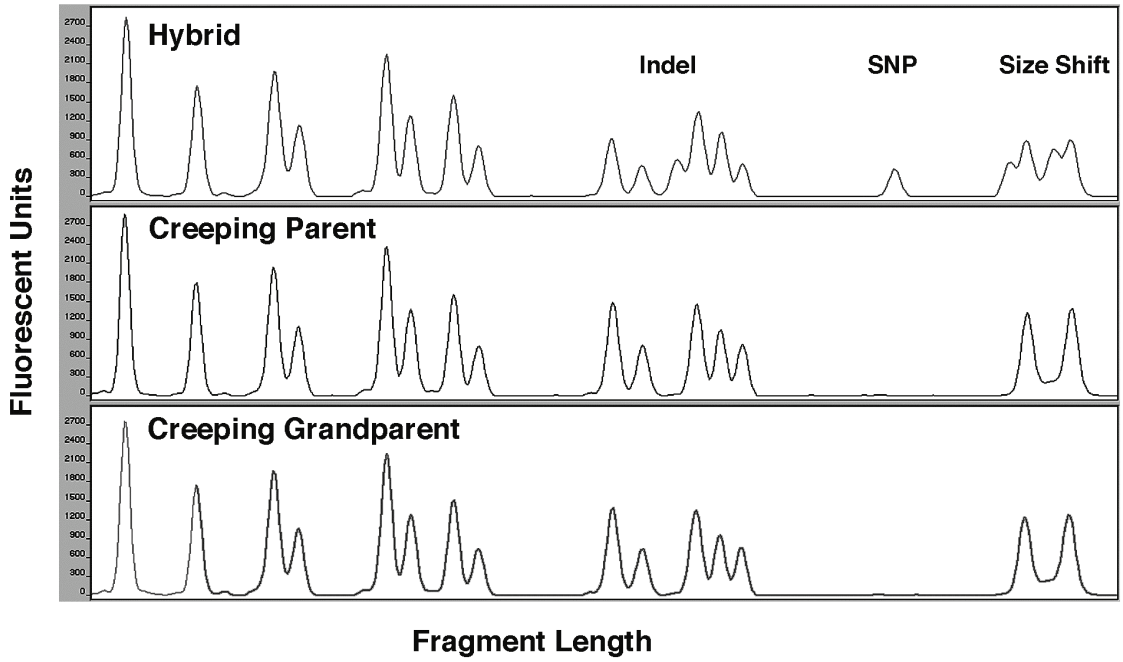
A



B

Hybrid	TAGTGCCTTAGCTTCTTGAAGCCG-ATTTGCAGCAAATCAAACTAT
	TAGTGCCTTGGCTTCTTGAAGCCTTATTTGCAGCAAACCAAACTAT
Creeping grandparent	TAGTGCCTTGGCTTCTTGAAGCCTTATTTGCAGCAAACCAAACTAT
Creeping parent	TAGTGCCTTGGCTTCTTGAAGCCTTATTTGCAGCAAACCAAACTAT

Figure 3.12. *ABI 3730 trace files of ddPS reactions on the parental DNAs of the same samples as shown in Figure 3.12A. The region of the trace files is the same as that shown in Figure 3.12A.*



Chapter 4

Colonial bentgrass genetic linkage mapping and identification of chromosomal locations possibly associated with dollar spot resistance

Chapter 4

Colonial bentgrass genetic linkage mapping and identification of chromosomal locations possibly associated with dollar spot resistance

Introduction

The previous chapters describe the relationship between creeping and colonial bentgrass. We have used a creeping x colonial bentgrass interspecific hybrid to create a mapping population that segregated for dollar spot resistance. There are no creeping bentgrass cultivars that have the type of dollar spot resistance observed in colonial bentgrass. Therefore this interspecific hybridization approach may be a useful strategy for introducing dollar spot resistance into creeping bentgrass. The primary objective of this thesis was to investigate the genetic nature of dollar spot resistance in colonial bentgrass. The approach to answer this question was to create a genetic linkage map of colonial bentgrass using the interspecific backcross population. By combining the information from this map with field data we identified genomic regions that are common in resistant individuals.

This chapter will describe the steps taken to utilize the EST libraries described in Chapter 2 as well as the method described in Chapter 3 to create a linkage map of colonial bentgrass.

Two hundred seventy one backcross individuals were replicated four times and field tested for dollar spot resistance over two years. A randomly selected subset of this population (93 individuals) eventually became the mapping population. The segregation of dollar spot within this population suggests that three genes may be necessary for the type of resistance observed in colonial bentgrass.

Methods

Plant materials and field evaluation of dollar spot resistance

A dollar spot resistant colonial bentgrass x creeping bentgrass interspecific hybrid was crossed with a creeping bentgrass plant in 2002 (Belanger et al., 2004). Individual seeds recovered from the cross were germinated in the greenhouse and vegetatively propagated. Clonally propagated replicates of all the progeny individuals were maintained in the greenhouse.

A field trial of the backcross plants was established at the Rutgers Turfgrass Research Facility at North Brunswick, NJ, on June 6, 2005. The trial was established as a randomized complete block design with four vegetatively propagated replications of 271 backcross individuals as well as four replicates of the colonial bentgrass x creeping bentgrass interspecific hybrid parent, the creeping bentgrass parent and the creeping bentgrass grandparent of the population. The colonial bentgrass grandparent of the population is not available and so could not be included in the field evaluation. The plants were placed 45 cm apart and maintained as mowed spaced plants at a height of approximately 2.5 cm. Weeds were controlled by a combination of herbicide sprays and manual removal. The preemergence herbicide Dithiopyr was applied on 6/10/05, 10/6/05, and 4/25/06. The broadleaf herbicide Trimec® Bentgrass Formula (which contains 2,4-D, Dicamba and MCPP) was applied on 6/20/05, 4/25/06, and 7/23/06. Fungicides were used to control diseases other than dollar spot. Flutolanil was applied on 7/20/05,

8/1/05, 8/16/05, 6/27/06 and 8/15/06. Azoxystrobin was applied 7/7/06 and 7/23/06.

A mixture of three isolates of the dollar spot fungus was used to inoculate the field trial. The isolates and the preparation of the inoculum were as described previously (Belanger et al., 2004). The inoculum was applied to the field with a drop spreader at a rate of 1.75 g m^{-2} on June 16, 2005 and June 23, 2006. The inoculated plants were rated weekly for percentage diseased turf for each replicate using a 1-9 scale. Nine represented 0-5% diseased turf, eight represented approximately 10% diseased turf, seven represented approximately 15-20% diseased turf, six represented approximately 30-40% diseased turf, five represented 40-50% diseased turf, four represented approximately 60-70% diseased turf, three represented approximately 75-85% diseased turf, two represented approximately 90% diseased turf, and one represented 95-100% diseased turf.

Amplified fragment length polymorphic (AFLP) marker analysis

For AFLP analysis, genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) extraction from 100 mg lyophilized plant leaf tissue (Saghaimaroof et al., 1984). DNA digestions, adaptor ligation, and pre-selective and selective amplifications were performed according to instructions provided with an AFLP analysis kit purchased from Invitrogen (Carlsbad, CA, USA), and standard AFLP procedures (Vos et al., 1995). The selective amplifications with three or four selective bases per primer were carried out by using 6-carboxy

fluorescein fluorescent primers labeled on the 5' nucleotide. The amplified fragments were detected with an ABI3730xl instrument (PE Applied Biosystems, Foster City, CA, USA). Each sample lane included the GeneScan 500-LIZ internal lane standard (Larson et al., 2001). The capillary electrophoresis procedures were performed by the Bovine Functional Genomics Lab (Beltsville, MD, USA). Fluorescent fragments between 50-500 nucleotides were identified by GeneScan 3.1 software (PE Applied Biosystems). GeneScan trace files were then analyzed for the presence or absence of AFLP products, in 1-nt intervals using the computer program Genographer (Benham et al., 1999).

EST marker development and analysis

For EST markers, genomic DNA was isolated by using a commercial kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO). PCR-based markers developed from the colonial bentgrass ESTs were analyzed by using high-resolution agarose gels, minisequencing, or dideoxy polymorphism scanning (Rotter et al., 2007a). Oligonucleotides for PCR amplification were designed from the 3' untranslated regions of the ESTs to amplify fragments of approximately 100-200 bp. Primers were designed to have a T_m of 60°C. Whenever possible, primers specific to a colonial bentgrass EST were designed based on sequence alignments of the similar colonial bentgrass and creeping bentgrass ESTs. Such alignments often revealed SNPs and indels that could be used for development of colonial bentgrass specific primers. The M13 (-21) sequence (5'-TGTAAAACGACGGCCAGT-3') was added to the 5' position of the forward primers. The primer sequences used for PCR

amplification and the method of polymorphism detection for the EST markers are presented in Supplemental Table 1. PCR reaction conditions were as described previously (Rotter et al., 2007b). PCR products from the parental DNA samples were checked on a 3% high-resolution agarose gel (MetaPhor, Cambrex BioScience Rockland, Inc., Rockland, ME) to confirm that a single DNA fragment was amplified. If a +/- polymorphism or a length polymorphism was present, then the mapping population was screened on a high-resolution agarose gel. If a single PCR product was amplified but no polymorphism was detected on the agarose gel, then the parental PCR products were evaluated for polymorphisms by using the dideoxy polymorphism scanning method as described previously (Rotter et al., 2007b). Five EST markers were developed by using the minisequencing method as previously described (Carvalho & Pena, 2005; Rotter et al., 2008).

Linkage analysis

JoinMap 3.0 software was used to carry out the linkage analysis using the doubled-haploid population model since the marker segregation is expected to be 1:1 (van Ooijen & Voorrips, 2001). The determination of linkage groups of markers was performed with log-of-odds (LOD) ratio thresholds of 5 (groups 1A1, 1A2, 2A2, 3A1, 3A2, 4A1, 4A2, 5A2, and 7A2), 6 (groups 5A1, 6A1, and 6A2), and 7 (groups 2A1 and 7A1). Different LOD scores were used to avoid excessively long linkage groups. The calculations of the linkage map were done using all pairwise recombination estimates < 0.400 and a LOD score of > 1.0 (ripple value = 1, jump threshold = 5, and a triplet threshold = 5). The Kosambi mapping function was used to convert

recombination units into genetic distances (Kosambi, 1944). Interval mapping analysis was employed to detect possible QTL's by using MapQTL 5.0 (van Ooijen, 2001).

Comparative genomics

The 12 chromosomes that represent the complete rice genome sequence were retrieved from NCBI (accessions NC_008394, NC_008395, NC_008396, NC_008397, NC_008398, NC_008399, NC_008340, NC_008341, NC_008342, NC_008343, NC_008344, NC_008405). To determine the physical location of the most similar rice gene to each colonial bentgrass EST a perl script was developed which utilized the Blastn function to sequentially search all colonial ESTs with each rice chromosome. The program returned the location and significance of the best match. Only ESTs that returned e values lower then e^{-10} were considered. SigmaPlot (Systat Software, Inc., San Jose, CA) was used to construct the final rice chromosome map figures.

Results

Colonial bentgrass genetic linkage mapping

We used 93 individuals randomly chosen from the backcross population as a mapping population for genetic linkage mapping of the colonial bentgrass genome. Our mapping strategy was to identify polymorphic markers that are found in the hybrid parent of the population and not in the creeping bentgrass grandparent or parent. Such markers originated from the colonial bentgrass grandparent of the population and thus can be used to map the colonial bentgrass genome in the hybrid. Such markers have an expected 1:1 (presence:absence) segregation ratio in the backcross population.

The colonial bentgrass linkage map covers 1156 cM and is composed of 212 AFLP markers and 110 gene-based markers (Figure 4.1). The characteristics of the AFLP markers are summarized in Table 4.1. The gene identification and rice chromosomal location of the mapped ESTs are presented in Table 4.2. The overall segregation distortion of the mapped markers was 37%.

The linkage group assignments were based on the Triticeae system, using the established rice-wheat chromosomal relationships (La Rota & Sorrells, 2004). The subgenome assignments of the linkage groups were based on our understanding of the relationships of the colonial bentgrass and creeping bentgrass subgenomes to each other. We previously used phylogenetic comparisons among conserved

orthologous set (COS) colonial and creeping bentgrass EST sequences to make subgenome assignments and to estimate the divergence times between the subgenomes of the two species (Fulton, Van der Hoeven, Eannetta, & Tanksley, 2002; Rotter et al., 2007b). The four colonial bentgrass ESTs previously assigned to the A_1 subgenome, DV853200, DV854798, DV854551, and DV858879, were mapped, establishing those linkage groups as from the A_1 genome. These four genes mapped to the $2A_1$, $3A_1$ and $7A_1$ linkage groups. The homoeologous A_2 sequences could not be mapped since no polymorphisms were found among the parental plants. However, for linkage groups $3A_1$ and $7A_1$, homoeologous sequences to other mapped genes on those groups were mapped to other linkage groups, establishing those other groups as the $3A_2$ and $7A_2$ linkage groups. It is clear from Figure 4.1 that the density of markers is considerably different between the $3A_1$ and $7A_1$ linkage groups and the homoeologous $3A_2$ and $7A_2$ linkage groups. This difference in marker density between the rest of the homoeologous linkage groups was used to make assignments to either the A_1 or A_2 subgenome. Each of the colonial bentgrass linkage groups is discussed below.

Linkage groups $1A_1$ and $1A_2$

Wheat linkage group 1 is comprised largely of genes from rice linkage groups 5 and 10 (La Rota & Sorrells, 2004). The assignment of colonial bentgrass linkage group $1A_1$ was based on having 7 of the 9 genes on the group having similar genes on either rice chromosomes 5 or 10. Two of the 3 genes on colonial bentgrass linkage group $1A_2$ had similar genes on rice chromosomes 5 or 10. Also, colonial bentgrass

EST DV857259 was similar to anchor probe CDO1160, which has been mapped to wheat chromosomes 1A and 1B (Van Deynze et al., 1998).

Two colonial bentgrass ESTs similar to 12-oxo-phytodienoic acid reductase were mapped to linkage group $1A_1$. There were several distinct 12-oxo-phytodienoic acid reductase ESTs in our collection and the PCR primers for the two mapped ESTs were each designed to amplify only a particular sequence. Since they both mapped to the same linkage group, they represent duplicate genes on the same chromosome.

One colonial bentgrass EST (FD933142) was mapped for which there was no similar gene in rice. No similar sequences were found in either BLASTN or BLASTX searches of the NCBI nonredundant databases. Significant matches were found to another colonial bentgrass EST (DV858128), a tall fescue (*Festuca arundinacea* Schreb.) EST (DT714521), and an oat (*Avena sativa* L.) EST (CN821054) when searched with the EST-others database. These genes may thus represent as yet uncharacterized genes unique to the Pooideae lineage of the grasses.

Linkage groups $2A_1$ and $2A_2$

Wheat linkage group 2 is comprised largely of genes from rice linkage groups 4 and 7 (La Rota & Sorrells, 2004). Fifteen of the sixteen genes on colonial bentgrass group $2A_1$ were most similar to genes found on rice chromosomes 4 or 7. Similar to the case of the two duplicate genes on colonial bentgrass linkage group $1A_1$, two similar colonial bentgrass ESTs (DV859086 and DV858748), which code for

unknown proteins, mapped to group $2A_1$. One of the two genes on colonial bentgrass group $2A_2$ was most similar to a gene on rice chromosome 4. The similarity to a gene on rice chromosome 4, rather than the similarity to a gene on rice chromosome 1, was used to make this linkage group assignment as $2A_2$ rather than $3A_2$, since another colonial bentgrass linkage group was more likely $3A_2$ (discussed below).

Linkage groups $3A_1$ and $3A_2$

Wheat linkage group 3 is comprised largely of genes from rice linkage group 1 (La Rota & Sorrells, 2004). Ten of the sixteen genes on colonial bentgrass linkage group $3A_1$ were most similar to genes on rice chromosome 1. The single gene on colonial bentgrass linkage group $3A_2$ was most similar to a gene on rice chromosome 1. The homoeologous sequence (DV858615), mapped to group $3A_1$, strengthening the assignment of the $3A_2$ linkage group.

Linkage groups $4A_1$ and $4A_2$

Wheat linkage group 4 is comprised largely of genes from rice linkage groups 3 and 11 (La Rota & Sorrells, 2004). Fifteen of the eighteen genes on colonial bentgrass linkage group $4A_1$ were most similar to genes on rice chromosomes 3 or 11. Also, two ESTs, DV857974 and DV854393, were similar to two anchor probes, CDO795 and CDO1387, which map to wheat chromosomes 4B and 4A, respectively (Van Deynze et al., 1998). For one of the mapped ESTs, DV857768, the only similar sequences so far reported are other ESTs from colonial and creeping bentgrass and one EST from *Agrostis scabra* Willd. These sequences may therefore be unique to

Agrostis spp. Two of the three genes on colonial bentgrass linkage group $4A_2$ are most similar to genes on rice chromosome 3. Also, for two of the genes on group $4A_2$, DV859294 and DV855831, homoeologous sequences, DV854440 and DV855114, were mapped to colonial bentgrass linkage group $4A_1$.

Linkage groups $5A_1$ and $5A_2$

Wheat linkage group 5 is comprised largely of genes from rice chromosomes 9 and 12 (La Rota & Sorrells, 2004). Ten of the twelve genes on colonial bentgrass linkage group $5A_1$ were most similar to genes on rice chromosomes 9 and 12. One EST, DV853194, is similar to the anchor probe CDO1338, which maps to wheat chromosome 5B. One colonial bentgrass EST, DV858575, does not have a significantly similar gene in rice, but is similar to a cDNA, EV519616, that was induced in response to heat in the related species *A. scabra* (Tain et al., 2008). All the three genes on colonial bentgrass linkage group $5A_2$ are most similar to genes on either rice chromosomes 9 or 12. For one of the genes, DV856365, the homoeologous sequence, DV855709, mapped to colonial bentgrass linkage group $5A_1$.

Linkage groups $6A_1$ and $6A_2$

Wheat linkage group 6 is comprised largely of genes from rice chromosome 2 (La Rota & Sorrells, 2004). Six of the seven genes on colonial bentgrass linkage group $6A_1$ were most similar to genes on rice chromosome 2. For one mapped EST sequence, FE597042, no similar sequences have yet been reported in any other organisms. This gene may therefore represent another gene possibly unique to

Agrostis. Four of the five genes on colonial bentgrass linkage group $6A_2$ were most similar to genes on rice chromosome 2. For one of the genes, DV855964, the homoeologous sequence, DV856448, mapped to colonial bentgrass linkage group $6A_1$.

Linkage groups $7A_1$ and $7A_2$

Wheat linkage group 7 is comprised largely of genes from rice chromosomes 6 and 8 (La Rota & Sorrells, 2004). Eleven of the thirteen genes on colonial bentgrass linkage group $7A_1$ were most similar to genes on rice chromosomes 6 or 8. Two colonial bentgrass ESTs, DV853439 and DV859455, which encode similar FAD-linked oxidoreductases, mapped to linkage group $7A_1$. Several similar genes are also present on rice chromosome 6. Both of the genes on colonial bentgrass linkage group $7A_2$ were most similar to genes on rice chromosome 8. For one of the genes, DV858291, the homoeologous sequence, DV857377, mapped to colonial bentgrass linkage group $7A_1$.

Field evaluation of the backcross population

The backcross population was evaluated for dollar spot resistance in a two-year field trial. The distribution of the means of the genotypes for each year is shown in Figure 4.2. The dates chosen for comparison in each year are those of the highest dollar spot pressure in the susceptible creeping bentgrass parental plants. The dollar spot disease severity of the parental plants is similar to what was previously observed for these genotypes (Belanger et al., 2004). The means of the individuals of the backcross population ranged from complete disease (dead) to no disease. The

wide distribution range of the backcross population reflects the combined effects of inheritance of good resistance from colonial bentgrass in some genotypes and the variation in degree of susceptibility from creeping bentgrass in other genotypes (Bonos et al., 2003). Of the 271 backcross genotypes in the field trial, 31 (11%) had good dollar spot resistance with mean ratings of 7 or higher in both years.

Identification of colonial bentgrass genomic regions possibly associated with dollar spot resistance

Among the 93 mapping population individuals in the backcross population, 8 were among the 31 plants exhibiting good resistance to dollar spot in both years of the field evaluation. QTL interval mapping analysis of the mapping population did not reveal any major loci significantly associated with dollar spot resistance. Another possibility is that several genes conferring minor effects are underlying the trait. If this is the case, then they would not be detectable as QTLs with this relatively small population size.

Another possibility is that the trait is qualitative rather than quantitative. The results of the field data are a good fit to a model of qualitative 3-gene recessive epistasis as defined by classical genetics (Table 4.3) (Aylor & Zeng, 2008). In the classical genetics model of recessive epistasis, recessive homozygotes at one locus have the same phenotype as recessive homozygotes at all epistatic loci. In this model the colonial bentgrass alleles are considered as dominant and all three proposed alleles are required for the resistance phenotype. Lack of inheritance of the colonial bentgrass allele at any one or more of the proposed loci would result in

the succeptiable phenotype. Software is not currently available to search for three way epistatic interactions.

As an alternative analysis to identify colonial bentgrass chromosomal regions possibly associated with dollar spot resistance, we searched for colonial bentgrass markers found in all of the resistant plants. To do this the percentage of resistant plants in the mapping population carrying each marker was compared with the percentage of all the rest of the plants carrying each marker (Figure 4.3). A cluster of several markers on linkage group $2A_1$ and a single marker on group $3A_1$ were found in 100% of the resistant plants, suggesting these regions contain colonial bentgrass genes associated with dollar spot resistance. Fourteen of the common markers were found on group $2A_1$ spanning a region of 27 cM and a single common marker was found on group $3A_1$. Those 15 markers are listed in Table 4.4.

In the field test there were 23 additional backcross individuals, not included among the 93 mapping population individuals, which exhibited dollar spot resistance. Eighteen of these were still available since some had died in the greenhouse. These 18 plants were evaluated for the presence of the 10 colonial bentgrass EST markers that were present in all the resistant individuals in the mapping population. Table 4.4 includes the percentage of dollar spot resistant individuals that were not part of the mapping population that had those 10 EST markers. These individuals were not evaluated for the presence of the AFLP markers. The presence of all the EST markers in these additional 18 individuals was significantly higher than expected,

based on the segregation observed for each marker in the mapping population. Although there is segregation distortion in the mapping population at these loci, this was taken into account in the analysis and these colonial bentgrass alleles are strikingly overrepresented in the additional resistant plants. These data suggest that colonial bentgrass genes on those regions of linkage groups $2A_1$ and $3A_1$ may be associated with dollar spot resistance.

Discussion

Many crop species, such as tomato, wheat, and sugarcane, contain genes from related species that have been introduced through breeding for the purpose of conferring specific beneficial phenotypic traits (Hajjar & Hodgkin, 2007; Osborn et al., 2007). One objective of this study was to determine if dollar spot resistance from colonial bentgrass could be introduced into creeping bentgrass. The results of the field study on the backcross population indicate that it is possible since a relatively high percentage (11%) of the population had the desired phenotype.

As a step towards marker assisted selection for introgression of dollar spot resistance into creeping bentgrass we used a randomly selected subset of the backcross population as the mapping population for developing the first genetic linkage map of colonial bentgrass. Our population structure was such that we were mapping the colonial bentgrass genome in a colonial bentgrass x creeping bentgrass interspecific hybrid. The genome complexity was thus reduced to 1N. This population structure was used in mapping allotetraploid *Leymus* wildryes and allotetraploid cotton (Lacape et al., 2003; Wu et al., 2003). The colonial bentgrass map length was 1156 cM, which is similar to the map length of 1125 cM of the related allotetraploid species creeping bentgrass (Chakraborty et al., 2006b).

The rice/wheat chromosomal relationships were used to make linkage group assignments (La Rota & Sorrells, 2004). Although there are numerous exceptions,

the wheat chromosomes are largely derived from specific rice chromosomal regions (La Rota & Sorrells, 2004). It has been well established that there is considerable conservation in chromosomal organization among members of the Pooideae (Devos, 2005). Genetic linkage maps of meadow fescue (*Festuca pratensis* Huds.), perennial ryegrass (*Lolium perenne* L.), and creeping bentgrass have revealed that these Poeae species have the same general chromosomal relationship to rice as does wheat , and we therefore expected the same for colonial bentgrass (Alm et al., 2003; Chakraborty et al., 2005; Sim et al., 2005). We chose to use the rice/wheat chromosomal relationship to make linkage group assignments rather than a direct comparison to wheat since many of the mapped colonial bentgrass genes have not been mapped in wheat. Our colonial bentgrass ESTs were also searched for sequences similar to the anchor probes which have been used in RFLP comparative mapping in several grass species (Van Deynze et al., 1998). Four of the mapped colonial bentgrass ESTs were similar to the CDO anchor probes, and their chromosomal location in wheat was consistent with the colonial bentgrass linkage group assignments made using the rice-wheat relationship. Most (81%) of the mapped ESTs conformed to the expectation of chromosomal location based on the location of the most similar genes in rice.

The map length was nearly evenly distributed between the A_1 and A_2 subgenomes at 590 cM and 566 cM, respectively. However, the density of markers between the two subgenomes was different. The average distance between markers on the A_1 subgenome is 2.5 cM and on the A_2 subgenome is 6.3 cM. The difference in marker

density is due to the fact that in this mapping population structure there is a single copy of the A_1 subgenome but both the colonial bentgrass A_2 and the creeping bentgrass A_2 subgenomes are present. We previously determined that the colonial and creeping bentgrass A_2 subgenomes were quite similar and only recently diverged from a common ancestor approximately 2.2 million years ago (Rotter et al., 2007a). Since the A_2 subgenome sequences of colonial and creeping bentgrass are quite similar to each other, even in the 3' untranslated regions of the EST sequences, it was more difficult to identify polymorphic markers for mapping the colonial bentgrass A_2 linkage groups. In our population structure, a polymorphism between the colonial bentgrass A_2 sequence and the creeping bentgrass A_2 sequence is required for mapping, since the hybrid and the mapping population contain both types of sequences. In contrast, a single allele of any specific colonial bentgrass A_1 gene is present in the hybrid parent and the mapping population making detection of polymorphisms easier. More polymorphic markers could be identified for the colonial bentgrass A_1 subgenome, and so the A_1 linkage groups are denser than the A_2 linkage groups.

The EST markers were designed to specific colonial bentgrass alleles that were then assigned to either the A_1 or A_2 subgenome linkage groups. This identification of specific colonial bentgrass A_2 subgenome alleles and the established relationship between the colonial bentgrass and creeping bentgrass A_2 sequences could be used in the future to assist in the assignment of the creeping bentgrass linkage groups to

either the A_2 or A_3 subgenomes (Chakraborty et al., 2005; Chakraborty et al., 2006b; Rotter et al., 2007a).

The segregation distortion of the mapped markers was 37%. This is similar to what has been found in other linkage maps based on interspecific crosses. In an annual x perennial ryegrass population 34-42% of the markers exhibited segregation distortion and in the *Leymus* maps 39-42% of the markers showed segregation distortion (Warnke et al., 2004; Wu et al., 2003). Clusters of markers with segregation distortion were found on linkage groups $1A_1$, $2A_1$, $5A_1$ and $7A_1$. Most of the markers with segregation distortion were found on the A1 linkage groups. This is likely due to the reported partial pairing of the A_1 and A_3 genomes in the interspecfic hybrid genome (Jones, 1956abc; Zhao et al., 2007).

Since no QTL for dollar spot resistance was detected even though the mapping population included resistant genotypes, our hypothesis is that there is qualitative epistatic gene interaction and the presence of possibly three colonial bentgrass genes is required for the observed dollar spot resistance. Fifteen colonial bentgrass markers were found in all the resistant plants in the mapping population. They were located on linkage groups $2A_1$ and $3A_1$, suggesting these linkage groups as the location of the genes associated with dollar spot resistance. These markers were also present at levels significantly higher than expected in 18 resistant backcross genotypes that were not included in the mapping population. Targeted mapping of additional genes onto the $2A_1$ and $3A_1$ linkage groups could be done to narrow the

genomic regions of interest and to eventually develop markers that could be used in marker assisted selection for introgression of the colonial bentgrass genes into creeping bentgrass. Also, additional interspecific backcross populations should be evaluated to confirm the association of colonial bentgrass linkage groups $2A_1$ and $3A_1$ with dollar spot resistance.

Field evaluation of a creeping bentgrass mapping population segregating for degree of dollar spot susceptibility revealed a highly significant QTL on one of the creeping bentgrass group 7 linkage groups (Chakraborty et al., 2006b). To develop creeping bentgrass cultivars with the most durable resistance to dollar spot, in the future it would be desirable to combine the approaches of selecting individuals for the presence of the creeping bentgrass QTL and the colonial bentgrass genes for dollar spot resistance.

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Table 4.1. *Summary of the characteristics of the mapped AFLP markers.*

Primer combination	Number of mapped polymorphic markers
EcoRI-AAG / MseI-CAA	2
EcoRI-AAG / MseI-CTC	6
EcoRI-ACC / MseI-CAA	12
EcoRI-ACC / MseI-CAT	12
EcoRI-ACC / MseI-CTG	13
EcoRI-ACG / MseI-CAA	10
EcoRI-ACG / MseI-CAC	3
EcoRI-ACG / MseI-CAG	14
EcoRI-AGC / MseI-CAA	2
EcoRI-AGC / MseI-CAG	4
EcoRI-AGG / MseI-CTC	1
EcoRI-ACC / MseI-CAC	5
EcoRI-ACC / MseI-CAG	5
EcoRI-ACC / MseI-CTA	5
EcoRI-ACC / MseI-CTC	7
EcoRI-ACC / MseI-CTT	11
EcoRI-ACT / MseI-CAA	6
EcoRI-ACT / MseI-CAG	5
EcoRI-ACT / MseI-CTG	15
EcoRI-ACT / MseI-CTT	7
EcoRI-ACG / MseI-CTC	6
EcoRI-ACG / MseI-CTT	3
EcoRI-ACT / MseI-CAC	7
EcoRI-ACT / MseI-CAT	3
EcoRI-ACT / MseI-CTA	8
EcoRI-ACT / MseI-CTC	6
EcoRI-AGC / MseI-CAAC	6
EcoRI-AGC / MseI-CAC	4
EcoRI-AGC / MseI-CAT	9
EcoRI-AGC / MseI-CTA	8
EcoRI-AGC / MseI -CTC	7
Total	212

***Table 4.2**. Gene identification and rice chromosomal location of colonial bentgrass mapped EST markers.*

Accession	Gene Identification	Rice Chromosome
Linkage Group 1A1		
DV855265	1-Aminocyclopropane-1-carboxylate oxidase	5
FD933142	No match	NA
FE597043	DVL	5
DV855651	Phosphate/phosphoenolpyruvate translocator	5
DV858475	Chloroplast beta-amylase	10
DV857969	12-oxo-phytodienoic acid reductase	1
DV857701	12-oxo-phytodienoic acid reductase	1
DV853684	bZIP protein	10
FD933072	DegP protease	5
Linkage Group 1A2		
DV857259	Ketol-acid reductoisomerase	5
DV853371	Cytoplasmic malate dehydrogenase	10
FD933079	Unknown protein	9
Linkage Group 2A1		
DV859501	Harpin-induced protein 1 (Hin1)	4
DV855554	10kD PSII protein	7
DV853200	Rieske iron-sulfur protein, chloroplast	7
DV859086	Unknown protein	4
DV855844	Inositol phosphatase	7
DV856678	Unknown protein	4
DV855696	Unknown protein	7
DV853949	Glutamine synthetase	4
DV858748	Unknown protein	4
DV855820	Endonuclease	7
DV857390	Sulfolipid synthase	7
DV856074	Unknown protein	4
DV855961	60 kDa chaperonin subunit alpha	12
FE597041	Unknown protein	7
DV855508	snRNP protein	4
DV859491	ATPase family (AAA)	4
Linkage Group 2A2		
DV856409	Ubiquinol-cytochrome c reductase iron-sulflur	4
DV855155	Oxygen-evolving enhancer protein	1

Linkage Group 3A1		
FD933070	Hypothetical protein	7
DV857855	Class III chitinase	1
DV856578	NPR1 interactor	1
DV859423	Phosphoprotein	1
DV858615	Unknown protein	1
DV852756	Unknown protein	10
DV857654	Aspartate aminotransferase	1
DV857853	Glycine rich RNA binding protein	1
DV857428	LEA5 protein	1
DV855086	Triose phosphate/phosphate translocator	5
DV857779	Unknown protein	1
DV856585	Ribosomal protein L12	5
DV854798	Unknown protein	8
DV861142	GTP-binding protein	1
DV859646	Metallothionine	5
DV858070	PRL1-interacting factor N	1
Linkage Group 3A2		
DV858327	Unknown protein	1
Linkage Group 4A1		
DV855114	Photosystem II reaction center W	1
FD933073	Unknown protein	3
DV854440	Unknown protein	3
DV859794	Phospholipid hydroperoxide glutathione peroxidase	3
FD932992	Unknown protein	3
DV858611	Ankyrin-repeat protein	3
DV857974	Seed maturation protein PM23	3
DV857960	Unknown protein	11
DV854834	Phospholipid transfer protein	11
DV856710	ZIM motif family protein	3
DV859720	AAA-type ATPase family protein	3
DV854393	Endosomal protein	3
DV856614	Zinc finger, C2H2 type family protein	3
DV857768	No match	NA
DV853833	Trypanothione-dependent peroxidase	3
DV853574	Ribosomal protein S1	3
DV859476	Stress enhanced protein	10
DV859874	Rubisco activase short isoform	11
Linkage Group 4A2		
DV859294	Unknown protein	3

DV858967	ADP-Ribosylation factor	3
DV855831	Photosystem II reaction center W	1
Linkage Group 5A1		
DV853194	Glucosamine-fructose-6-phosphate	11, 12
DV858575	No match	NA
DV857414	Peptidyl-prolyl cis-trans isomerase	9
DV859329	One helix protein	12
DV853105	Phosphatidylinositol phosphodiesterase	9
DV859108	Unknown protein	12
DV855709	Heat shock protein 80	9
DV858550	Monosaccharide transporter	9
DV853746	Fatty acid desaturase	9
DV852755	Thioredoxin M-type	12
DV855644	Ribosomal protein P1	8
DV856306	Unknown protein	12
Linkage Group 5A2		
DV858974	Unknown protein	12
DV856365	Heat shock protein 80	9
DV857545	Unknown protein	9
Linkage Group 6A1		
DV856448	Class IV chitinase	2
DV855732	Dehydrin	2
DV856314	Ribosomal protein S21	2
FD933075	Unknown protein	2
FE597042	No match	NA
DV855897	Thioredoxin-like protein	2
DV854250	Unknown protein	2
Linkage Group 6A2		
DV855964	Class IV chitinase	2
DV854103	Cyclophilin	2
DV853829	Unknown protein	2
DV853564	Sterol carrier protein 2	2
DV852843	Unknown protein	8
Linkage Group 7A1		
DV857284	smr domain protein	6
DV853439	FAD-linked oxidoreductase	6
DV859329	One helix protein	12
DV853828	NADPH-dependent reductase	8
DV858291	Photosystem II 10 kD protein	8

DV858879	Unknown protein	8
DV852753	Unknown protein	8
DV858277	Mitochondrial ATP synthase	8
DV857095	Unknown protein	8
DV859455	FAD-linked oxidoreductase	6
DV857083	Photosystem I subunit N	12
DV859270	Mt aldehyde dehydrogenase	6
DV856180	Caffeic acid O-methyltransferase	8
Linkage Group 7A2		
DV857377	Photosystem II 10KD	8
DV857991	Serine-glyoxylate aminotransferase	8

Table 4.3*. Goodness of fit of a 3-gene recessive epistasis model for inheritance of dollar spot resistance from colonial bentgrass in the backcross progeny.*

Genotype models of parents (interspecific hybrid x creeping bentgrass)	***N***	**Observed**		**Expected**		X^2	***P***
		Resistant†	Susceptible	Resistant	Susceptible		
AaBbCc‡ x aabbcc	271	31	240	34	237	0.30	0.58

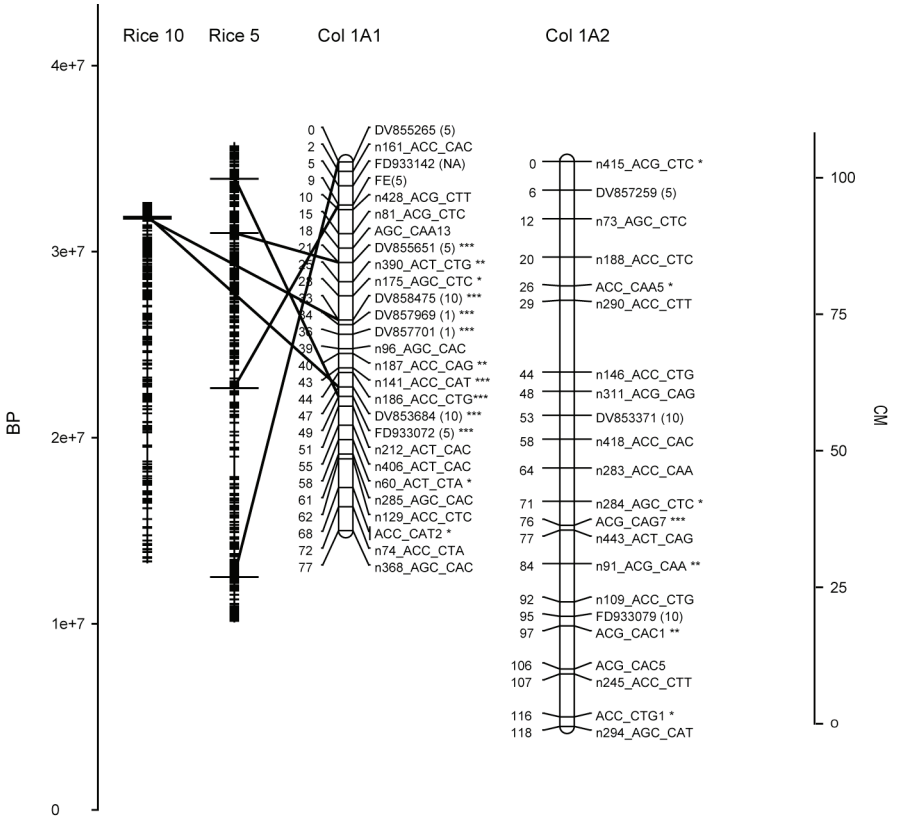
† Resistance defined as having a dollar spot average of 7.0 or higher in both years

‡ Genotype AxBxCx required for resistance

Table 4.4*. Colonial bentgrass markers present in all of the resistant individuals of the mapping population. The percentage of the additional 18 dollar spot resistant backcross individuals not in the mapping population, as well as the overall percentage of individuals in the mapping population, that have each marker is indicated. The X^2 and P values for the expected presence of each marker in the additional resistant plants is indicated.*

Marker	Additional resistant plants with marker (%)	Mapping population plants with marker (%)	X^2	*P*
Group 2A1				
DV853200	100	63.4	10.42	0.0012
DV859086	100	64.5	9.93	0.0016
DV855844	88.9	64.5	4.69	0.0302
DV856678	100	67.7	8.55	0.0034
DV853949	94.4	67.7	5.86	0.0154
DV855820	100	65.6	9.45	0.0021
DV857390	100	65.6	9.45	0.0021
FE597041	88.9	63.4	5.06	0.0244
n341_ACG_CTG	-	-	-	-
AGG_CTC2	-	-	-	-
n73_ACG_CAA	-	-	-	-
DV855508	83.3	61.3	3.74	0.0531
n367_AGC_CAT	-	-	-	-
AGC_CAT2	-	-	-	-
Group 3A1				
DV854798	88.9	51.6	9.98	0.0015

Figure 4.1. *Genetic linkage map of colonial bentgrass. Genetic length in cM for the colonial bentgrass linkage groups is indicated on the left and marker identification is indicated on the right. The AFLP markers are named according to marker number followed by the selective nucleotides. The EST markers are named by their GenBank accession numbers, with the rice chromosomal location of the most similar gene in parentheses. Loci labeled with asterisks showed distorted segregation (*P<0.05, ** P<0.01, ***P<0.001). To the left of the A1 linkage groups are diagrams of the rice chromosomes having the genes most similar to the mapped ESTs. The colonial bentgrass EST collection was compared to the rice genome and the positions of the best matches are indicated as small horizontal lines on the rice chromosomal diagrams. The position of the most similar rice gene to the mapped colonial bentgrass ESTs is indicated by the longer horizontal lines on the rice chromosome diagrams and lines are drawn between the colonial bentgrass EST and the most similar rice gene.*


Rice 10
Rice 5
Col 1A1
Col 1A2
BP
4e+7
3e+7
2e+7
1e+7
0
0 DV855265 (5)
2 n161_ACC_CAC
5 FD933142 (NA)
9 FE(5)
10 n428_ACG_CTT
15 n81_ACG_CTC
18 AGC_CAA13
21 DV855651 (5) ***
25 n390_ACT_CTG **
28 n175_AGC_CTC *
33 DV858475 (10) ***
34 DV857969 (1) ***
36 DV857701 (1) ***
39 n96_AGC_CAC
40 n187_ACC_CAG **
43 n141_ACC_CAT ***
44 n186_ACC_CTG***
47 DV853684 (10) ***
49 FD933072 (5) ***
51 n212_ACT_CAC
55 n406_ACT_CAC
58 n60_ACT_CTA *
61 n285_AGC_CAC
62 n129_ACC_CTC
68 ACC_CAT2 *
72 n74_ACC_CTA
77 n368_AGC_CAC
0 n415_ACG_CTC *
6 DV857259 (5)
12 n73_AGC_CTC
20 n188_ACC_CTC
26 ACC_CAA5 *
29 n290_ACC_CTT
44 n146_ACC_CTG
48 n311_ACG_CAG
53 DV853371 (10)
58 n418_ACC_CAC
64 n283_ACC_CAA
71 n284_AGC_CTC *
76 ACG_CAG7 ***
77 n443_ACT_CAG
84 n91_ACG_CAA **
92 n109_ACC_CTG
95 FD933079 (10)
97 ACG_CAC1 **
106 ACG_CAC5
107 n245_ACC_CTT
116 ACC_CTG1 *
118 n294_AGC_CAT
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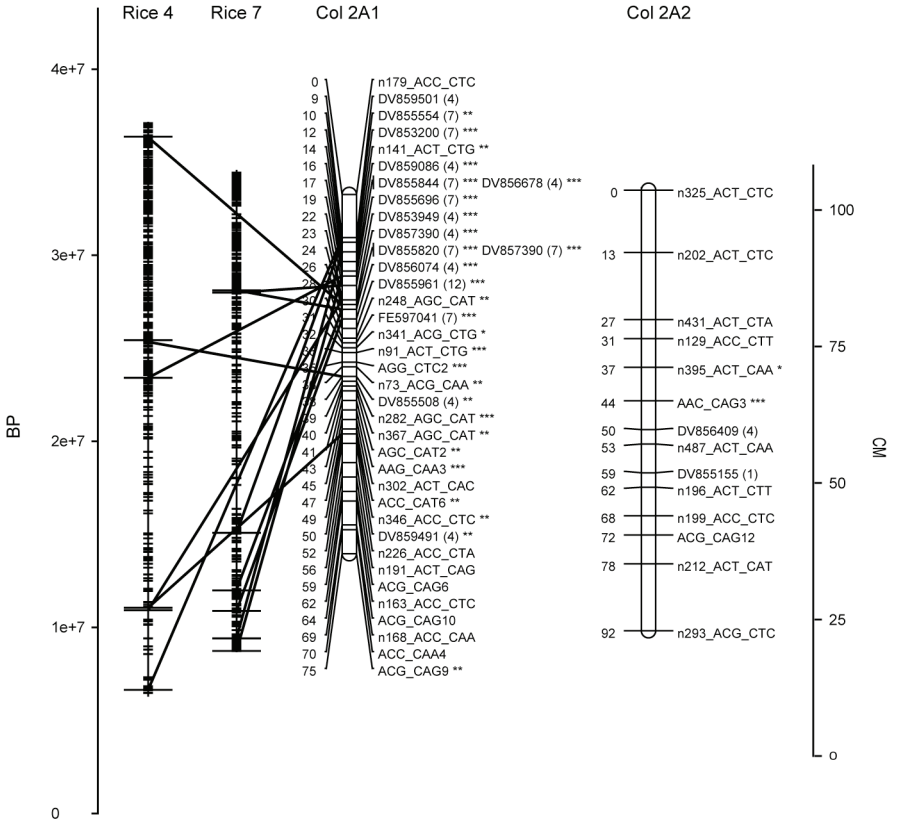
75
50
25
0
CM


Rice 4
Rice 7
Col 2A1
Col 2A2
BP
4e+7
3e+7
2e+7
1e+7
0
0 n179_ACC_CTC
9 DV859501 (4)
10 DV855554 (7) **
12 DV853200 (7) ***
14 n141_ACT_CTG **
16 DV859086 (4) ***
17 DV855844 (7) *** DV856678 (4) ***
19 DV855696 (7) ***
22 DV853949 (4) ***
23 DV857390 (4) ***
24 DV855820 (7) *** DV857390 (7) ***
26 DV856074 (4) ***
28 DV855961 (12) ***
30 n248_AGC_CAT **
32 FE597041 (7) ***
34 n341_ACG_CTG *
36 n91_ACT_CTG ***
38 AGG_CTC2 ***
39 n73_ACG_CAA **
39 DV855508 (4) **
40 n282_AGC_CAT ***
40 n367_AGC_CAT **
41 AGC_CAT2 **
43 AAG_CAA3 ***
45 n302_ACT_CAC
47 ACC_CAT6 **
49 n346_ACC_CTC **
50 DV859491 (4) **
52 n226_ACC_CTA
56 n191_ACT_CAG
59 ACG_CAG6
62 n163_ACC_CTC
64 ACG_CAG10
69 n168_ACC_CAA
70 ACC_CAA4
75 ACG_CAG9 **
0 n325_ACT_CTC
13 n202_ACT_CTC
27 n431_ACT_CTA
31 n129_ACC_CTT
37 n395_ACT_CAA *
44 AAC_CAG3 ***
50 DV856409 (4)
53 n487_ACT_CAA
59 DV855155 (1)
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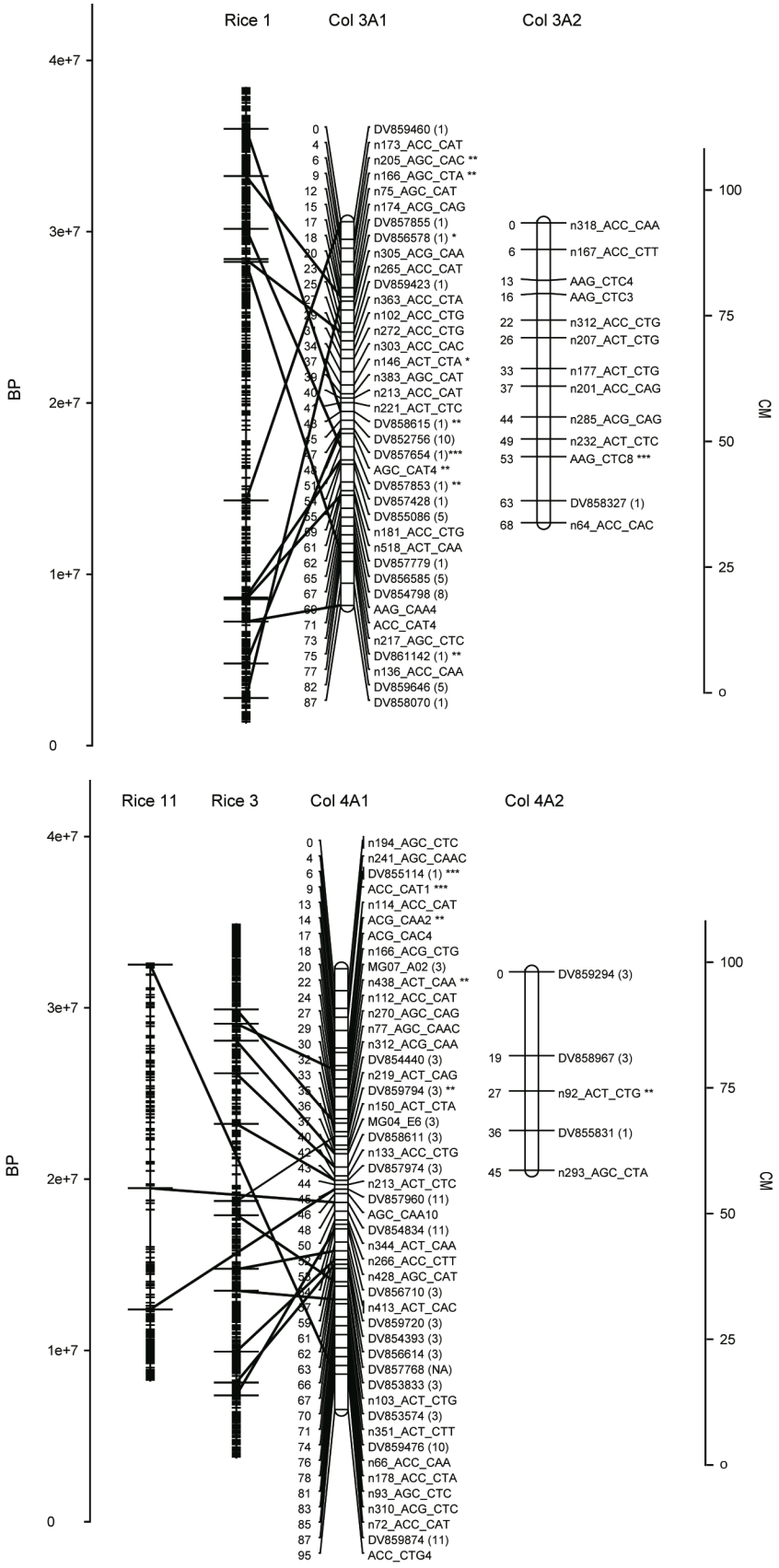
72 ACG_CAG12
78 n212_ACT_CAT
92 n293_ACG_CTC
100
75
50
25
0
CM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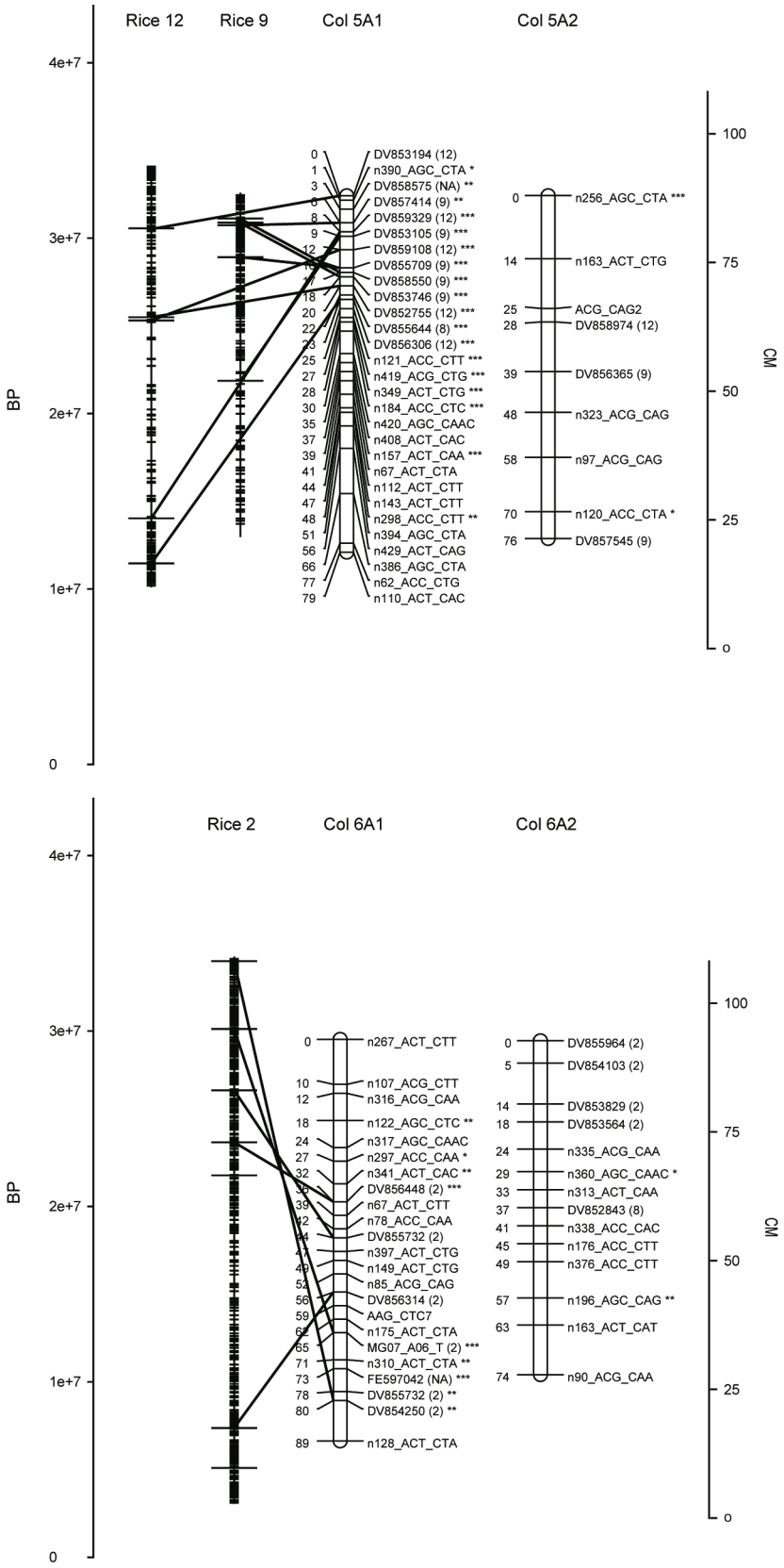
Col 3A1
Col 3A2
BP
CM
4e+7
3e+7
2e+7
1e+7
0
100
75
50
25
0
0 DV859460 (1)
4 n173_ACC_CAT
6 n205_AGC_CAC **
9 n166_AGC_CTA **
12 n75_AGC_CAT
15 n174_ACG_CAG
17 DV857855 (1)
18 DV856578 (1) *
20 n305_ACG_CAA
23 n265_ACC_CAT
25 DV859423 (1)
27 n363_ACC_CTA
29 n102_ACC_CTG
31 n272_ACC_CTG
34 n303_ACC_CAC
37 n146_ACT_CTA *
39 n383_AGC_CAT
40 n213_ACC_CAT
41 n221_ACT_CTC
43 DV858615 (1) **
45 DV852756 (10)
47 DV857654 (1)***
49 AGC_CAT4 **
51 DV857853 (1) **
53 DV857428 (1)
55 DV855086 (5)
59 n181_ACC_CTG
61 n518_ACT_CAA
62 DV857779 (1)
65 DV856585 (5)
67 DV854798 (8)
69 AAG_CAA4
71 ACC_CAT4
73 n217_AGC_CTC
75 DV861142 (1) **
77 n136_ACC_CAA
82 DV859646 (5)
87 DV858070 (1)
0 n318_ACC_CAA
6 n167_ACC_CTT
13 AAG_CTC4
16 AAG_CTC3
22 n312_ACC_CTG
26 n207_ACT_CTG
33 n177_ACT_CTG
37 n201_ACC_CAG
44 n285_ACG_CAG
49 n232_ACT_CTC
53 AAG_CTC8 ***
63 DV858327 (1)
68 n64_ACC_CAC
Rice 11
Rice 3
Col 4A1
Col 4A2
BP
CM
4e+7
3e+7
2e+7
1e+7
0
100
75
50
25
0
0 n194_AGC_CTC
4 n241_AGC_CAAC
6 DV855114 (1) ***
9 ACC_CAT1 ***
13 n114_ACC_CAT
14 ACG_CAA2 **
17 ACG_CAC4
18 n166_ACG_CTG
20 MG07_A02 (3)
22 n438_ACT_CAA **
24 n112_ACC_CAT
27 n270_AGC_CAG
29 n77_AGC_CAAC
30 n312_ACG_CAA
32 DV854440 (3)
33 n219_ACT_CAG
35 DV859794 (3) **
36 n150_ACT_CTA
37 MG04_E6 (3)
40 DV858611 (3)
42 n133_ACC_CTG
43 DV857974 (3)
44 n213_ACT_CTC
45 DV857960 (11)
46 AGC_CAA10
48 DV854834 (11)
50 n344_ACT_CAA
52 n266_ACC_CTT
53 n428_AGC_CAT
56 DV856710 (3)
57 n413_ACT_CAC
59 DV859720 (3)
61 DV854393 (3)
62 DV856614 (3)
63 DV857768 (NA)
66 DV853833 (3)
67 n103_ACT_CTG
70 DV853574 (3)
71 n351_ACT_CTT
74 DV859476 (10)
76 n66_ACC_CAA
78 n178_ACC_CTA
81 n93_AGC_CTC
83 n310_ACG_CTC
85 n72_ACC_CAT
87 DV859874 (11)
95 ACC_CTG4
0 DV859294 (3)
19 DV858967 (3)
27 n92_ACT_CTG **
36 DV855831 (1)
45 n293_AGC_CTA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Rice 9
Col 5A1
Col 5A2
BP
CM
4e+7
3e+7
2e+7
1e+7
0
100
75
50
25
0
0 DV853194 (12)
1 n390_AGC_CTA *
3 DV858575 (NA) **
6 DV857414 (9) **
8 DV859329 (12) ***
9 DV853105 (9) ***
12 DV859108 (12) ***
15 DV855709 (9) ***
17 DV858550 (9) ***
18 DV853746 (9) ***
20 DV852755 (12) ***
22 DV855644 (8) ***
23 DV856306 (12) ***
25 n121_ACC_CTT ***
27 n419_ACG_CTG ***
28 n349_ACT_CTG ***
30 n184_ACC_CTC ***
35 n420_AGC_CAAC
37 n408_ACT_CAC
39 n157_ACT_CAA ***
41 n67_ACT_CTA
44 n112_ACT_CTT
47 n143_ACT_CTT
48 n298_ACC_CTT **
51 n394_AGC_CTA
56 n429_ACT_CAG
66 n386_AGC_CTA
77 n62_ACC_CTG
79 n110_ACT_CAC
0 n256_AGC_CTA ***
14 n163_ACT_CTG
25 ACG_CAG2
28 DV858974 (12)
39 DV856365 (9)
48 n323_ACG_CAG
58 n97_ACG_CAG
70 n120_ACC_CTA *
76 DV857545 (9)
Rice 2
Col 6A1
Col 6A2
BP
CM
4e+7
3e+7
2e+7
1e+7
0
100
75
50
25
0
0 n267_ACT_CTT
10 n107_ACG_CTT
12 n316_ACG_CAA
18 n122_AGC_CTC **
24 n317_AGC_CAAC
27 n297_ACC_CAA *
32 n341_ACT_CAC **
35 DV856448 (2) ***
39 n67_ACT_CTT
42 n78_ACC_CAA
44 DV855732 (2)
47 n397_ACT_CTG
49 n149_ACT_CTG
52 n85_ACG_CAG
56 DV856314 (2)
59 AAG_CTC7
62 n175_ACT_CTA
65 MG07_A06_T (2) ***
71 n310_ACT_CTA **
73 FE597042 (NA) ***
78 DV855732 (2) **
80 DV854250 (2) **
89 n128_ACT_CTA
0 DV855964 (2)
5 DV854103 (2)
14 DV853829 (2)
18 DV853564 (2)
24 n335_ACG_CAA
29 n360_AGC_CAAC *
33 n313_ACT_CAA
37 DV852843 (8)
41 n338_ACC_CAC
45 n176_ACC_CTT
49 n376_ACC_CTT
57 n196_AGC_CAG **
63 n163_ACT_CAT
74 n90_ACG_CAA

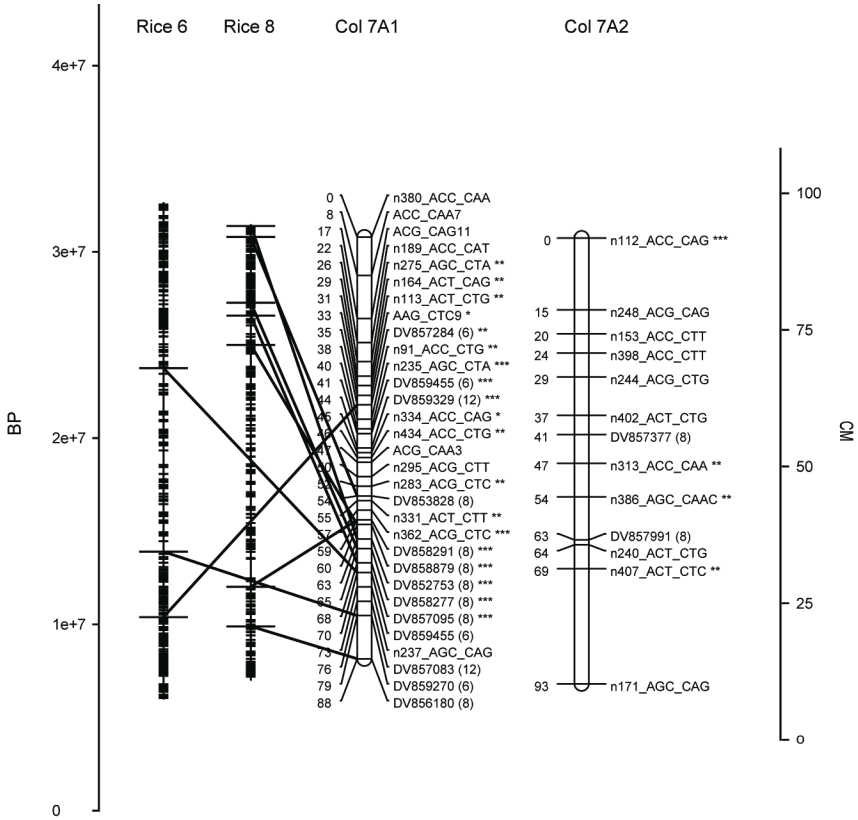

Rice 6
Rice 8
Col 7A1
Col 7A2
BP
4e+7
3e+7
2e+7
1e+7
0
0 n380_ACC_CAA
8 ACC_CAA7
17 ACG_CAG11
22 n189_ACC_CAT
26 n275_AGC_CTA **
29 n164_ACT_CAG **
31 n113_ACT_CTG **
33 AAG_CTC9 *
35 DV857284 (6) **
38 n91_ACC_CTG **
40 n235_AGC_CTA ***
41 DV859455 (6) ***
44 DV859329 (12) ***
45 n334_ACC_CAG *
46 n434_ACC_CTG **
47 ACG_CAA3
50 n295_ACG_CTT
53 n283_ACG_CTC **
54 DV853828 (8)
55 n331_ACT_CTT **
57 n362_ACG_CTC ***
59 DV858291 (8) ***
60 DV858879 (8) ***
63 DV852753 (8) ***
65 DV858277 (8) ***
68 DV857095 (8) ***
70 DV859455 (6)
73 n237_AGC_CAG
76 DV857083 (12)
79 DV859270 (6)
88 DV856180 (8)
0 n112_ACC_CAG ***
15 n248_ACG_CAG
20 n153_ACC_CTT
24 n398_ACC_CTT
29 n244_ACG_CTG
37 n402_ACT_CTG
41 DV857377 (8)
47 n313_ACC_CAA **
54 n386_AGC_CAAC **
63 DV857991 (8)
64 n240_ACT_CTG
69 n407_ACT_CTC **
93 n171_AGC_CAG
100
75
50
25
0
CM

Figure 4.2. *Distribution of dollar spot severity among genotypes of the backcross population as well as those of the parental plants on 8-29-05 and 8-18-06.*

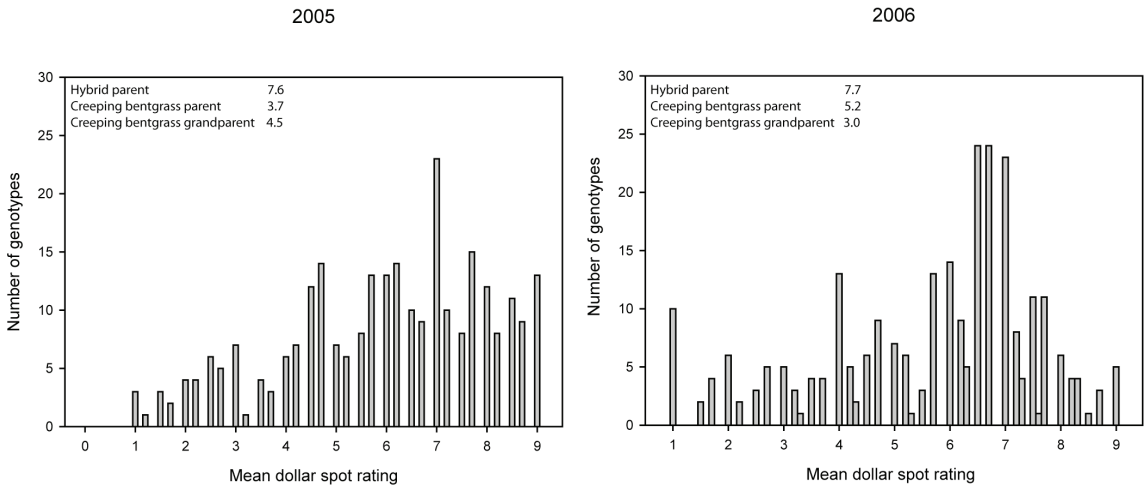
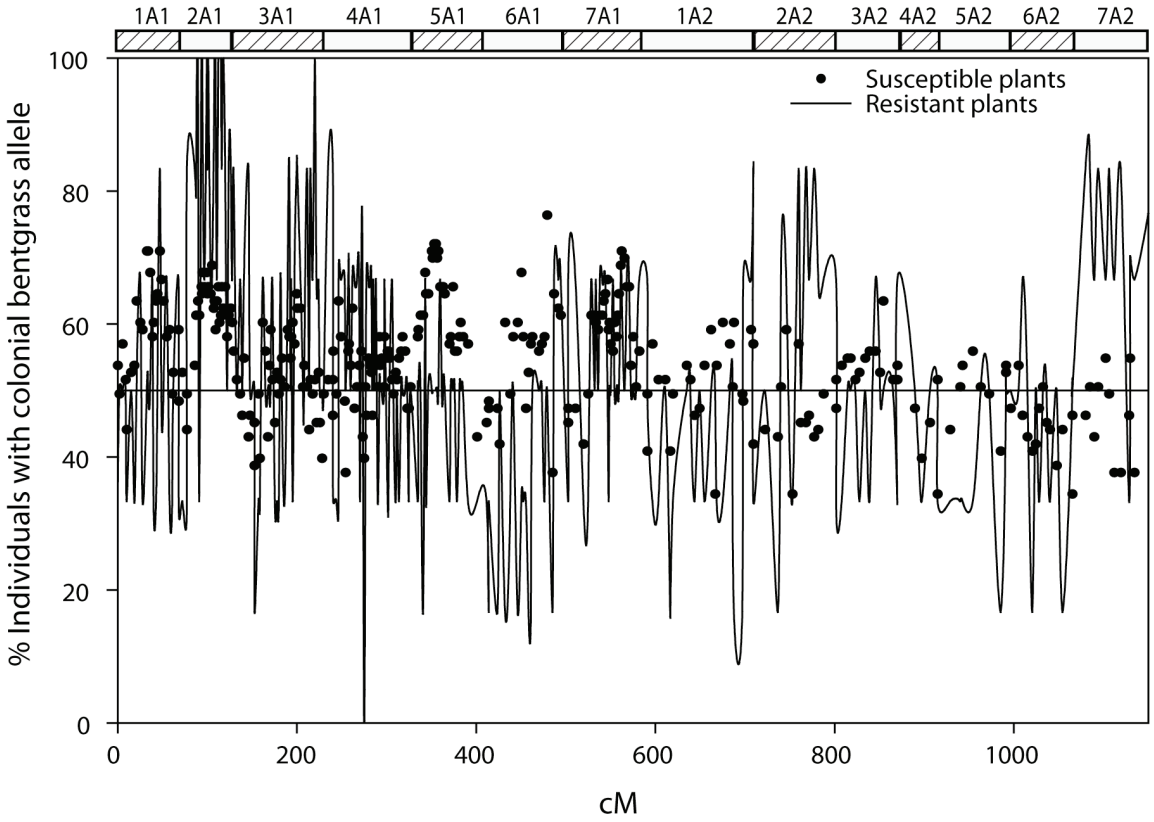


Figure 4.3. *Percentage of individuals of the mapping population possessing each mapped colonial bentgrass marker. The percentage the resistant plants possessing each marker is indicated as a line and the percentage of the susceptible plants possessing each marker is indicated with dots. The linkage groups are indicated along the top bar.*



Chapter 5

Summary.

Chapter 5

Summary

Creeping bentgrass is a popular turfgrass that is very susceptible to dollar spot. Colonial bentgrass is a related species that is known for having good resistance. Previous work generated fertile colonial x creeping bentgrass hybrids that possessed much of the aesthetic qualities of creeping bentgrass while retaining colonial bentgrasses resistance to dollar spot infection. This hybrid was backcrossed with another creeping bentgrass plant to create a mapping population which segregated for dollar spot resistance.

The goal of this thesis was to identify potential regions of the colonial bentgrass genome which may be associated with dollar spot resistance. This was achieved by using AFLPs and gene based makers to create a genetic linkage map of colonial bentgrass. The data obtained from this map was correlated with field data to find colonial bentgrass genomic regions held in common among the resistant plants in the backcross population.

As part of this thesis, we generated EST libraries for creeping and colonial bentgrass. The annotation and analysis of these genes proved to be very useful in developing markers for mapping. By aligning common genes between creeping and colonial bentgrass EST libraries we were able to differentiate linkage groups

belonging to the A1 or A2 subgenomes. We named the colonial bentgrass linkage groups using the nomenclature used for wheat.

To further facilitate the identification of polymorphic gene based markers we developed a new marker method, which we termed dideoxy polymorphism scanning. It was an efficient and low cost method of mapping genes and contributed 45% of the gene based markers on the colonial bentgrass genetic linkage map.

The analysis of field data led to the identification of regions possibly associated with dollar spot resistance. The segregation of resistance in the backcross population suggests a three gene recessive epistasis model for dollar spot resistance in colonial bentgrass. By comparing genotypes we identified colonial bentgrass loci on groups 2A1 and 3A1 that are common in all resistant individuals. We hypothesize that genes related to dollar spot resistance may lie on these groups. Other resistant backcross individuals not included in the mapping population also shared these loci at a proportion significantly higher then expected. This lends additional support to our model of dollar spot resistance in colonial bentgrass.

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