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GENETIC LINKAGE MAP OF CREEPING BENTGRASS AND CHARACTERIZATION OF *LpCBF3* GENE FOR COLD TOLERANCE IN PERENNIAL RYEGRASS

By

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ABSTRACT

GENETIC LINKAGE MAP OF CREEPING BENTGRASS AND CHARACTERIZATION OF *LpCBF3* GENE FOR COLD TOLERANCE IN PERENNIAL RYEGRASS

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Colonial bentgrass (*Agrostis capillaris* L.) is a potential source for genetic improvement of resistance to environmental stress and disease for other bentgrass species (*Agrostis* spp.). Genetic diversity in colonial bentgrass species was investigated using amplified fragment length polymorphism (AFLP) markers. Ten *EcoRI*/*Mse*I and six *PstI*/*Mse*I AFLP primer combinations produced 181 and 128 informative polymorphic bands, respectively. Cluster analysis of genetic similarity (GS) estimates revealed a high level of diversity in colonial bentgrass species with averages of 0.51 (*EcoRI*/*Mse*I) and 0.63 (*PstI*/*Mse*I). Greater genetic diversity was detected by the *EcoRI*/*Mse*I AFLP primer combinations. A low but significant positive correlation (r=0.44, p=0.0099) between the two Jaccard similarity matrices was obtained by the Mantel test.

To enhance creeping bentgrass disease resistance, interspecific hybrids between creeping bentgrass and colonial bentgrass were produced for introgression of resistance. The hybrids (2n=28) were confirmed by a species-specific sequence characterized amplified region (SCAR) marker for the colonial bentgrass genome. In addition, an average of seven ring bivalents, several univalents and multivalents were observed at meiosis of hybrids. Pollen fertility of the hybrids ranged from 4% to 34.5%. The hybrids were evaluated for gray snow mold disease resistance in the cold room and dollar spot disease resistance in the greenhouse. Some of the hybrids had enhanced snow mold and dollar spot resistance, exhibiting partially diminished disease symptoms.

A segregating pseudo-testcross population consisting of 188 progeny F_1 full-sib population was developed for linkage map construction and QTL detection in creeping bentgrass. In the male parent map, 93 markers were assigned in 14 linkage groups covering a total length of 793cM with an average interval of 8.2cM, while in the female parent map 139 markers were assigned in 14 linkage groups spanning 805cM with an average distance of 5.9cM between adjacent markers. Five putative QTLs were found in this segregation population, three QTLs for leaf width, one QTL for snow mold disease resistance, and one QTL for recovery rate after snow mold disease infection.

A *CBF* homologous gene, *LpCBF3*, was isolated and characterized from a cold-tolerant perennial ryegrass accession (*Lolium perenne* L.). *LpCBF3* encodes a protein of 237 amino acids with a predicted molecular mass of 25.5 kDa. It carries sequences of the typical AP2 DNA-binding domain and an acidic activation which are present in the majority of the plant CBF family. Northern blotting and RT-PCR analysis found *LpCBF3* reached the highest expression after 90 minutes of cold-treatment (4°C). In *Arabidopsis*, the over-expression of *LpCBF3* under control of the 35S promoter resulted in plants that were dwarf, later flowering, and freezing tolerant. These results lead us to propose potential implications and applications of *LpCBF3* gene in turfgrass cold-tolerance and quality breeding program.

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LITERATURE REVIEW

Introduction

Bentgrass species (*Agrostis* spp.), native to western Europe, belong to the Poaceae family and are cool season turfgrasses (Harlan, 1992). Distribution of the species spans from shade to open habitats, lowlands to highlands and cool to extremely arctic areas. Bentgrasses are normally out-crossing pollinated species and vary in ploidy levels. There are over 220 known species of bentgrass (Hitchcock, 1951). Five species are used commercially as turfgrass in the USA. These include creeping bentgrass (*A. stolonifera*), colonial bentgrass (*A. capillaris*), dryland bentgrass (*A. castellana*), velvet bentgrass (*A. canina*) and redtop bentgrass (*A. gigantean*).

Creeping bentgrass is best known for its fine texture and adaptation to mowing heights as low as 3mm, which makes it well suited for use on golf course tees, greens and fairways. The commercial value of creeping bentgrass makes it attractive for genetic improvement. However, breeding progress in this species has lagged behind other grass species. Creeping bentgrass species is susceptible to a wide range of diseases, including dollar spot (*Sclerotinia homoeocarpa*); (Viji et al., 2004), brown patch (*Rhizoctonia solani*) ;(Burpee and Goulty, 1984), and gray snow mold (*Typhula incarnate*); (Wu and Hsiang, 1998). No commercial cultivars show an acceptable resistance to these pathogens (Vincelli et al., 1997). Control of these diseases relies heavily on fungicide applications and cultivation management (Abernathy et al., 2001). The fungicides are expensive to apply, have limited efficacy, and may adversely affect the environment. Some fungal pathogens may develop resistance to fungicides after years of repeated applications

(Latin, 2006; Reicher and Throssell, 1997). In addition, disease control by cultivation management has been met with limited success due to the environmental effects.

Developing disease-resistant creeping bentgrass cultivars is believed to be an efficient alternative method to overcome these disease infections. However, disease-resistant germplasm should be identified for the eventual development of resistant cultivars (Boerma and Hussey, 1992). Fortunately, two types of disease resistance germplasm are available within creeping bentgrass breeding programs. The first resistance is derived from creeping bentgrass related species, such as colonial bentgrass. Some colonial bentgrass accessions show good resistance to dollar spot and snow mold diseases (Belanger et al., 2003). These resistant genes can be integrated into the creeping bentgrass genome by interspecific hybridization and genome introgression. However, this strategy requires multiple selfing and backcrossing to minimize the length of alien chromosome segments and eliminate deleterious gene combinations, thereby reducing linkage drag. The second approach is to screen the resistant turfgrass clones from old golf courses that have not been sprayed with fungicide or over-seeded for several years. Creeping bentgrass populations from the old golf courses are under a high selection level of disease. Some clones which may contain the resistant genes from natural mutations should be able to preserve and thrive. The resistant genes from these clones could be exploited and utilized to enhance disease resistance levels and diversifying the resistance base in bentgrass cultivars by interpollination.

Understanding the genetics of resistant genes of bentgrass species could accelerate the processes of resistance selection and improvement. The basic chromosome number of bentgrass species genome is $x=7$. Genome compositions of some *Agrostis* species were determined by examining chromosome pairing configurations of species or their hybrids (Jones, 1956a; Jones, 1956b). Creeping bentgrass species is a strict allotetraploid with a genome composition of C_2C_2SS, and shares a common genome C_2C_2 with colonial bentgrass. Colonial bentgrass is a segmental allotetraploid with a genome composition of $C_1C_1C_2C_2$. These two species are sexually compatible and capable of producing hybrids, which may result in introgression of the alien genes between common genomes after pairing. Redtop bentgrass is an allopolyploid with a genome composition of $C_1C_1C_2C_2SS$, which includes the three genomes of creeping bentgrass and colonial bentgrass. Redtop and creeping bentgrass are cross-compatible and capable of producing sterile pentaploid hybrids. More details on genome compositions of bentgrass species are listed in Table 1.1.

Table 1.1 Proposed genomic compositions of five common-used bentgrass species

Common name	**Species**	**Chromosome number**	**Chromosome composition**	
Creeping bentgrass	*A. stolonifera*	28	C_2C_2SS	Allotetraploid
Colonial bentgrass	*A. capillaris*	28	$C_1 C_1 C_2C_2$	Segmental allotetraploid
Dryland	*A. castellana*	42	$C_1 C_1 C_2C_2$??	Segmental allotetraploid
Velvet bentgrass	*A. canina*	14	$C_1 C_1$	Diploid
Redtop bentgrass	*A. gigantean*	42	$C_1 C_1 C_2C_2SS$	Allohexaploid

Although the ambiguities of genome composition from some species still need clarification, the cytogenetics research conducted a half century ago has already provided an excellent initial examination of the species relationship in this complex genus (Jones, K. 1956a). Molecular techniques can be used to add resolution in cytogenetics studies. Diversity analysis with AFLP provides an alternative approach to elucidate genome composition relationships in the not under-researched and complex genus. In our study, relatedness of creeping bentgrass and three other bentgrass species, colonial bentgrass, dryland bentgrass, and velvet bentgrass was elucidated by using 199 polymorphic AFLP markers. Both principal component analysis (PCA) and unweighted pair group method with arithmetic mean (UPGMA) dendrogram distinguished these four bentgrass species into three groups, which are consistent with their genome compositions. Colonial and dryland bentgrass were clustered into the same group based on their similarity of genome compositions. Although this method cannot completely replace the traditional meiosis research to determine genome composition, it can provide preliminary genome composition information based on the genetic distance of different species (Zhao and Bughrara, submitted).

I. Phenotypic and genetic diversity research

Bentgrass species have an abundance of phenotypic and molecular diversity that can be harnessed for gains in cultivar development. However, to utilize this diversity effectively, an understanding of the phenotypic and molecular variations of the germplasm are necessary because genetic diversity is considered as one of the criteria for germplasm conservation and parent selection for cultivars (Menkir et al., 2006; Thompson and Nelson, 1998). Diverse genetic backgrounds provide an ample supply of allelic variation that can be used to create a new, favorable and unique gene combination for maximizing genetic gain by hybridization (Carena and Wicks, 2006). In plants, two procedures are available to evaluate diversity among the genotypes: investigating morphological traits or estimating the genetic relatedness based on fingerprint of DNA markers (Ferriol et al., 2004). Morphological characteristics can be analyzed by Euclidian distance, and is applied to examine the morphological divergence in large collections of plant genotypes (Anderson et al., 2006). These traits can be evaluated *in situ* or in the greenhouse. Morphological characters are easiest to measure and may provide a primary classification of the collection. However, because these morphological characters are subject to considerable environmental influence and different plant growth stages, the data collected from different years and locations could vary. Estimating genetic distance based on DNA marker polymorphisms offers the unique capacity to describe genetic diversity, and distinguish heterotic groups within plant species due to their abundant polymorphism and independence of environmental effect. Several types of molecular markers are available for evaluating the extent of genetic diversity in crops. Microsatellite or simple sequence repeat (SSR) markers and restriction fragment length polymorphism (RFLP) markers are dependable marker systems for diversity research (Guarino et al., 2006; Paull et al., 1998). However, they are unavailable in some plant genera because of limited genomic sequence information. Random amplified polymorphic DNA (RAPD) markers can be applied in diversity research in turfgrass species, but the approach has been criticized because the results are lack of reliable or reproducible (Rajasekar et al., 2006). Amplified fragment length polymorphism (AFLP) markers provide a powerful tool for genetics diversity research among turfgrass species because their polymorphism and no prior sequence information is required for their application (Vergara and Bughrara, 2003, 2004). AFLP markers can be generated by CNG methylation-sensitive (*Pst*I/*Mse*I) or by CNG methylation-insensitive (*Eco*RI/*M*seI) restriction enzyme combinations that target different chromosome regions (Menz et al., 2002).

In theory, phenotypic diversity from morphological variations should reflect the genetic relationship drawn from the DNA markers. As the diversity of morphological traits increases in a population, accordingly, the diversity of genes involved in the control of morphological traits should increase. Several studies have attempted to correlate plant diversity drawn from DNA markers with a diversity of morphological traits with the expectation that morphological variations would be reflected by genetic diversity derived from molecular markers (Borba et al., 2002; Bruschi et al., 2003). However, in most cases, information on genetic diversity from molecular markers does not appear to correlate with differences in morphological traits in other species (Tar'an et al., 2005; Tommasini et al., 2003). Genomic similarity which does not necessarily reflect resemblance of morphological traits may be the results of the following. First, a small number or common set of genes/QTLs may control several correlated morphological traits observed in the experiment. The genome coverage represented by morphological traits is likely to be extremely low. Second, most of the morphological traits used as genetic markers were controlled by QTLs. Different morphological traits may be controlled by a different numbers of genes that are difficult to distinguish by applying different statistical weights in diversity calculation. When comparing AFLP markers, every marker is given the same statistical weight. This could directly lead to a lack of correlation between two dendrograms drawn from DNA markers and morphological traits. Third, a significant proportion of environmental variation can be present in the estimation of morphological traits. Fourth, an absolute measure of genetic difference is not technically feasible without the entire genome sequences. Consequently, any molecular markers used to study differences will represent a subset of the variation sampled. The limited proportions and different specific regions of the genome scanned by DNA markers will lead to deviation of estimations. Lastly, AFLP fragments with the same size could originate from different loci and may have different locus origins. This could be the case in segmental allotetraploid colonial bentgrass due to its genome composition and multiple homologous loci. The approaches used to attempt to overcome the above problems are as follows. Markers developed from expressed sequences would be used that may be directly responsible for morphological traits of genotypes. Single nucleotide polymorphisms (SNPs) could be applied to genetic diversity research. SNPs has great potential for the detection of associations between allelic forms of a gene and phenotypes (Rafalski, 2002).

Genetic dendrograms may be used for universal taxonomic studies because the molecular markers offer the possibility to screen a large number of anonymous loci, and provide the unique capacity to classify genotypes regardless of environmental condition and plant growth stage. Since most desirable traits in plants are the result of the interaction among expressed genes, they cannot be easily elucidated by DNA markers. Morphology studies are still critical in germplasm description, serving as an efficient tool for selection of morphologically diverse parents for the breeding programs. Therefore, determination of molecular diversity can be considered as complementing the morphological classification.

II. Linkage construction and QTL detection in out-pollinating plant species

Linkage maps provide researchers insights with into genome structure and evolution by comparing the genetic synteny among related species. Linkage maps are useful for identifying QTLs or genes of interest by providing the framework to understand the biological basis of complex traits. In out-crossing heterozygous turfgrass species, such as creeping bentgrass species, F_2 or backcross segregation populations are rarely available due to self-incompatibility. Genetic linkage construction is performed commonly on F_1 progeny (pseudo F_2) derived from the crossing between two heterozygous parents (Chakraborty et al., 2005; Inoue et al., 2004). Since genetic segregations in the F_1 progeny are the result of meiotic recombination from both parents (not between two parents), segregation data can be analyzed as a double pseudo-testcross strategy and used in developing two separate maps (female and male parent maps). AFLP (Amplified Fragment Length Polymorphism), which does not require the prior knowledge of the DNA sequence, is considered to be a highly productive and reproducible DNA marker system. AFLP has been applied to rapidly create genetic linkage maps and detect the QTLs in some grass species (Saha et al., 2005). QTL, an association between marker loci and the phenotypic variation in the segregation population, can be studied in terms of the magnitude of effects on phenotype, the parental origins of the favorable QTL alleles and the interactions between different QTLs. Several examples have been reported for QTL mapping in out-pollinating plant species. In grapevine (*Vitis vinifera*), putative QTLs for seedlessness, berry weight, and fruit yield were identified (Doligez et al., 2002; Fanizza et al., 2005). In perennial ryegrass (*Lolium perenne*), three potential QTLs of resistance to gray leaf spot were characterized (Curley et al., 2005) and four QTLs controlling crown rust resistance were detected (Muylle et al., 2005). For turfgrass species in general, and creeping bentgrass in particular, QTL analysis has met with limited success. No documented information is currently available on documented QTL analysis in creeping bentgrass species.

Linkage information from well-studied major crops provides a promising approach to elucidating turfgrass linkage structure and arrangement. Some crop species such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oat (*Avena sativa*), maize (*Zea mays*) and other Gramineae species have been studied separately by different linkage construction projects. Comparative mapping using homologous sequences across the species can merge the separate genetic linkages and maps which would be useful for cross-referencing genetic information (Lagercrantz, 1998). RFLP (cDNA probe) and SSR (derived from EST sequence from the homologous regions across different species) markers in linkage map often reveal extensive conservations of syntenic relationships among related species. Comparative maps between species can provide insights into the chromosome, even chromosome segments arrangement, which cannot be solved by using traditional cytogenetical methods. Conserved chromosomal segments identified in these species show the translocation, conservation, deletion and duplication that occurred during evolution and selection process (Beales et al., 2005). In addition, by comparing the genetic changes responsible for evolving similar traits in widely separated populations, it should be possible to determine whether there are many different approaches to evolve a new characteristic.

Developing a platform to rapidly dissect complex traits by utilizing both association and linkage based approaches is playing a critical role in QTL analysis. To conduct these analyses, linkage and association populations that capture much of the natural variation inherent in the plant genome need to be developed. Extensive phenotyping and surveys of tens of thousands of candidate gene sequences will then be employed. This approach should allow the rapid dissection of complex traits at the gene (DNA sequence) level. Although QTL mapping has been successful in describing the genetic architecture of complex traits, the molecular basis of quantitative variation is less well-understood. Besides gene sequence differences which directly lead to functional variations, the intergenic sequences (non-coding sequence) also have an impact on phenotypic polymorphism by changing gene expression patterns and levels. Clark and his colleagues (2006) provide an excellent example of elucidating the intergenic DNA sequence basis for phenotypic variation and the role of cis-regulatory evolution by studying a distant upstream enhancer of *tb1* gene in maize. Recently, expression QTL (eQTL) analyses have provided a new approach to dissect QTL (Farrall, 2004). eQTL can be split into two categories, *cis*-acting and *trans*-acting classes, based on the relative genomic locations of the transcript and its phenotypic QTL. This has provided a glimpse into some basic principles regarding the relative contributions of *cis*-acting versus *trans*-acting loci. Significantly more *trans*-acting QTLs (N=110) than *cis*-acting QTLs (N=17) were found in human lymphoblastoid cell lines study (Morley et al., 2004). Some of the *trans*-acting QTLs were found to aggregate in genomic 'hotspots'. These hotspots presumably contain the 'master regulators', each controlling a large number of transcripts. A global understanding of genetic variations will provide new insights into the vast amount of genetic linkage and gene expression data accumulated over time.

III. CBF and cold tolerance

Plants from temperate regions can increase their cold tolerance in response to low, non-freezing temperature. This process is called cold acclimation which contributes to an ability to survive, grow, and acclimate at low temperatures. Cold acclimation process involves physiological and biochemical adaptations which are regulated by at least two pathways, abscisic acid (ABA) and ABA independent (Shinozaki and Yamaguchi-Shinozaki, 2000, Nordin et al., 1991). The plant hormone abscisic acid (ABA) modulates a wide spectrum of responses, including gene activation and repression, guard cell closure, cell cycle blockage, and photosynthesis inhibition, under multiple environmental stress conditions such as drought, cold, and salinity (Sheen, 1998). In the ABA-independent process, cold temperatures trigger the transcription of the CBF family by an upstream transcription factor, *ICE1* (inducer of CBF expression 1); (Chinnusamy et al., 2003). The expression of CBF factors activates the transcription *COR* genes encoding a diverse array of proteins with a presumed function in tolerance to dehydration caused by freezing (Mohapatra et al., 1989). Cold signaling for freezing tolerance requires a cascade of transcriptional regulations. In *Arabidopsis*, the transcription of *COR* genes has been shown to be regulated by cis-acting drought and cold responsive elements (DRE) containing a core CCCGAC (CRT) sequence (Yamaguchi-Shinozaki and Shinozaki, 2001, Stockinger et al., 1997). The trans-acting factor CBF (Core Binding Factor) that could bind to DRE and activate *COR* genes' expression was firstly isolated from *Arabidopsis*. The *CBF* genes in *Arabidopsis* are a multigene family, with a potential nuclear localization sequence (NLS), an AP2-DNA-binding domain and an acidic activation domain (Gilmour et al, 1998). Three members of the *CBF/DREB1* family, *CBF1*, *CBF2*, and *CBF3* (or *DREB1b*, *DREB1c*, and *DREB1a*, respectively), are induced within 15 minutes in cold temperatures, followed by expression of the *CBF* regulon of target genes, such as *COR* genes (Gilmour et al., 1998; Liu et al., 2004).

Overexpression of the *CBF/DREB*1 transcription factors in transgenic *Arabidopsis* plants resulted in the accumulation of compatible solutes that have cryoprotective activities, including proline, sucrose, and raffinose accumulation(Gilmour et al., 2000), improvement of cold tolerance by driving expression of *COR* genes at the whole plant level in both nonacclimated and cold-acclimated plants (Thomashow et al., 2001), and the enhancement of the tolerance of plants to dehydration caused by either imposed water deficit or exposure to high salinity (Kasuga et al., 1999). Transgenic plants with overexpressed *CBF* gene exhibit phenotypic traits such as dwarf, stunting growth, and later flowering (Gilmour et al., 2000). The dwarf and stunting growth are desired in turfgrass breeding because of reducing mowing frequency and labor input to lawn maintenance. The later flowering trait can improve the forage quality of ryegrass by decreasing the fiber content.

Understanding the mechanism controlling cold tolerance by connecting phenotypes to the molecular pathway is a critical first step toward the eventual unraveling of the complex interplay among gene, regulator, and environment.

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CHAPTER 1

GENETIC DIVERSITY IN COLONIAL BENTGRASS (*Agrostis capillaris* L.) REVEALED BY ECORI/MSEI AND PSTI/MSEI AFLP MARKERS

Abstract

The colonial bentgrass species (*Agrostis capillaris* L.) is a potential source for genetic improvement of resistance to environmental stress and disease for other bentgrass species (*Agrostis* spp.). To conserve and study the existing genetic resources of colonial bentgrass for use in breeding, genetic diversity in the colonial bentgrass species was investigated using amplified fragment length polymorphism (AFLP) markers. Included in this study were twenty-two accessions from USDA germplasm collected from 11 countries, consisting of fourteen accessions from northern Spain, and three commercial cultivars. Ten *Eco*RI/*Mse*I and six *Pst*I/*Mse*I AFLP primer combinations produced 181 and 128 informative polymorphic bands, respectively. Cluster analysis of genetic similarity estimates revealed a high level of diversity in colonial bentgrass species with averages of 0.51 (*Eco*RI/*Mse*I) and 0.63 (*Pst*I/*Mse*I). Greater genetic diversity was detected by the *Eco*RI/*Mse*I AFLP primer combinations. A low but significant positive correlation (r=0.44, p=0.0099) between the two Jaccard similarity matrices was obtained by the Mantel test. Commercial cultivars of bentgrass showed a narrow genetic background. The assessment of genetic diversity among colonial bentgrass accessions suggested the potential value of the colonial bentgrass germplasm in turfgrass cultivar improvement.

Key words: Colonial bentgrass, Genetic diversity, AFLP, Cluster analysis

Introduction

Colonial bentgrass (*Agrostis capillaris* L. 2n=4x=28), native to Europe and temperate Asia, is commonly used for tennis courts, high-grade lawns, fairways and erosion control (Hubbard, 1984). Colonial bentgrass prefers mowing height between 1.0 to 2.5 cm, which limits its use on the golf course to fairways and tees (Ruemmele, 2003). For this reason colonial bentgrass has been partially replaced by creeping bentgrass (*Agrostis stolonifera* L. 2n=4x=28), which has a fine texture, high density, and good adaptation to low mowing height (Warnke, 2003). However, creeping bentgrass is susceptible to a wide range of fungal diseases such as dollar spot, caused by *Sclerotinia homoeocarpa* (Murphy et al., 2000), and gray snow mold caused by *Typhula incarnata* (Wu and Hsiang, 1998). Dollar spot is a foliar disease favored by high humidity, warm days, and cool nights. Gray snow mold is common in cold, humid conditions, especially under snow cover. These diseases are particularly important in northern regions of the United States where they cause considerable turf loss. Every year, at least one hundred million dollars worth of fungicides are applied to control these diseases, much of it for snow mold and dollar spot (Watson et al., 1992).

Certain colonial bentgrass accessions are widely recognized as having good resistance to gray snow mold (Vergara and Bughrara, 2005) and dollar spot (Belanger et al., 2004). Transferring resistance from colonial to creeping bentgrass is a promising goal in bentgrass breeding programs. Hybridization between creeping and colonial bentgrass species can occur naturally in the field (Jones, 1956) or in the greenhouse (Belanger et al., 2003), which makes it possible to utilize the colonial bentgrass disease resistance by genome introgression to the creeping bentgrass species. Assessment of genetic diversity among accessions of colonial bentgrass species could contribute to the elimination of undesirable duplications in the germplasm collection and increase the efficiency of research efforts. Researchers could select potential diverse resistant genes from different colonial bentgrass sources, and incorporate and pyramid them into creeping or other bentgrass species to enhance levels and durability of disease resistance.

Estimates of genetic distance based on molecular markers have proven a useful method for describing genetic diversity and distinguishing heterotic groups within plant species because of their abundant polymorphism and the fact they are independent of environmental effect. Several types of molecular markers are available for evaluating the extent of genetic diversity in crops. These include restriction fragment length polymorphism (RFLP); (Paull et al., 1998), random amplified polymorphic DNA (RAPD); (Garcia-Mas et al., 2001), amplified fragment length polymorphism (AFLP); (Vergara and Bughrara, 2003; Mian et al., 2002), and microsatellite or simple sequence repeat (SSR); (Burnham et al., 2002). Species-specific RFLP and SSR markers are unavailable in colonial bentgrass due to a lack of existing genomic information. AFLP involving the use of random, largely dominant markers is efficient and cost-effective and requires no prior sequence knowledge. AFLP markers can be generated by CNG methylation-sensitive (*PstI*/*MseI*) or by CNG methylation-insensitive (*Eco*RI/*M*seI) restriction enzyme combinations. AFLP analysis has been applied increasingly for genetic diversity research, and is suitable for evaluating genetic diversity among turfgrass species in which the genetic information is limited (Guthridge et al., 2001; Vergara and Bughrara, 2003).

The genetic diversity of the colonial bentgrass species has not been studied previously.

The objectives of this study were to identify new collected colonial bentgrass germplasm and confirm correct species by mitotic analysis, to investigate the genetic diversity in a collection of the colonial bentgrass species by using AFLP markers (*Eco*RI/*Mse*I and *Pst*I/*Mse*I enzyme combinations), and to compare the correlation between estimates of genetic diversity derived from these two enzyme combinations.

Materials and Methods

Plant materials

Twenty-two different accessions of colonial bentgrass collected from 11 countries were obtained from the USDA Regional Plant Introduction Station (Pullman, Washington). Another fourteen colonial bentgrass accessions were collected from northern Spain. Three commercial cultivars 'Golfin', 'SR 7150', 'Tiger' were also included in this study (Table 2.1).

AFLP analysis

Total genomic DNA was extracted from fresh leaves of 25 plants per accession using the protocol of Gill et al. (1991), and was quantified by DYNA Quant 200 Fluorometer (Pharmacia Biotech, San Francisco, CA). Approximately 200 ng of DNA was digested with two restriction enzyme combinations (*Eco*RI/*Mse*I and *Pst*I/*Mse*I). The *Pst*I and *Eco*RI are six base pair cutters that are methylation-sensitive and methylation-insensitive, respectively. The *Mse*I is a four base pair cutter. The AFLP protocol applied in this research was as described by Vos et al. (1995) with some modifications. Preamplification was done on PTC-100 thermal cycler (MJ Research, Waltham, MA) using 30 cycles of 94℃ 45s, 52℃ 45s, 72℃ 1min, followed by elongation at 72℃ 10min. Combinations of fluorescent dye labeled *Pst*I and non-labeled *Mse*I primers, each with three selective nucleotides at the 3' ends, were used for selective amplification with 56℃ annealing temperature. Adapters and pre-primers used include *Mse*I adapter, *M00* (universal primer), *Eco*RI adapter, *E00* (universal primer), *Pst*I adapter, *P00* (universal primer) as listed in Table 2.2. The PCR products were separated on 5% denaturing polyacrylamide gels at a constant 800V for 6h at 50℃ and analyzed with a LI-COR DNA Analyzer A200 (LI-COR Inc., Lincoln, NE). The PCR products from the other *Eco*RI /*Mse*I primer combinations with three selective nucleotides at the 3' ends were separated on 5% denaturing polyacrylamide gels and were visualized with silver staining following the protocol of the Promega silver DNA sequencing system™ (Promega Inc., Madison, WI).

Mitotic Analysis

Plants were grown in the greenhouse at 20 ± 5°C. Roots of the plants were collected at 1:00 pm. They were pretreated in 0°C water for 25 h to accumulate metaphase cells, fixed in 3:1 ethanol (95%, v/v), acetic acid (Farmer's fixative), and stored at 4°C. Root tips were macerated in 45% acetic acid for 10 to 20 min, were squashed on a glass slide and flame-dried. Slides were viewed under a phase contrast microscope to determine the number of chromosomes in the root-tip cell. In the metaphase, the chromosome numbers of the 14 accessions from northern Spain were counted. Images of chromosomes were recorded on film using a single-lens reflex camera attached to an Olympus (BX-51) microscope system at 1,500 X magnification.

Data Analysis

Gels were visualized by means of Gene ImagIR 4.0 (Scanalytics, Inc., VA). Bands that showed clear polymorphisms were scored visually as present ('1') or absent ('0'). Bands of different electrophoretic mobilities were assumed to be non-allelic, while bands of the same mobility were assumed to be allelic. Some ambiguous bands were ignored. Genetic diversity analyses were conducted by using Numerical Taxonomy and the Multivariate Analysis System, NTSYSpc v.2.2 (Rohlf, 1993). Genetic similarities based on Jaccard's coefficients (Jaccard, 1908) were computated among all possible pairs with the SIMQUAL option and ordered in a similarity matrix. The similarity matrix was run by Sequential, Agglomerative, Hierarchical, Nested clustering (SAHN); (Sneath and Sokal, 1973) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as an option (Sokal and Michener, 1958). The dendrogram and cluster groupings were constructed by the UPGMA clustering algorithm from the SAHN option of NTSYSpc v2.2. Cophenetic correlation was calculated to measure goodness of fit between the similarity and cophenetic matrices. Principle coordinate analysis was run with the Common Principal Components Analysis (CPCA) option (NTSYS) to identify the number of groups based on eigenvectors. The principal coordinates' analysis result was displayed by the Mod3D plot module. Correlation between the two matrices obtained with two marker sets was estimated by means of the Mantel matrix correspondence test (Mantel, 1967).

Results

Chromosome numbers of 14 accessions of colonial bentgrass collected from northern Spain.

Five root-tip cells per accession showed that all 14 of the accessions have 28 chromosomes at metaphase. These chromosomes are uniform in length (Fig.2.1). Based on the chromosome number, we can distinguish these accessions from dryland bentgrass (*Agrostis castellana*) which has 42 chromosomes.

Cluster analysis determined by EcoRI/MseI AFLP markers.

The AFLP fragment size ranged from 50 bp to 1000 bp, but only the bands from 150 bp to 500 bp were selected. A total of 181 unequivocally recognizable polymorphic bands were obtained. The number of polymorphic bands varied from seven (*Eco*RI-CTG/*Mse*I-ATG) to 25 (*Eco*RI-AGC/*Mse*I-CAC) among the accessions by using different primer combinations. Pair-wise comparison of genetic similarity among accessions revealed a wide genetic diversity within the colonial bentgrass species. The genetic similarity (Jaccard) coefficients (GS_j) ranged from 0.34 to 0.70 with a mean of 0.51. The most similar colonial bentgrass accessions were SP 1274 collected from Spain and PI 491264 collected from Finland (GS_j =0.70), followed by PI 290708 and PI 420235 (GS_j=0.69) both collected from England. The least similar accessions (GS_j =0.34) were detected among SP 1270 from Spain and PI 325194 collected from the Russian Federation.

A dendrogram among 39 colonial accessions based on their cluster analysis of GS_j coefficients showed that no major 'ball cluster' (Rohlf, 1993) was found (Fig.2.2). The 14 colonial bentgrass accessions collected from Spain formed three main groups that were separated into other colonial bentgrass PI accessions from the USDA germplasm collection. The first group consisted of accession numbers SP 1274, SP 1275, SP 1285, SP 1278, SP 1286, SP 1273 and SP 1276 with an average GS_j of 0.63. Three commercial colonial bentgrass cultivars (Golfin, SR 7150 and Tiger) were assigned to this group (GS_j =0.65). SP1265 and SP1266 were clustered into the second group (GS_j=0.64). The third group possessed accessions SP 1267, SP 1269, SP 1268 and SP 1271 (GS_j=0.56). SP 1270 was separated from the other colonial bentgrass accessions in the dendrogram by a finer leaf when grown in the greenhouse. The three accessions collected from England, PI 420235, PI 420236 and PI 290708 clustered with each other (GS_j =0.66), but none of the four accessions from Turkey clustered together and had a low GS_j (0.47). Three PI accessions from the United States, PI 469217, PI 578528 and PI 578528 which we were unable to group, showed a wide genetic diversity (GS_j =0.43). PI 469217 and PI 578528 were reclassified as dryland bentgrass on the basis of their morphological characteristics (Steiner and Lupold, 1978). However in this research, these two accessions showed no evidence of deviation from other colonial accessions. PI 469217 was grouped with

PI 252045, PI 538785, PI 237717 and PI 578528 from Italy, the Former Soviet Union, Germany and the US respectively with an average GS_j of 0.60.

Cluster analysis determined by PstI/MseI AFLP markers.

Six AFLP primer pairs produced 128 polymorphic bands based on the selection criteria mentioned above (150 bp-500 bp), resulting in an average of 20 polymorphic loci per primer pair. Values of GS_j ranged from 0.99 between commercial cultivar SR 7500 and the Spanish accession SP 1267 to 0.45 between PI 325194 from Stavropol, the Russian Federation, and the Spanish accession SP 1270. Using the CPCA subroutine programs of NTSYS, a rotated PCA with the AFLP markers was used to determine the number of groups based on Eigen values >1. Three groups were formed with an average GS_j=0.63 (Fig. 2.3). Thirty-three accessions were clustered in the first group. The second group consisted of five accessions and the third group comprised only PI 325194. Fig.2. 4 shows the dendrogram generated from the UPGMA cluster analysis with one possible tie found between the closest pair. In the first group, a mean GS_j for all colonial bentgrass accessions was 0.71 consisting of more than 80% tested materials. Thirteen out of 14 accessions from Spain were clustered in this group except accession SP 1270. No main subgroup was formed among these 13 accessions. Within this group, three cultivars, Golfin, SR 7150 and Tiger, clustered in a subgroup with a mean GS_j =0.95, which is similar to *Eco*RI/*Mse*I AFLP analysis. Commercial cultivar SR 7150 and Spanish accession SP 1276 shared the highest genetic similarity with a GS_j=0.99. The second group consisted of five other accessions collected from five different countries with a mean of GS_j =0.62. PI 469217 from the United States and four other accessions (PI

237717, PI 252045, PI 440109 and SP 1270) were clustered in this group. In the third group, PI 325194 showed the largest genetic distance from accession SP 1270 in the whole dendrogram, which is consistent with the results obtained from the *Eco*RI/*Mse*I enzyme combination.

Comparison between two sets of AFLP restriction enzyme combinations.

The co-phenetic correlation coefficients (*r*-value) for *EcoRI*/*Mse*I was 0.94 and *PstI*/*Mse*I data was 0.95, suggesting a good fit between the dendrogram clusters and the similarity matrices from which they were derived. AFLP markers from *PstI*/*Mse*I (methylation sensitive) produced higher estimates of GS_j with a narrower distribution than those by *Eco*RI/*Mse*I (methylation insensitive) in the colonial bentgrass species. A low but significantly positive correlation between the genetic similarity coefficients obtained through these two restriction enzyme combinations was observed (r=0.44 p=0.0099, one-tailed Mantel test). The pattern of clustering of the genotypes remained more or less the same between the two enzyme combinations in both dendrogram figures. The three cultivars showed the closest genetic relationship based on the GS_j in both experiments. PI 325194 and SP 1270 showed the least genetic similarity coefficients, with SP 1270 separated from other Spanish accessions in both enzyme combinations. However, the different classification of accessions was also detected between two figures. Three major groups were clustered by using the *PstI*/*Mse*I AFLP markers, but the same groups were not formed using the *Eco*RI/*Mse*I combination.

Discussion

Verification of colonial bentgrass accessions collected from northern Spain.

Colonial bentgrass is taxonomically related to dryland bentgrass (*Agrostis castellana*), which makes it difficult to distinguish between the two species based on phenotype. However, the colonial bentgrass species has 28 chromosomes and the dryland bentgrass species has 42 chromosomes (Jones, 1953; Bjorkman, 1954), making it possible to distinguish between the two species based on the number of chromosomes. The identity of 14 Spanish accessions was confirmed in this study by counting chromosome numbers. The C-banding technique can also provide more cytological information on chromosome descriptions (Friebe et al., 1992) by comparing the C-banding patterns between the Spanish accessions and the USDA PIs for further analysis of their relationship.

Comparison between two enzyme combination results.

In this study, a set of *Eco*RI/*Mse*I and *Pst*I/*Mse*I AFLP markers were utilized to determine the genetic similarity among colonial bentgrass accessions and produced two dendrograms which shared a low correlation (r=0.44, p=0.0099). The explanation for the difference might be that markers from two restriction enzyme combinations target different regions of the genome. The *Eco*RI/*Mse*I combination can produce three kinds of fragments that include the *Eco*RI/*Eco*RI, the *Eco*RI/*Mse*I and the *Mse*I/*Mse*I types originating from both the methylation sensitive and insensitive regions. All of these bands can be detected with silver staining technique, and are prone to cluster along the hypermethylation regions of the genome, such as the region around the centromere (Menz et al., 2002). The *Pst*I/*Mse*I combination also can produce three kinds of fragments including *Pst*I/*Pst*I, *Pst*I/*Mse*I and *Mse*I/*Mse*I types. In this experiment, we labeled the *Pst*I primer. The *Mse*I/*Mse*I fragments do not have fluorescent dye labeled primer ends, and therefore cannot be visualized and scored. The *Pst*I/*Pst*I and *Pst*I/*Mse*I fragments produced from unmethylated regions of the genome can be visualized because of their labeled ends. Bands generated by the *Pst*I/*Mse*I combination are distributed along the genome except for the methylated regions of the chromosomes (Young et al., 1999). The genome region distribution of the AFLP markers did affect the classification of 39 accessions although there is a correlation between two clustering results. The lower estimates of GS_j from the *EcoR*I/*Mse*I combination compared with *Pst*I/*Mse*I can be explained by the higher variation within the hypermethylated regions compared to hypomethylated regions of colonial bentgrass species. Barrett and Kidwell (1998) evaluated the genetic diversity of wheat cultivars using AFLP markers produced from *Pst*I/*Mse*I and *Eco*RI/*Mse*I combinations, and a low correlation (r=0.53 p=0.001) was observed between two genetic diversity estimate matrices. With the advent of high-density genetic maps and high-throughout marker systems for turfgrass species (Jones et al., 2002), it is possible to estimate genetic diversity with a large number of markers that are evenly distributed across the plant genome. The advantage of using markers distributed over the entire genome with known map position instead of by random selection should make it possible to avoid over- or under-representation of certain regions of the genetic map, thus avoiding inaccurate estimates of genetic similarities among individuals.

Genetics diversity among the colonial bentgrass species.

Information about genetic diversity permits the classification of germplasm into heterotic groups, which affects the potential genetic gain through breeding selection (Casler et al., 2003). Genetic diversity among the accessions provides the information for core germplasm collection. AFLP-based genotyping was effective in revealing DNA polymorphism for fingerprinting among colonial bentgrass accessions representing 12 countries. A low level of genetic similarity among colonial bentgrass accessions is a result of their inter-pollination characteristics. The wide genetic background in this species provides diverse germplasm for exploiting and pyramiding disease resistance into the bentgrass cultivars. Highly related as well as diverse accessions can be grouped and distinguished by AFLP marker analysis, and the diverse disease resistant accessions from heterotic groups will be selected and used in breeding. Further allelism tests are needed to determine if loci controlling disease resistance have a high level of diversity in selected accessions. Utilizing interspecific and intraspecific hybridization between these diverse resistant colonial bentgrass accessions and creeping bentgrass species is the promising goal of bentgrass breeding.

AFLP analysis revealed its usefulness for assessing the germplasm collection. The 14 accessions collected from Spain were included in this study. AFLP clustering results showed that 14 accessions were distributed in the colonial bentgrass accessions from the USDA germplasm collection, and revealed a broad genetic diversity with high similarities observed between some accessions. Spain is one of the native areas of origin of the colonial bentgrass species and is well suited for exploitation of diverse germplasm. In this study, the accessions from different countries showed different levels of genetic diversity. For example, three accessions from England have high genetic similarities, whereas the four accessions from Turkey showed wide genetic diversity. The genetic diversity of three commercial cultivars released within the past 20 years in the U.S. revealed that genetic backgrounds were more closely related to accessions from Spain, than from other countries. In contrast to the broad diversity existing among the colonial bentgrass accessions, these three cultivars share a very narrow genetic base. Variability of germplasm will play a key role in future turfgrass (bentgrass) breeding selection and genomic research.

Table 2.1 List of PIs, species, geographic origin and chromosome number of colonial bentgrass accessions

Colonial bentgrass accessions	*Origin*	*chromosome number*
PI 171470 *Agrostis capillaris*	Turkey	28
PI 172698 *Agrostis capillaris*	Turkey	28
PI 204397 *Agrostis capillaris*	Turkey	28
PI 206626 *Agrostis capillaris*	Turkey	28
PI 234685 *Agrostis capillaris*	Denmark	28
PI 237717 *Agrostis capillaris*	Germany	28
PI 252045 *Agrostis capillaris*	Italy	28
PI 283173 *Agrostis capillaris*	Czechoslovakia	28
PI 290708 *Agrostis capillaris*	England, UK	28
PI 325194 *Agrostis capillaris*	Stavropol, Russian Federation	28
PI 392338 *Agrostis capillaris*	Former Soviet Union	28
PI 420235 *Agrostis capillaris*	England, UK	28
PI 420236 *Agrostis capillaris*	England, UK	28
PI 440109 *Agrostis capillaris*	Stavropol, Russian Federation	28
PI 469217 *Agrostis capillaris*	Oregon, US	28
PI 491264 *Agrostis capillaris*	Finland	28
PI 494120 *Agrostis capillaris*	Germany	28
PI 494121 *Agrostis capillaris*	Netherlands	28
PI 509437 *Agrostis capillaris*	Romania	28
PI 538785 *Agrostis capillaris*	Former Soviet Union	28
PI 578527 *Agrostis capillaris*	Oregon, US	28
PI 578528 *Agrostis capillaris*	Rhode Island, US	28
SP1286 *Agrostis capillaris*	Spain	28*
SP1265 *Agrostis capillaris*	Spain	28*
SP1266 *Agrostis capillaris*	Spain	28*
SP1267 *Agrostis capillaris*	Spain	28*
SP1268 *Agrostis capillaris*	Spain	28*
SP1269 *Agrostis capillaris*	Spain	28*
SP1270 *Agrostis capillaris*	Spain	28*
SP1271 *Agrostis capillaris*	Spain	28*
SP1273 *Agrostis capillaris*	Spain	28*
SP1274 *Agrostis capillaris*	Spain	28*
SP1275 *Agrostis capillaris*	Spain	28*
SP1276 *Agrostis capillaris*	Spain	28*
SP1278 *Agrostis capillaris*	Spain	28*
SP1285 *Agrostis capillaris*	Spain	28*
Tiger *Agrostis capillaris*	Cultivars	28
Golfin *Agrostis capillaris*	Cultivars	28
SR7150 *Agrostis capillaris*	Cultivars	28

*Number of chromosome was determined in this study.

Table 2.2 List of adapters and pre-primers used

Primer/adapters	*Sequences*
*Mse*I adapter	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
M00 (universal primer)	GATGAGTCCTGAG TAA
*Eco*RI adapter	5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
E00 (universal primer)	GACTGCGTACCAATTC
*Pst*I adapter	5′-ACGCAGTCTACGAGTGCA-3′ 3′-CCATGCGTCAGATGCTC-5′
P00 (universal primer)	CAGTCTACGAGTGCAG

Fig. 2.1 Mitosis of colonial bentgrass accession of SP1275 from Spain. 28 chromosomes were observed.

Figure 2.2 UPGMA dendrogram of 39 colonial bentgrass accessions revealed by *Eco*RI/*Mse*I AFLP markers

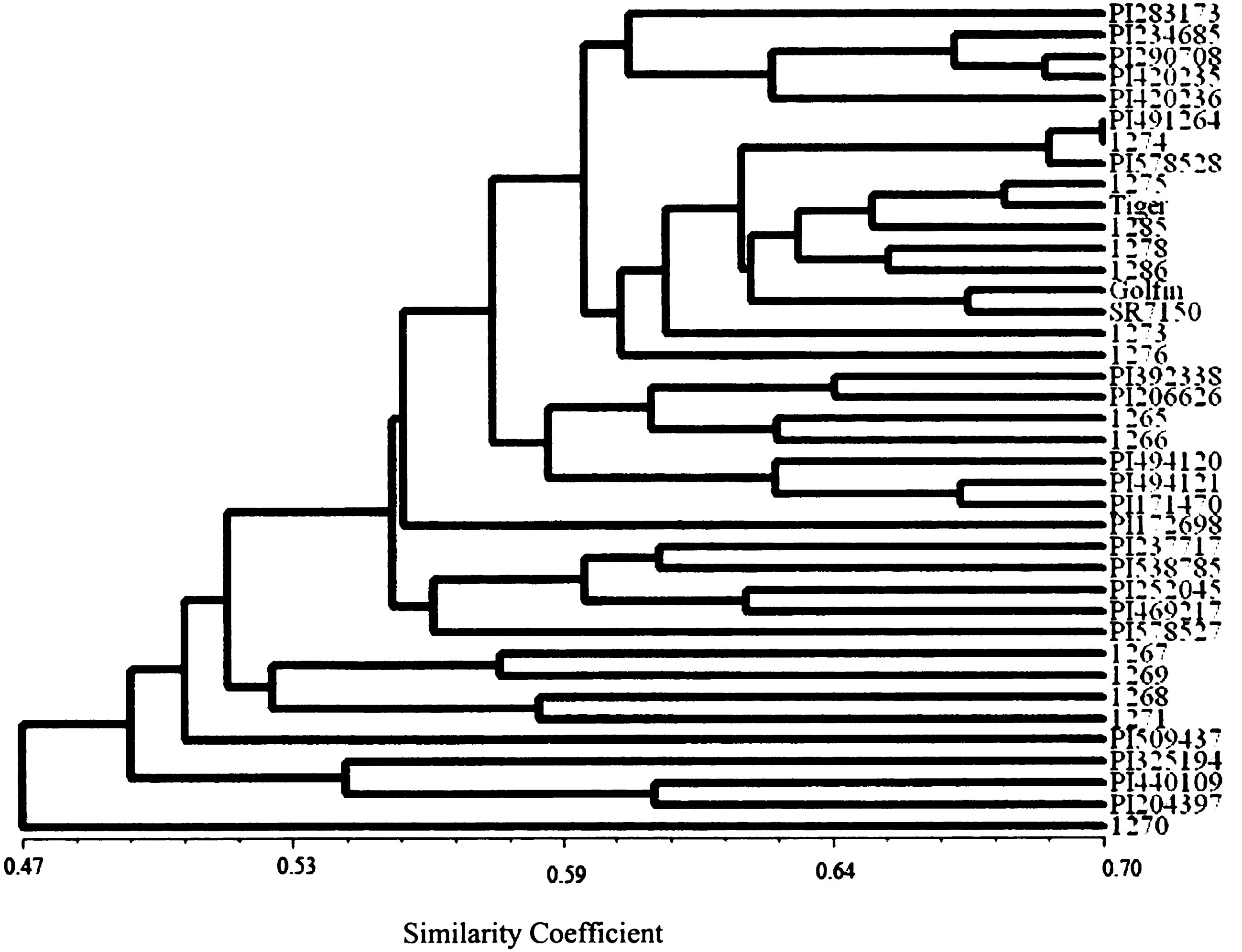


Figure 2.3 Three-dimensional plot of principal component analysis with 128 amplified fragment length polymorphism markers and 39 colonial bentgrass accessions defining three groups marked as 1, 2, and 3 from the CPCA and plot options of NTSYS v. 2.1.

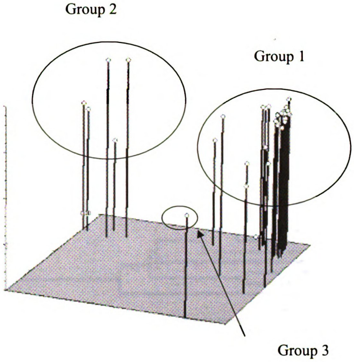
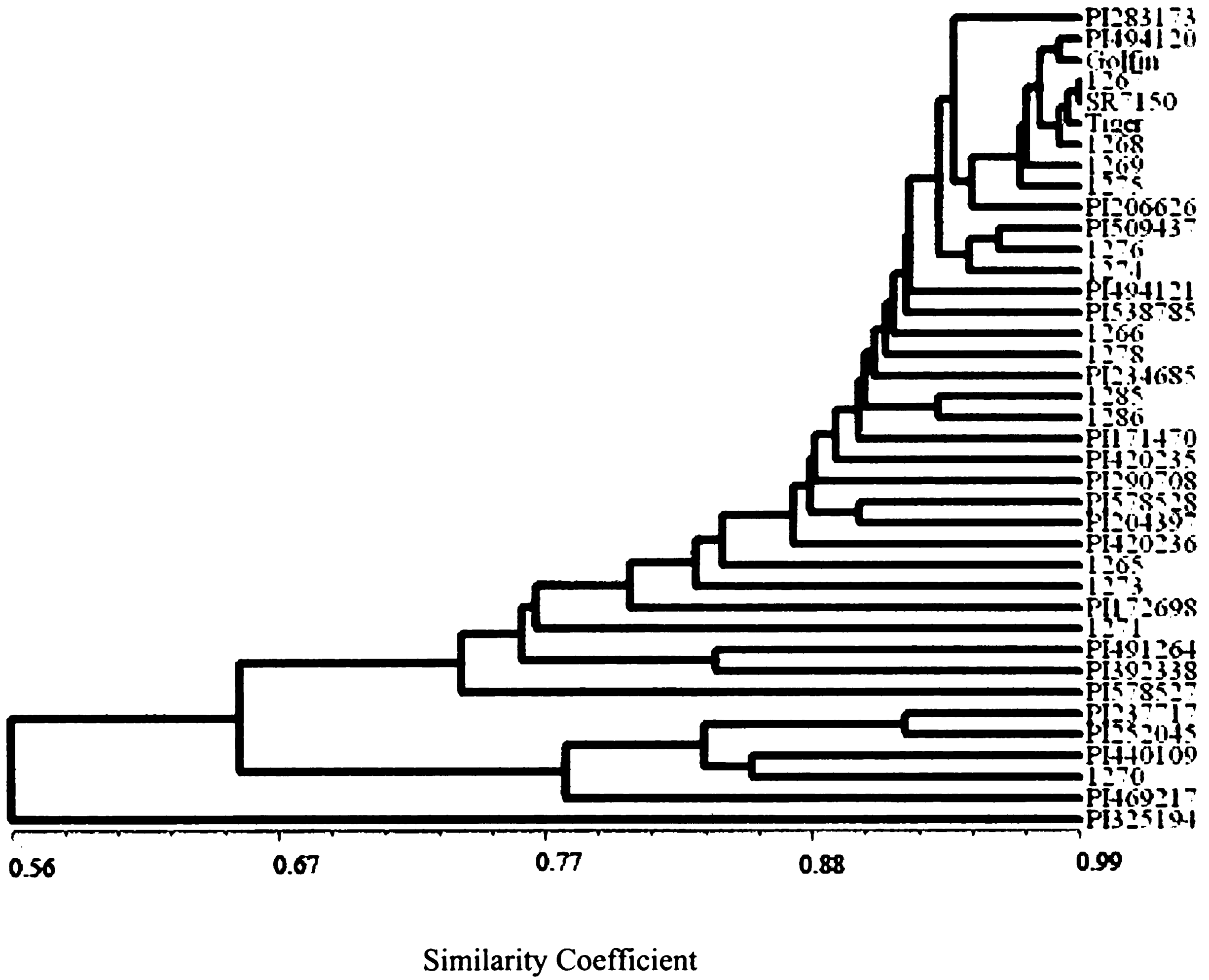


Figure 2.4 UPGMA dendrogram of 39 colonial bentgrass accessions revealed by *PstI*/*MseI* AFLP markers.



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CHAPTER 2

GENERATION AND CHARACTERIZATION OF INTERSPECIFIC HYBRIDS BETWEEN COLONIAL AND CREEPING BENTGRASS

Abstract

Creeping bentgrass (*Agrostis stolonifera* L.) is commonly used in putting greens, tees and fairways. No commercial cultivars exhibited an adequate tolerance to various fungal pathogens such as dollar spot (*Sclerotinia homeocarpa*), gray snow mold (*Typhula incarnata*), and brown patch (*Rhizoctonia solani*). The related species, colonial bentgrass (*A. capillaris* L.), has good level of resistance to dollar spot and snow mold. To improve resistance on creeping bentgrass, interspecific hybrids between creeping bentgrass and colonial bentgrass were generated for introgression of resistance genes. The hybrids (2n=28) were confirmed by a species-specific sequence characterized amplified region (SCAR) marker present in the colonial bentgrass genome. In addition, cytogenetic analysis revealed an average of seven ring bivalents, several univalents and multivalents at meiosis in the hybrids. Pollen fertility of the hybrids ranged from 4% to 34.5%. The hybrids were evaluated for gray snow mold disease resistance in the cold room and dollar spot disease resistance in the greenhouse. A few of the hybrids had moderate level of resistance to snow mold and dollar spot, and exhibited partially enhanced disease resistance.

Key words: Colonial bentgrass, Creeping bentgrass, Interspecific hybridization, Snow mold disease, Dollar spot disease

Introduction

Creeping bentgrass (2n=4x=28 C_2C_2SS Allotetraploid) is a cool-season grass and is the predominant turfgrass species in temperate climates of North America. It is commonly used in putting greens, tees and fairways due to its fine texture, high density, and adaptation to low mowing height (Warnke et al., 1998). However, creeping bentgrass is susceptible to a wide range of diseases such as dollar spot, gray snow mold, and brown patch. Each year, approximately a hundred million dollars worth of fungicides is applied to golf courses in the United States (Latin, 2006). Currently, no cultivars show a desirable level of resistance to these pathogens. Breeding resistant creeping bentgrass cultivars is therefore one of the main objectives in breeding programs.

Colonial bentgrass (2n=4x=28 $C_1C_1C_2C_2$ Segmental allotetraploid), a related species of creeping bentgrass, shows good level of resistance to dollar spot and snow mold diseases (Vergara and Bughrara, 2003). Colonial bentgrass was originally used for golf greens, bowling greens, and tennis courts. Because of the higher mowing height, its use is limited to tees. However, it was widely recognized by breeders as having high resistance to gray snow mold and dollar spot. Transferring resistance from colonial to creeping bentgrass provides an opportunity to improve disease resistance in creeping bentgrass.

Introgression of alien chromosomes, or chromosome segments, carrying useful genes by interspecific hybridization, is a valuable method for crop improvement. Hybridization between crops and wild relatives species followed by backcrossing and selfing is commonly used to integrate desirable genes into crop genomes (Qi et al., 1996), and has been used in breeding for improving resistance, quality and stress tolerance of numerous species (Li et al., 2005). Hybridization between creeping and colonial bentgrass can naturally occur in the field, which makes it easy to utilize the colonial bentgrass disease resistance by genome introgression. Jones (1956) found naturally occurred hybrids between the creeping and colonial bentgrass species and examined them for chromosome pairing configuration with 14 bivalents at metaphase I of meiosis. Belanger et al. (2003) produced hybrids between these two species and field-tested them against dollar spot. Some of the hybrids had excellent dollar spot resistance, exhibiting essentially no disease symptoms. In addition to dollar spot resistance in colonial bentgrass, Vergara (2003) found that some colonial bentgrass accessions possessed an adequate resistance to snow mold disease, a prevalent turfgrass winter disease in the Northern U.S.

The principle aim of our work was to produce interspecific hybrids between a snow mold resistant colonial bentgrass accession and a creeping bentgrass cultivar. The interspecific hybrids were characterized by cytogenetics and molecular methods, and the disease resistant reaction was evaluated in the greenhouse and cold room.

Materials and methods

Plant materials

The colonial bentgrass accession PI578528 was obtained from the USDA Plant Introduction Station at Pullman, Washington. It has been tested in our lab for dollar spot and snow mold diseases for two years and shown to be resistant to both diseases (Vergara, unpublished data). PI578528 was used as male parents in crossing with creeping bentgrass. The second parent, 'ASR368', was transgenic glyphosate-resistant creeping bentgrass plants provided by Scotts Company (Marysville, OH). The creeping bentgrass plants were resistant to glyphosate via the expression of a *cp4-epsps* gene, which encodes a glyphosate-resistant form of EPSP from the CP4 strain of *Agrobacterium* species. The procedure used for generating the transgenic plants is described by Hartman et al (1994) and Lee (1996). The F_1 seeds collected from creeping bentgrass were grown and maintained in the greenhouse. Two creeping bentgrass commercial cultivars, 'Penncross' and 'Crenshaw' were included as susceptible controls for snow mold and dollar spot disease evaluations, respectively.

Disease evaluation and rating procedure

Snow mold disease evaluation

Sclerotia of gray snow mold (*Typhula incarnata*) was collected from Hancock Turfgrass Research Center at Michigan State University and was grown on sterilized potato dextrose agar (PDA). The pathogen was grown on PDA at 5 ℃ for 2 months. PDA and was cut into 1- by 1-cm pieces and transferred to sterilized cornmeal mixture for multiplication. Growth of fungus in cornmeal was continued at 5℃ for another two months (Vergara and Bughrara, submitted). The plants with three replications of each F1 hybrids were brought into the cold room (4~5 ℃) for seven days for acclimation prior to disease inoculation using a completely randomized design. Equal amount of infected cornmeal (1 g) containing the pathogen was put in the center of each pot and covered with moistened cheesecloth. The pots were placed into trays which were filled with approximately 1cm water and covered with plastic bags to maintain high humidity and optimize disease severity. Visually scoring for resistant and susceptible plants was performed at 6 weeks. Nine represented 100% to 95% diseased turf, eight represented approximately 90% diseased turf, seven represented approximately 75% to 85% diseased turf, six represented approximately 60% to 70% diseased turf, five represented 40% to 50% diseased turf, four represented approximately 30% to 40% diseased turf, three represented approximately 15% to 25% diseased turf, two represented approximately 10% diseased turf, and one represented 0 to 5% diseased turf (Bonos et al., 2003). The disease scores obtained by two evaluators were averaged for analysis. One-way CRD with three replications was used to investigate the disease index. ANOVA analysis was conducted using PROC GLM function in SAS system V8 (SAS Institute, 2002, Cary, NC).

Dollar spot disease evaluation

An isolate of *Sclerotinia homoeocarpa* was used as inoculum in the greenhouse (East Lansing, MI) to simulate natural infection. This isolate (A) was obtained from the department of Plant Pathology at Michigan State University (East Lansing, MI). The isolate was grown on sterilized Kentucky bluegrass seed for inoculation. Two hundred grams of Kentucky bluegrass seed were autoclaved for 15 min. at 151°C. Seventy-five milliliters of dH_2O was added to the Kentucky bluegrass seed in an Erlenmeyer flask, and was let sit overnight. One half of a Petri dish containing a single isolate was cut into 1- by 1-cm pieces and transferred to the flask. The isolate (isolate A) was grown in flasks for approximately 3 weeks at room temperature. The inoculum was dried on newspaper for 3 days. Equal amounts of seeds (3 grains) infested with the isolate pathogen were put in the center of each pot of eleven-week old plants which were grown in the greenhouse. The pots were randomly placed into trays and covered with plastic bags to maintain high humidity and optimize disease severity. After three days, the plants were scored for disease symptom. Nine represented 100% to 95% diseased turf, eight represented approximately 90% diseased turf, seven represented approximately 75% to 85% diseased turf, six represented approximately 60% to 70% diseased turf, five represented 40% to 50% diseased turf, four represented approximately 30% to 40% diseased turf, three represented approximately 15% to 25% diseased turf, two represented approximately 10% diseased turf, and one represented 0 to 5% diseased turf (Bonos et al., 2003). The disease scores obtained by two evaluators were averaged for analysis. One-way CRD with three replications was used to investigate the disease index. ANOVA analysis was conducted using PROC GLM function in SAS system V8 (SAS Institute, 2002, Cary, NC).

DNA extraction

Fresh leaves (about 3g) were ground in liquid nitrogen and then the fine powder was transferred to 50 ml polypropylene tubes. The extraction buffer (0.1 M Tris pH8.0, 0.05 M EDTA pH 8.0, 0.5 M NaCl, 1.24% SDS) was added to the powdered tissue in a volume of 1:1. After incubating at 65℃ for approximately 1 hour, the samples were purified with chloroform/isoamyl alcohol (24:1; v/v), and centrifuged at 2000 rpm for 25 min. The supernatant was added into pre-cooled 100% ethanol. DNA was washed in 70% ethanol and diluted in TE buffer after removing excess ethanol. RNAase was added and DNA was quantified.

DNA amplification and analysis

A SCAR marker was amplified in creeping and colonial bentgrass plants following procedure of Scheef et al., 2003. PCR products were run on a 1.5% (w/v) agarose gel and stained with ethidium bromide. Presence and absence of the SCAR band in the F1 hybrids was visually scored and compared with each parent species.

Cytological analysis

Plants were grown in a greenhouse at 20 ± 5℃. Roots of the hybrids were collected at 1:00 pm. and were pretreated in 0℃ water for 25 h to accumulate prometaphase cells, fixed in methanol-acetic acid (3:1), and stored at 4℃. Root tips were macerated in 2.5% cellulase at 37℃ for 1.5 h. Squashes were made in the fixative on a glass slide and flame-dried. The young panicles of the hybrids were fixed in ethanol:chloroform:acetic acid (3:2:1) for 2 days at 4℃ and transferred to ethanol: acetic acid (3:1). Anther squashes were made in 1% acetocarmine. Chromosome pairing was analyzed on semipermanent slides sealed with a gelatine-acetic acid medium. Images of chromosomes were recorded on film using a single-lens reflex camera attached to an Olympus (BX-51) microscope system at 1,500 X magnification.

Pollen Viability Determination

Plants were transferred to the laboratory for pollen staining from 11:00 am to 3:00 pm on a sunny day when the flowers first opened. Pollen viability of all hybrids and their parents was determined by staining the pollen grains from four flowers with 1% carmine in 45% acetic acid. Stained and unstained pollen grains were counted in 10 fields per slide. Pollen viability, based on pollen stainability, was expressed as an average percentage of stained pollen grains on a slide with four replicate slides. Photographs were taken with an Olympus microscope with Magna Fire-Sp® software.

Results

Phenotype of F1 hybrids of colonial and creeping bentgrass

Bentgrass species are generally considered to be self-incompatible. However, some creeping bentgrass clones exhibiting high levels of self-fertility have been reported (Warnke et al., 1998). Therefore, the seeds produced from crossing need to assessed to eliminate any individuals that may have been generated from self-pollination. A SCAR marker was used to amplify a specific band (about 400 bp) from the genome of colonial bentgrass accessions. This band was not amplified in creeping bentgrass species with the same SCAR marker. After testing all F1 progeny, six seedlings were determined to result from self-pollination because the SCAR marker was not observed. The other 10 plants produced that specific band and were planted in the greenhouse for further research and backcrossing (Fig. 3.1).

The growth habit of the hybrids was similar to that of colonial bentgrass, being more upright and having fewer stolons than creeping bentgrass. Phenotypes between these 10 interspecific hybrids were also variable for stature height, stolons number and vernalization sensitivity. The difference of the morphology reflects their parents' heterozygosity.

Cytological analysis

Somatic metaphase chromosomes of colonial (2n=28) and creeping (2n=28) bentgrass are shown in Fig. 3.2. The chromosomes of both species are relatively small and morphologically uniform, which creates difficulty in identifying any chromosome differences between these two bentgrass species. Root-tip cells of all 10 hybrids showed 28 chromosomes at mitosis.

Meiosis

Seven plants were selected from the F_1 population. Chromosomes pair configurations of the F_1 hybrid cells were examined at the first meiotic metaphase. The most striking feature of the F_1 hybrids was that homologous chromosomes paired regularly in ring bivalents in all pollen mother cells (PMCs); (Fig. 3.3). Chromosome pairing at metaphase I was typified by the mean values of 6.7 univalents, 8.1 bivalents, 1.1 trivalents, and 0.45 quadrivalents per PMC, which indicates that the C_2 genome of creeping and colonial bentgrass are highly homologous; C_1 and S genomes were partially homologous based on the multivalents formed. The parental chromosomes were evenly segregated into daughter cells because no lagging chromosomes were observed at anaphase and telophase.

Pollen viability of hybrids

Utilization of wild germplasm depends on the production of fertile interspecific hybrids. Pollen viability from seven F_1 hybrids was estimated by pollen stainability. Pollen viability of the hybrids ranged from 4% to 34.5% with an average of 19.6%, which was significantly lower than that of the colonial and creeping bentgrass parents, for which the pollen viabilities were 94 % and 96%, respectively.

Disease resistance evaluation

Three vegetative clones of each F_1 hybrid and their parents were subjected to screening for snow mold and dollar spot diseases. In the snow mold evaluation, disease rating means and analysis of variance showed that differences were significant across the population at six weeks after inoculation. The colonial bentgrass parent showed moderate resistance compared to the creeping bentgrass parent after disease evaluation (Table 3.1). Eight hybrids exhibited greater snow mold disease resistance than the creeping bentgrass control, 'Penncross', but two of them (CC1 and CC6) did not exhibit significant improved resistance compared to 'Penncross'. In the dollar spot disease screening evaluation, eight hybrids showed greater resistance than creeping bentgrass control, 'Crewshaw', and CC6 had a high level of resistance comparing to the colonial bentgrass parent. However, CC6 did not show any desirable resistance to snow mold disease.

Discussion

Creation of interspecific hybrids of creeping and colonial bentgrass is the first step to eventually releasing resistant creeping bentgrass cultivars with alien genes (Sukno et al., 1999). Considering the partial self-fertility of creeping bentgrass, the hybrids should be confirmed with DNA marker analysis or other phenotypic traits to eliminate selfing seeds. Belanger and his colleagues (2003) used herbicide resistance gene (*bar*) as selection marker. In their study, colonial bentgrass was used as female parent, and transgenic creeping bentgrass served as the donor parent. The F_1 hybrids were confirmed by herbicide selection. This method is straightforward with high efficiency, however, it requires a transgenic parent, and the hybrids contain colonial cytoplasm which may have a deleterious effect on the further recovery of creeping bentgrass background. In our experiment, a specific SCAR as a selectable maker for colonial bentgrass genome was used. From 16 potential hybrids (seeds were collected from creeping bentgrass), 10 interspecific hybrid plants were confirmed by this marker. However, in further continuous backcrossing with creeping bentgrass, this SCAR marker may be limited because it may not represent entire colonial genome. Some backcross progeny having partial colonial bentgrass genome can not be detected by a single SCAR marker. More colonial bentgrass specific SCAR markers representing the entire genome should be developed for detecting backcross progeny which only carries the partial colonial bentgrass genome.

The karyotype of parents and their hybrids were analyzed by measuring chromosome length and centromeres location. No significant differences exist in these parameters. The C-banding can not produce desirable information because of the small chromosomes and faint bands (data not shown), which creates difficulty to distinguish chromosome originality by C-banding method. The multi-color genomic *in situ* hybridization (McGISH) method provides an alternative solution to paint species specific genome or fragment instead of individual chromosome. All the chromosomes from the different genomes could be identified by different fluorescent colors. The McGISH method has been used for hybrid confirmation, and is effective in identifying chromosomes and examining the distribution of each genome in the nucleus. Ali et al. (2002) employed the McGISH method for the identification of alien chromosomes in the backcross progeny of fusion hybrids between potato and tomato, where the chromosomes were similar and small. They discriminated tomato chromosomes from potato chromosomes and showed the usefulness of this method for plant breeding. In future studies, two genomic probes (creeping bentgrass and colonial bentgrass) simultaneously and label them with two different colors respectively for discriminating their genomes in hybrids will be used. Because these two parents share one genome (C_2) and some fragments in the other genome according to hybrid meiosis pairing configuration, three colors will be detected, one color indicting the C_1 genome from colonial, the other color indicating the S genome from creeping and the mixture of the above colors presenting C_2 genome or homologous fragments from both colonial and creeping bentgrass genomes.

At meiosis, an average of seven ring bivalents was observed. This result is consistent with Jones's (1956) conclusion that creeping and colonial bentgrass probably have one ancestral diploid species in the common and show good homology in one pair of their genomes and partial homology in the other one. The resistant genes could be introgressed into creeping bentgrass genome from colonial bentgrass genome if genes located the homologous chromosomes. However, if a resistant gene(s) was on colonial bentgrass chromosome (s) which can not pair with creeping bentgrass' chromosome(s) at meiosis, the biological and physical approaches to induce small segmental chromosome translocations would be applied (Yuan et al., 1998). Short arm of rye (*Secale cereale* L.) chromosome 1R is useful in wheat (*Triticum aestivum* L.) breeding because it confers resistance to several pests and diseases and improve yield, but it reduces bread making quality. To remedy this defect, segmental chromosome translocations of rye chromosome arm 1R with wheat 1BL or 1DL was induced by the ph1b mutation to eliminate linkage drag (Lukaszewski, 2000).

Some of the F_1 plants exhibited hybrid vigor for certain morphological traits. However some of the hybrids grew weakly although all hybrids were planted in same environment, and not all of the hybrids showed improved resistance. Since 28 chromosomes were observed in the hybrids at mitosis, no chromosomes were eliminatied. Locus (gene) interactions may lead to the hybrids' morphological and resistant difference. The plants that are the most resistant to both dollar spot and snow mold will be used in backcrossing program with a creeping bentgrass cultivar as a recurrent parent for recovering creeping bentgrass elite phenotypes. Also interpollination between these hybrids provide an opportunity to combine the snow mold resistance and dollar spot resistance.

The 2001 National Bentgrass Test sponsored by USDA and National Turfgrass Federation findings showed that from the snow mold complex ratings of 26 bentgrass cultivars grown on fairway or tee, only one cultivar of colonial bentgrass, SR 7100 was found to be resistant to snow mold. In this study, the colonial bentgrass parent, PI578528, whose genetic background derived from genetic distance analysis is different from SR7100 (Zhao et al., 2006), was used to improve the disease resistance in creeping bentgrass. In further research, we will combine the resistance of SR7100 with our resistant hybrids to broaden resistance basis and pyramid the potential different resistance into new creeping bentgrass cultivars.

Table3.1 Snow mold and dollar spot disease index of 10 hybrids (CC1 to CC7) and their parents and cultivars.

	PI578528	Penncross	Crenshaw	CC1	CC2	CC6	CC7	CC10	CC12	CC13	CC15	CC16	CC17
SM resistance	5.3de	8.7a	NA	7.7ab	5de	7.3abc	6.3bcde	6.7bcd	5.7cde	6.7bcd	6.3bcde	6bcde	4.7e
DS resistance	3.0ef	NA	7.7ab	7.7ab	3.3ef	2.7f	9.0a	6.0c	3.0ef	5.7cd	5.7cd	6.3bc	4.3de

1 = 0-5% disease, 9 = 95%-100% disease turf. SM means snow mold and DS means dollar spot.

Means followed by the same letter within the same row are not significantly different by pair-wise *t*-test at $P<0.05$

ASR368 has disease index of 6.0(SM) and 5.0(DS)

Snow mold: 1. Two parents are significantly different. 2. CC17 and CC2 are similar to PI578528. 3. Dollar spot: 1. Two parents are significantly different. 2. CC2, CC6, CC12 and CC17 are similar to PI578528.

NA not available

Figure 3.1 The image of SCAR marker of ten hybrids and their parents. From CC1 to CC17 are 10 hybrids. ASR368 is creeping bentgrass and PI 578528 is colonial bentgrass.

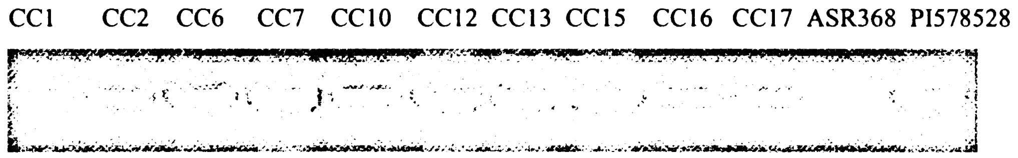


Figure 3.2 Mitosis of (left) colonial bentgrass accession of PI578528, (center) creeping bentgrass 'ASR368', and (right) hybrid CC6.

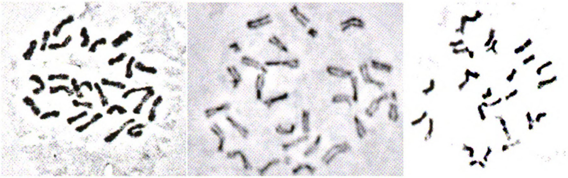
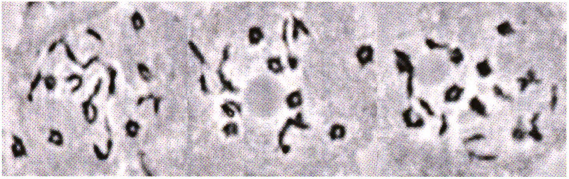


Figure 3.3 Meiosis of hybrid (CC6), seven bivalents several, some univalents and multivalents at meiosis were observed.



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CHAPTER 3

GENETIC LINKAGE MAPS AND QTL ANALYSIS OF CREEPING BENTGRASS (*Agrostis stolonifera* L.)

Abstract

Creeping bentgrass is the most widely cultivated turfgrass species in United States. Genetic linkage maps of creeping bentgrass were constructed for QTL analysis of gray snow mold resistance (*Typhula incarnate*), recovery after *Typhula* infection and leaf width. A segregating population of 188 F_1 progeny was developed by two-way pseudo-testcross mapping strategy. Amplified fragment length polymorphism (AFLP), Random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers, corresponding to DNA polymorphisms heterozygous in one parent and null in the other, were scored and placed on the two separate genetic linkage maps, representing each parent. In the male parent map, 93 markers were mapped to 14 linkage groups covering a total length of 793cM with an average interval is 8.2cM. In the female parent map, 139 markers were clustered in another 14 linkage groups spanning 805cM with an average distance of 5.9cM between adjacent markers. In the same population, we investigated the associations between the genetic markers with three agronomically quantitative traits: leaf width, snow mold disease resistance and recovery after *Typhula* infection. Three quantitative trait loci (QTL) explained 23% of the phenotypic variation for leaf width. One QTL explained 9% of the phenotypic variation for snow mold disease resistance, and one QTL explained 7% of the phenotypic variation for recovery after *Typhula* infection. The construction of linkage map and QTL analysis in this study provide a useful tool for the genetic dissection of some complex quantitative traits in creeping bentgrass.

Keywords: Creeping bentgrass; Linkage; QTL; Leaf width; Snow mold resistance; Recovery after *Typhula* infection

Introduction

Creeping bentgrass (*Agrostis stolonifera* L. 2n=28), native to Western Europe, belongs to genus *Agrostis* which consists of approximately 220 species (Hitchcock, 1951). It has fine leaf texture, high density, and good adaptation to low mowing heights, which make it widely used on golf course tees, greens and fairways. Creeping bentgrass is reported to have a moderate genome size (5.65 pg/2C); (Arumuganathan et al., 1999). Jones (1955) examined chromosome pairing configuration of tetraploid creeping bentgrass and found 14 bivalents formed at metaphase I in the pollen mother cell. Creeping bentgrass is considered to be a strict allotetraploid (C_2C_2SS). Isozyme analysis provided genetic evidence to support disomic segregation in creeping bentgrass (Warnke et al., 1998). Despite commercial importance of creeping bentgrass, understanding of its genetic mechanisms has received little attention and lags behind other plant species partially due to its self-incompatibility which makes traditional genetic analysis difficult.

In the last decade, with the advent of DNA-based markers, genetic studies have been greatly facilitated and hundreds of genetic linkage maps have been published in many crops (Collard et al., 2005). Molecular marker-based genetic linkage maps allow researchers to further elucidate inheritance mechanisms of important agronomical traits (Kolkman and Kelly, 2003), isolate genes (even QTLs) by providing the framework to understand the biological basis of complex traits (Ashikari et al., 2005), and determine chromosome synteny and evolution (Jung et al., 2006). A segregating population is necessary to construct a genetic linkage map. In out-crossing plant species, such as creeping bentgrass, F_2 or backcross segregation populations are rarely available because of self-incompatibility. Genetic linkage construction in such species is commonly performed in the F_1 progeny (pseudo F_2) derived from a cross between two heterozygous parents (Wu et al., 2000, Chakraborty et al., 2005;). Because of genetic segregation in the F_1 progeny is the result of meiotic recombination from both parents, marker data can be analyzed by a double pseudo-testcross strategy, and used in developing two separate linkage maps for the female and male parents (Grando et al., 2003). However, as a pseudo-F_2 mapping population, no crossovers can occur between the parents' homologous chromosomes in the F_1 generation. The chromosome recombination events detected by segregation of DNA marker were from previous generation. Several types of molecular markers are available for constructing genetic linkage maps in plant species. These included RFLP (Devey et al., 1996), RAPD (Boiteux et al., 2000), AFLP (Qin et al., 2005), and SSR (Okogbenin et al., 2006) markers. Species-specific RFLP and SSR, as co-dominant marker systems, need some existing genomic information, which is unavailable in some turfgrass species. RAPD markers have been criticized because their banding patterns are not always reliable or reproducible. AFLP is considered to be a highly productive and reproducible DNA marker system and does not require prior genome sequence knowledge. It is widely applied to rapidly create genetic linkage maps and detect QTLs in some grass species (Saha et al., 2004) and diversity research. QTL, as an association between marker loci and phenotypic variation of a quantitative trait in the mapping population, is a valuable parameter for the genetic dissection of complex traits.

QTL are studied in terms of the magnitude level of the effects on the phenotype or the parental origins of the favorable QTL alleles, or the interactions between different QTL. Several examples have been documented for QTL mapping in out-crossing plant species, such as in grapevine (*Vitis vinifera*). Several putative QTLs for seedlessness, berry weight and fruit yield were identified (Doligez et al., 2002; Fanizza et al., 2005). In perennial ryegrass (*Lolium perenne* L.), three potential QTLs for gray leaf spot disease resistance (Curley et al., 2005), and four QTLs determining crown rust resistance were characterized (Muylle et al., 2005). Based on the information on QTL, marker-assisted selection (MAS) can complement the traditional breeding tools and help implement more efficient breeding strategies in out-crossing species (Studer et al., 2006; Lima et al., 2006). QTL analysis also can unveil masked and interesting wild alleles, such as disease resistance, and makes for easier introduction of desirable and specific genetic materials from related and unrelated wild species for germplasm enhancement (Bai et al., 2003; Singh et al., 2005; Villamon et al., 2005). However, limited information is available on QTL analysis in turfgrass species in general and creeping bentgrass in particular.

Gray snow mold, caused by *Typhula incarnata*, is a major disease of creeping bentgrass (Wu et al., 2000). Gray snow mold is prevalent in cold humid conditions with snow cover for over 90 days (Burpee et al., 1987) and is particularly important in northern regions of the United States where it can cause considerable turf loss. Improvement of host plant resistance is therefore one of the practical objectives in current creeping bentgrass breeding programs (Belanger et al., 2003). Mapping QTL controlling disease resistance is an approach to providing information on the locations and effects of the locus influencing the traits, and help direct the breeding efforts for accelerating selection and pyramiding resistance genes or loci (Jones et al., 2002).

The objectives of the present study were: (1) to develop the genetic linkage of creeping bentgrass based on a two-way pseudo-testcross strategy using AFLP, RAPD, and SSR markers, and (2) to identify QTL controlling leaf width, snow mold disease resistance and recovery after *Typhula* infection.

MaterialS and methods

Plant materials

The mapping population was derived from a cross between two heterozygous creeping bentgrass genotypes, 'ASR368' and 'MSU#8', with contrasting phenotypes for leaf width, snow mold disease resistance and recovery rate after disease infection. 'MSU#8', used as a female parent, has wide leaf, desirable resistance to snow mold disease, good recovery after *Typhula* infection (Vergara and Bughrara, 200x) and glyphosate-sensitivity compared to male parent 'ASR368'. 'ASR368' is a transgenic glyphosate-resistant creeping bentgrass plant provided by Scotts Company (Marysville, OH). The creeping bentgrass plants were resistant to glyphosate due to the expression of a gene *cp4-epsps,* which encodes a glyphosate-resistant form of EPSP from the CP4 strain of *Agrobacterium* species. The procedure used for generating the transgenic plants is described by Hartman et al (1994) and Lee (1996). 'MSU#8' and 'ASR368' have high

DNA polymorphisms based on AFLP analysis (Appendix A). About 700 F_1 seeds were germinated on wet filter paper for seven days at 25℃. 600 seedlings were transferred into soil filled pots and grown in greenhouse. After excluding plants resulted from self-fertilization, which were identified after spraying glyphosate herbicide (Simarmata et al., 2005), 188 randomly selected progeny were used as the mapping population. All the progeny and their parents were propagated vegetatively into several clones for further DNA isolation and phenotype evaluation.

DNA isolation

Total genomic DNA was extracted from 5 g fresh leaves of the parents and their progeny using the procedure reported by Gill et al. (1991). DNA was quantified by DYNA Quant 200 Fluorometer (Pharmacia Biotech, San Francisco, CA).

AFLP analysis

The AFLP analysis was conducted according to the protocol provided by the AFLP Plant Mapping Kit of Perkin-Elmer Applied Biosystems (Foster City, Ca.). The pre-amplification products were diluted 20-fold for template DNA for selective amplifications. Selective amplification was performed with the fluorescently labeled *Pst*I selective primers (Saha et al., 2004). PCR reactions were run under standard conditions for all primers using 1U AmpliTaq Gold with GeneAmp PCR buffer II (Applied Biosystems/Roche, Branchburg, N.J.), 3m*M* $MgCl_2$, 200u*M* dNTPs, 0.2m*M* of each primer and 20ng of template DNA in a 10ul reaction. Pre-amplification was done on

PTC-100 thermal cycler (MJ Research, Waltham, MA) using 30 cycles of 94℃ 45s, 52℃ 45s, 72℃ 1min, followed by elongation at 72℃ 10min. Combinations of fluorescent dye labeled *Pst*I and non-labeled *Mse*I primers, each with three selective nucleotides at the 3' ends, were used for selective amplification at 56℃ annealing temperature. The samples were electrophoresed on an ABI 3100 capillary genetic analyzer (Perkin-Elmer Applied Biosystems) with an injection time of 8s and a run time of 28min. Raw data were analyzed with GENESCAN (ver.2.1, Perkin-Elmer Applied Biosystems). The AFLP fragments 50–500 bp in length were scored as present (A) or absent (B). Scores were recorded and formatted for analyses using the CONVERT GENOGRAPHER software (Noble Foundation, Ardmore, OK.). Scores thus obtained were verified on the gel images. A total of 15 primer combinations were selected to assay the whole population (Table 4.1).

SSR analysis

A set of 31 tall fescue (TF)-EST-SSRs (NFFA) developed by the Noble Foundation was used for genetic mapping in this creeping bentgrass pseudo-testcross population (Saha et al., 2004); (Table 4.1). PCR reaction conditions consisted of 5 min at 95℃, followed by 40 cycles of 50s at an annealing temperature between 58℃ and 64℃ (optimum annealing temperature for each primer), 90s at 72℃, and a final extension step of 10min at 72℃. The SSR products were resolved by analyzing samples on 6% polyacrylamide denaturing gels run under standard conditions. The amplified products were visualized by silver staining. Polymorphism was determined by the presence or absence of a SSR locus according to the single-dose restriction fragment approach (Wu et al., 2000).

RAPD analysis

One hundred decamer oligonucleotides (Operon Technologies, Alameda, CA) were used to screen the parents to detect the polymorphism at annealing temperature 41°C. The RAPD profiles were generated in 2% (w/v) agarose gel with 0.003% (w/v) ethidium bromide. A 1-kb ladder was used to mark the size of fragments. The images of RAPD were obtained through an Eagle Eye II Still Video System V3.2 (Stratagene, La Jolla, CA). The 26 primers which can clearly reveal the polymorphism between parents and a subset of six F_1 progeny randomly selected from population were used for mapping the whole population.

Phenotypic measurements

Leaf morphology was measured on the parents and all F_1 progeny using 12-week old plants grown at 30°C under constant illumination in Baccto planting mix (Michigan Peat, Houston) at MSU greenhouse (East Lansing, MI). Leaf width was determined from measurements taken at the base of fully expanded mature leaf from randomly chosen tillers and measured across the leaf at its widest point. The data was collected from three replications per genotype and ten leaves per replication.

Snow mold disease evaluation

Sclerotia of gray snow mold (*T. incarnata*) was collected from Hancock Turfgrass Research Center at Michigan State University and was grown on sterilized potato dextrose agar (PDA). The pathogen was grown in PDA at 5 ℃ for 2 months. The pathogen was grown on PDA at 5 ℃ for 2 months. PDA and was cut into 1- by 1-cm pieces and transferred to sterilized cornmeal mixture for multiplication. Growth and incubation in cornmeal was made at 5℃ for another two months (Vergara and Bughrara, submitted). The plants with three replications of each F_1 progeny were brought into the cold room (4~5 ℃) for seven days for acclimation prior to disease inoculation in a completely randomized design. Equal amount of infected cornmeal (1 g) containing the pathogen was placed in the center of each pot and covered with moistened cheesecloth. The pots were placed into trays which were filled with approximately 1cm water and covered with plastic bags to maintain high humidity and optimize disease severity. Visually scoring for resistant and susceptible plants was performed at 6 weeks using the following scale. Nine represented 100% to 95% diseased turf, eight represented approximately 90% diseased turf, seven represented approximately 75% to 85% diseased turf, six represented approximately 60% to 70% diseased turf, five represented 40% to 50% diseased turf, four represented approximately 30% to 40% diseased turf, three represented approximately 15% to 25% diseased turf, two represented approximately 10% diseased turf, and one represented 0 to 5% diseased turf (Bonos et al., 2003). Recovery was scored after another 10 days (1, 3, 5, 7, 9, 10 scale, with 10 as 100% recovered). The disease scores and recovery rate obtained by two evaluators were averaged for analysis. One-way CRD with three replications was used to investigate the disease index. ANOVA analysis was conducted using PROC GLM function in SAS system V8 (SAS Institute, 2002, Cary, NC).

Data analysis

Data from each replication were statistically using PROC MIXED function in SAS system V8 (SAS Institute, 2002, Cary, NC). The mean values of three replications were then used to summarize the range and mean of each genotype, and derive a matrix of correlations between leaf width, snow mold resistance and recovery after *Typhula* infection.

Linkage map construction

Linkage analysis was carried out using JoinMap 3.0 (Van Ooijen and Voorrips, 2002). Segregation ratios of DNA markers in mapping population (3:1 and 1:1) were scored depending on the allelic state of the parents at analyzed loci. Only markers that fitted the expected ratios (1:1 for mono-parental markers, Aa×aa or aa×Aa) were further considered followed by Chi-square test ($P > 0.05$). Two parental maps were constructed using a LOD of 3.0 for the grouping of the markers by JoinMap 3.0. In each linkage group, the order of the markers was inferred using the pairwise data of only those loci that showed a recombination frequency less than 0.4 and a LOD value larger than 1.0. The Kosambi mapping function was used to convert recombination data to map distances. The linkage maps were drawn using MapChart 2.1 (Voorrips, 2002).

QTL analysis

QTL analysis was carried out using QTL Cartographer 1.13 (Basten et al., 1999). Two linkage maps of the parents using only the DNA markers with a segregation ratio of 1:1 were analyzed for QTL with the BC_1 algorithm. Composite Interval Mapping (CIM) analysis was performed separately on each map (from both maps) associated with the trait. CIM was run with model 6 of the program and a window size of 2 cM for all analysis. The number of markers for the background control was set to 5, which means that the 5 most significant markers outside the interval under the analysis were fitted to the model. The markers used for the background control were detected through forward and backward stepwise regression (Wang et al., 2000). The LOD thresholds generated by 1000 times permutation tests at a 0.05 significant level were used to determine putative QTL (Inoue et al., 2004).

Results

Phenotypic variation

In the pseudo F_2 population, all traits showed a continuous phenotypic variation typical of quantitative or polygenic inheritance. The frequency distributions for three traits are presented in Fig.4.1. Mean values of leaf width, snow mold disease resistance and recovery after *Typhula* infection for both parents and pseudo F_2 progeny are reported in Table 4.1. 'ASR368' showed significantly ($P<0.05$) narrower leaf width compared with 'MSU#8'. However, 'MSU#8' showed a significantly ($P<0.05$) higher snow mold disease resistance and better recovery after *Typhula* infection than 'ASR368'. Some progeny exhibited transgressive segregation for these three traits. The segregations of three traits were normally distributed when checked by skewedness and kurtosis parameters (Table 4.2); (Fig.4.1). A significant positive correlation was observed between disease resistance and recovery after *Typhula* infection ($r=0.58$, $P<0.001$), but no other significant correlations between leaf width and resistance or recovery were observed.

RAPD and SSR analysis

Twenty-six primers that generated polymorphisms between the parents and progeny were used to screen the mapping population. The selection was made based on clear and reproducible band patterns to provide unambiguous scoring. Of the 28 polymorphic markers amplified, 10 were inherited from the female parent 'MSU#8', 12 from the male parent 'ASR368', and 6 from both parents. Seventeen markers with distorted segregation that did not fit the expected single-dose restriction fragment ratios (1:1 or 3:1) were not included for further analysis based on the chi-square test ($P<0.05$) with 1:1 or 3:1 segregation ratio.

Thirty one EST-SSR primers successfully amplified 89 polymorphism bands between two parents and their progeny. Forty six polymorphic markers were inherited from the female parent 'MSU#8', and forty three from the male parent 'ASR368'. After the chi-square test ($P<0.05$) with 1:1 segregation ratio, 57 markers with distorted segregation were not included in linkage construction.

AFLP analysis

AFLP markers were generated from methylation-sensitive restriction enzyme *Pst*I. The 14 AFLP primer combinations (*Mse*I/*Pst*I) revealed a total of 383 polymorphic markers with optimally repeatable size of 50–450 bp, and an average of 28 markers per primer pair. Of the AFLP markers, 328 were heterozygous in one parent and null in another (202 from female parent and 126 from male parent, testcross configuration segregating 1:1), while 55 were segregating in both. Chi-square analysis revealed 252 markers (77 %) fitted a 1:1 segregation ratio and 28 (9%) fitted a 3:1 ratio. The remaining 48 (15 %) showed segregation distortion within the population.

Linkage (LG) map construction

Map construction was carried out according to the two-way pseudo-testcross procedure with JoinMap 3.0 based on 188 individuals. Of the 292 markers showing 1:1 Mendelian segregation, 232 (220 AFLPs, 5 RAPDs and 7 SSRs) markers were successfully mapped (79.5 %) on fourteen linkages for each of the two parents using a LOD score of 3.0. Sixty markers were unlinked. The linkage map of female map, 'MSU#8', consisted of 139 markers assigned to 14 LGs. Among these LGs, 11 LGs included 4 to 33 markers, and the remaining 3 LGs consisted of only two or three markers, respectively. The map spanned 805cM of the genome with an average inter-marker distance of 5.8 cM. The average size of the 14 LGs was 57.5 cM, ranging from 208cM (LG3) to 5cM (LG11); (Fig.4.2). In male parental map of 'ASR368', 93 markers were assembled into 14 LGs ranging from 12 markers per LG (LG10) to 2 markers per LG (LG6, LG9 and LG13). The sizes of these LGs varied from 121cM (LG12) to 9cM (LG9). The length of the male parental maps was covered 793 cM of an average 8.2 cM between two adjacent markers (Fig.4.3). Marker distribution in the map linkages seemed fairly even, and no pronounced clustering of any markers was found. However, some large gaps (above 20cM) still remained on some LGs.

QTL identification

To identify the position of genomic regions involved in leaf width, snow mold disease resistance, and recovery after *Typhula* infection, QTL analyses were performed on each parental linkage map ('ASR368' and 'MSU#8') seperately. Using composite interval mapping (CIM), the permutation test was conducted to establish an appropriate experiment-wise threshold of significance for trait-marker associations (Churchill and Doerge, 1994). This process was repeated 1,000 times at P=0.05 for each trait to determine the significant threshold for LR statistics. The threshold scores (LOD) of QTLs were 2.7 (leaf width), 3.1 (snow mold disease resistance) and 3.0 (recovery after *Typhula* infection). Three QTLs for leaf width (LW) were found on 'MSU#8' linkage map which has wide leaf parent. The proportion of variance explained by three QTLs detected was estimated. LW1 on LG2 (9%), LW2(6%) on LG7 and LW3 (7%) on LG12, explained phenotypic variance of leaf width (Table 4.3); (Fig.4.4a) . Multiple regression analyses using combinations of significant markers and their interactions revealed that epistatic interactions were not significant among these QTLs. In the second parent 'ASR368', no significant QTL for leave width was found. One QTL with LOD=3.81 explained 9% of the phenotypic variance for snow mold disease resistance was detected on LG10 from 'ASR368' (susceptible parent); (Table 4.3); (Fig.4.4b). No significant QTLs associated to resistance were located on the 'MSU#8' linkage map. One QTL affecting recovery after *Typhula* infection was found on LG1 of 'MSU#8' (higher recovery rate), explaining 7% of the phenotypic variance of the trait, and no significant QTLs were detected from 'ASR368' (Table 4.3); (Fig.4.4c).

Discussion

The development of a genetic linkage map in creeping bentgrass is the first step in understanding the genetic control of traits of agronomic interest. The double pseudo-testcross strategy was used in constructing the female and male linkage maps of creeping bentgrass based on AFLPs, SSRs, and RAPDs. Because AFLP are dominant markers, two types of segregation ratios (1:1 and 3:1) were observed in pseudo-F_2 mapping population with disomic inheritance. The markers which are heterozygous in only one parent and recessive homologous in the other parent segregate 1:1 in pseudo-F_2 mapping population (similar to backcross), whereas markers heterozygous in both parents should segregate in a 3:1 ratio. Markers segregating in a 3:1 ratio can be used as 'bridging locus' to align the homologous chromosomes of male and female linkage maps (Hanley et al., 2002). However, these markers (3:1 ratio) were not included in this study because double heterozygous markers are less informative than single heterozygous ones based on the fact that AFLP markers used as a main marker system in linkage map construction are dominant and can not distinguish AA, Aa, and aA genotypes in the progeny. Also, distorted markers (> 5%) were not used in linkage map construction to preserve linkage accuracy. The length of genetic map of the female parent was estimated to be 805cM and that of the male parent linkage was 793cM. Chakraborty et al. (2005) constructed a creeping bentgrass map which consists of 424 mapped loci covering 1,110 cM in 14 linkage groups by using RAPD, AFLP and RFLP markers. Since no common markers were shared among linkage maps, comparison can not be conducted. Mapping of additional co-dominant markers may help to combine these linkage maps and identify homoeologous linkage groups. A comparative map between creeping bentgrass and other members of the *Poaceae* such as, rice, wheat and maize is desired. The comparative genetic information will benefit creeping bentgrass researchers working on the improvement of agronomic traits.

QTL mapping is a common genetic analysis practiced in many plant species to study quantitative variations. However, no QTL analysis has been reported in creeping bentgrass species. To improve the understanding of the inheritance mechanism of some complex agronomical traits, and to facilitate creeping bentgrass breeding programs, five QTLs involved in leaf morphology, plant disease resistance and recovery after *Typhula* infection, which could have important roles in improvement of creeping bentgrass cultivar were identified. QTL mapping strategies of out-crossing plant species are different from self-pollination crops, such as wheat and rice. Separate QTL analysis for each parent was conducted to determine the QTL originality and magnification. To identify more major QTLs related to these traits, factors, such as, linkage coverage and saturation, size of mapping population and accuracy of phenotype data should be considered. Improved linkage coverage and saturation can increase the ability to detect QTLs and estimate their location. The creeping bentgrass linkage maps presented herein have some gaps between the markers. QTLs present in these gaps may not have been detected. More markers are desired to be integrated into linkage maps for bridging the gaps and increasing coverage and saturation. Size of mapping population is another factor to determine QTL analysis results. The small size of mapping population could lead to an underestimation of the number of QTL per trait and to an overestimation of the phenotypic effect associated with each QTL from simulation studies (Jansen et al., 2003). Large population sizes will increase the accuracy of QTL mapping in cases of sparse marker coverage (Jannink, 2005). Vales et al. (2005) detected more QTLs controlling barley stripe rust disease resistance by using a large segregation population (n=409), although they also found there was little improvement when population size is beyond 300. In this study, we selected 188 progeny out of 600 F_1 progeny to construct linkage and analyze QTL. To improve the power of QTL detection, more progeny could be included in the mapping population. Accurate phenotypic data also plays a key role in QTL analysis because QTL is derived from co-segregation analysis between markers and agronomic characters. To guarantee consistent phenotypic scoring, the artificial evaluation of snow mold disease was conducted in the cold rooms, in which provides a constant environment. However, further evaluations with field inoculation will provide additional information on QTL detection and environmental effect.

We detected three QTLs associated with leaf width that explained only 22% of the phenotypic variance in F_1 population. These data would indicate the presence of additional QTLs in creeping bentgrass. In rice, leaf width is inherited quantitatively and influenced largely by growth environments. More than a dozen genes, located on different chromosome regions with different functions, were involved in the genetic mechanisms of leaf development (Kobayashi et al., 2003). Utilizing DNA markers linked to QTLs for leaf width of other crops opens a new avenue for elucidating the genetic mechanism of leaf morphology and selecting leaf morphology in creeping bentgrass. Improvement of snow mold disease resistance is another creeping bentgrass breeding goal. Creeping bentgrass cultivars possess a limited resistance to gray snow mold which is prevalent in North America (Wang et al., 2005). 'MSU#8', which has the desirable resistance to gray snow mold disease, could be used as useful germplasm for improving resistance of modern cultivars. In addition disease resistance, recovery ability after disease infection also should be considered in the breeding. Pyramiding QTLs controlling resistance and recovery after *Typhula* infection should provide an opportunity to develop cultivars combining higher snow mold resistance with better recovery ability in creeping bentgrass breeding program.

Table 4.1 AFLP,SSR and RAPD primers used for mapping the creeping bentgrass population.

AFLPs *(PstI +MseI)*	**SSRs (Tall fescue EST-SSRa) a**	**RAPDs**
P-ACC/M-CACG	NFFA015 ,NFFA093	701
P-ACC/M-CAG	NFFA021 ,NFFA101	777
P-ACC/M-CGGC	NFFA022 ,NFFA106	BA01
P-ACC/M-CTCG	NFFA023 ,NFFA109	BA02
P-ACC/M-CTG	NFFA042 ,NFFA115	BA08
P-AGG/M-CAG	NFFA045 ,NFFA117	OPC09
P-AGG/M-CAT	NFFA050 ,NFFA121	OPG10
P-AGG/M-CGGA	NFFA056 ,NFFA126	OPY07
P-AGG/M-CGGC	NFFA062 ,NFFA132	OPY17
P-AGT/M-CAG	NFFA063 ,NFFA142	
P-AGT/M-CAT	NFFA066 ,NFFA147	
P-AGT/M-CCGG	NFFA072 ,NFFA148	
P-AGT/M-CGGC	NFFA075 ,NFFA150	
P-AGT/M-CTCG	NFFA082 ,NFFA151	
P-AGT/M-CTG	NFFA088 ,NFFA154	
	NFFA090 ,	

a The tall fescue EST-SSRs were develop at Noble Foundation, and sequences of these primer pairs were published in Saha et al. (2004)

Table 4.2 Phenotypic values of three quantitative traits (leaf with, snow mold disease resistance and recovery after *Typhula* infection) for parental lines (ASR368 and MSU#8) and 188 F1 progeny.

	Parental Lines		F1 Progeny		
Traits	'ASR368'	'MSU#8'	Range	Skewedness	Kurtosis
Leaf width(mm)	1.4	2.8	1--3	1.06	0.45
Resistance	7	4	2--9	0.7	-0.16
Recovery	3	7	1--7	0.09	-1.7

Resistance means snow mold disease resistance and Recovery means recovery after *Typhula* infection

Resistance 1 = 0-5% disease, 9 = 95%-100% disease turf

Recovery 1=10% turf recovery, 9=90% recovery

Table 4.3 Characteristics of the detected QTL for three traits (leaf with, snow mold disease resistance and recovery after *Typhula* infection). In linkage group column, Flg means female linkage and Mlg means male linkage.

QTLs	Linkage Group	Peak Position	Nearest Markers	LOD Score	LOD Threshold	R2%
Leaf Width1	Flg2	42.2cM	P-AGG/M-CAG*n218 / P-AGT/M-CCGG*n318	4.1	2.6	9%
Leaf Width2	Flg7	21.6cM	P-AGT/M-CAG*n356 / P-ACC/M-CGGC*n130	2.7	2.6	6%
Leaf Width3	Flg12	18.8cM	P-AGT/M-CTCG*n287 / P-ACC/M-CGGC*n161	3.0	2.6	7%
Recovery	Flg12	60.6cM	P-AGT/M-CAG*n275 / P-AGT/M-CAT*n303	3.2	3.0	7%
Disease Resistance	Mlg10	24.8cM	P-ACC/M-CTG*n422 / P-AGT/M-CGGC*n400	3.8	3.0	9%

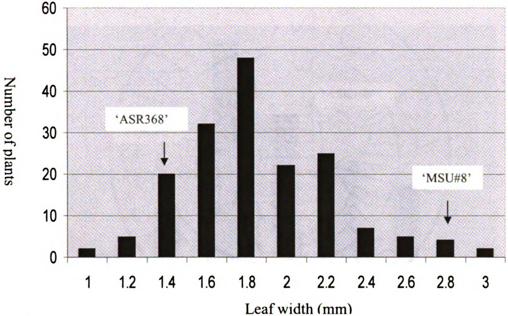

Figure 4.1a Frequency distribution in 188 F1 progeny from the cross between ASR368 and MSU#8 for a leaf width (mm). Parental trait values are indicated by arrows.

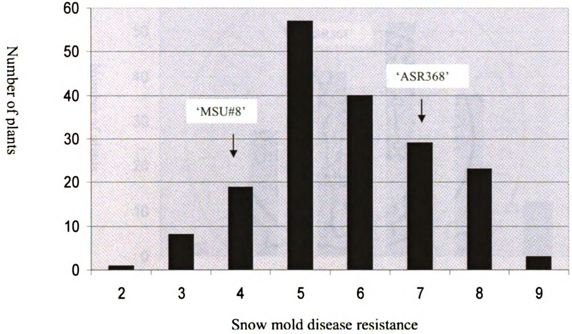

Figure 4.1b Frequency distribution in 188 F1 progeny from the cross between ASR368 and MSU#8 for resistance to snow mold. Parental trait values are indicated by arrows. Resistance: 1 = 0-5% diseased turf, 9 = 95%-100% diseased turf.

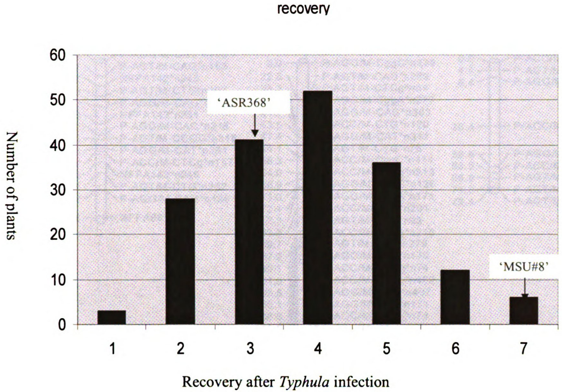

Figure 4.1c Frequency distribution in 188 F1 progeny from the cross between ASR368 and MSU#8 for recovery after *Typhula* infection. Parental trait values are indicated by arrows. Recovery: 1=10% turf recovery, 9=90% turf recovery.

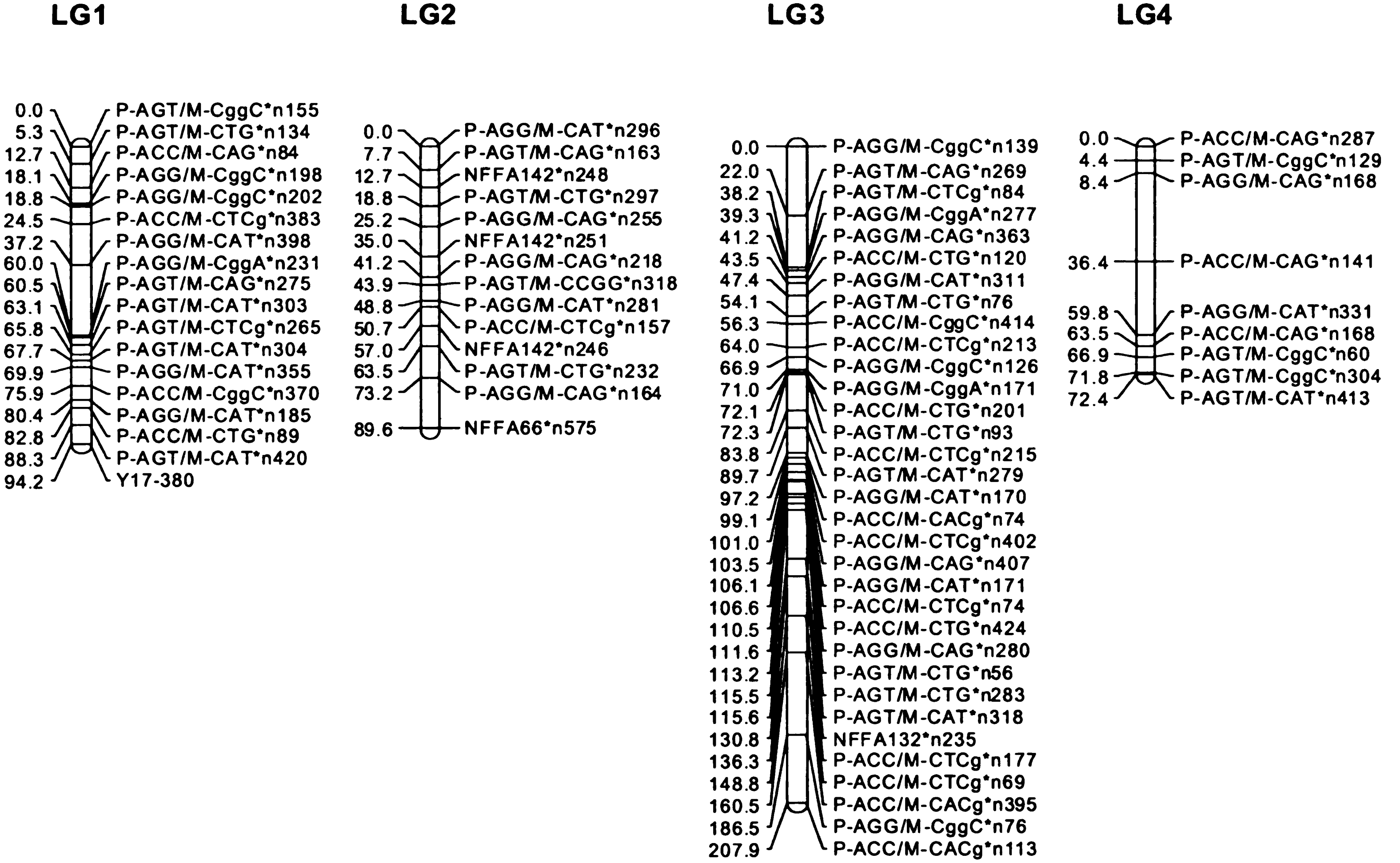

Figure 4.2a A genetic linkage map of the female parent (MSU# 8) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right.

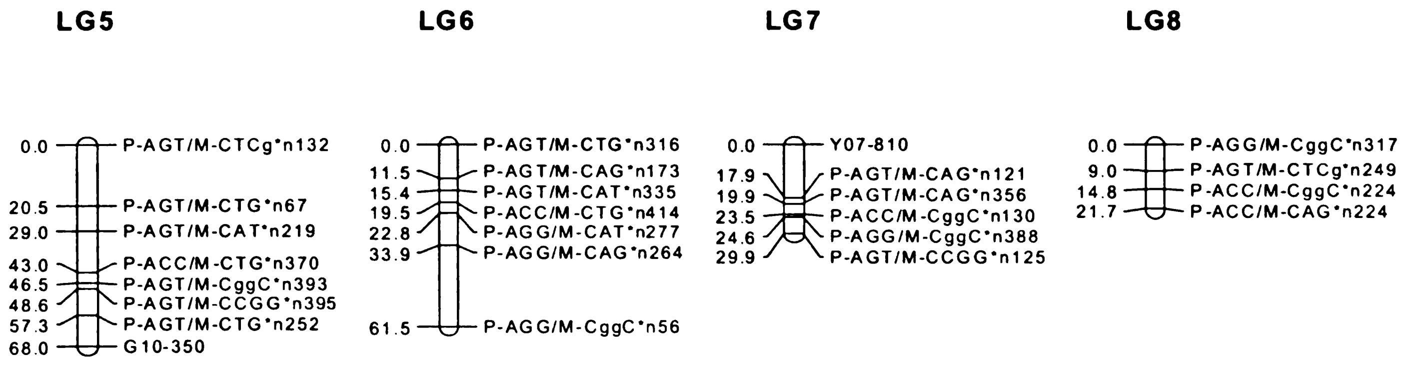

Figure 4.2b A genetic linkage map of the female parent (MSU# 8) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

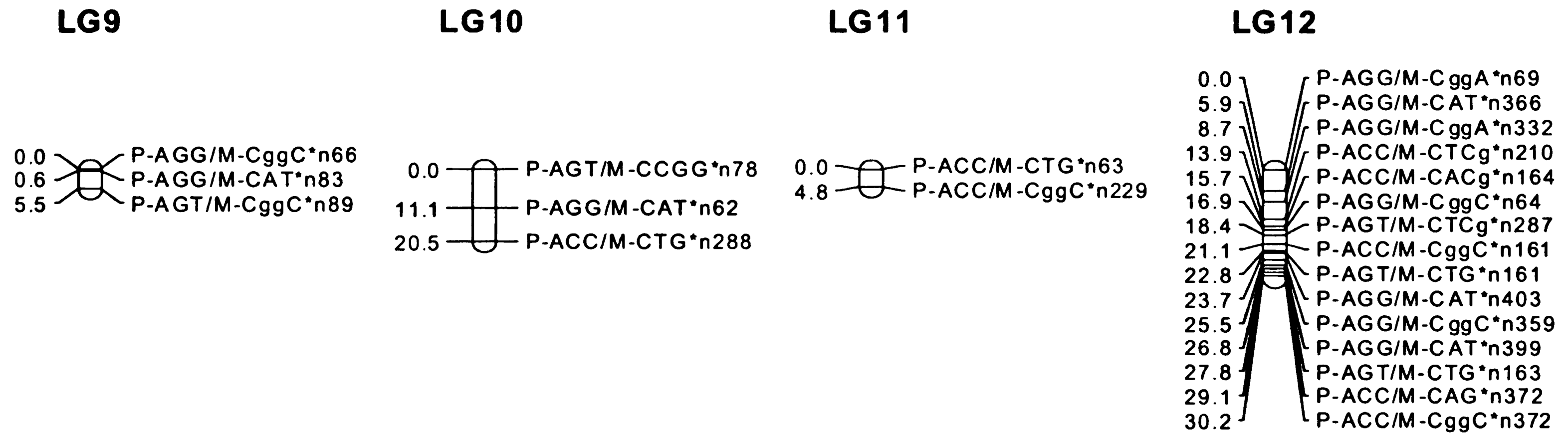

Figure 4.2c A genetic linkage map of the female parent (MSU# 8) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

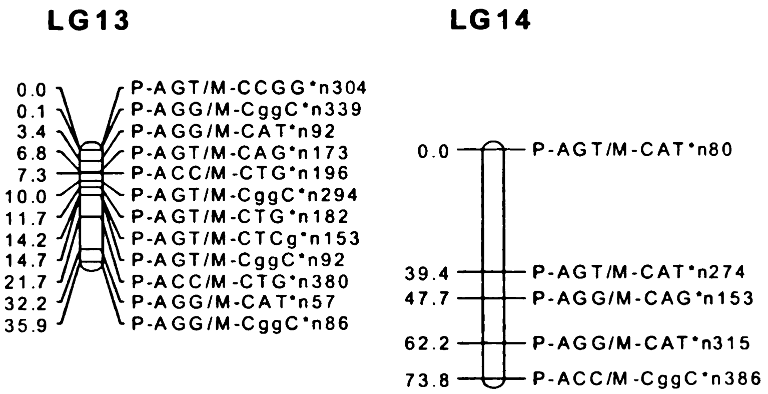

Figure 4.2d A genetic linkage map of the female parent (MSU# 8) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

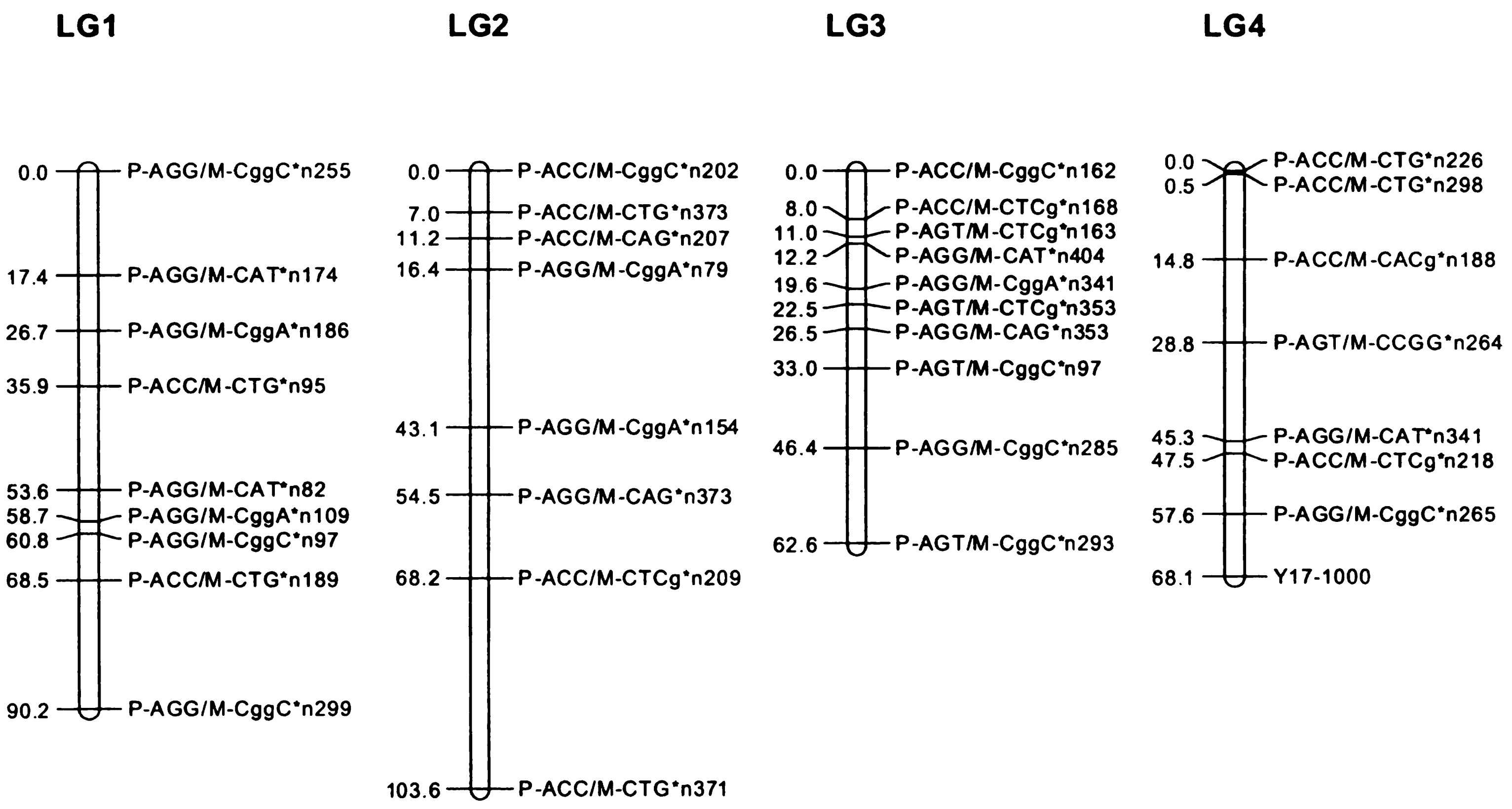

Figure 4.3a A genetic linkage the map of male parent (ASR368) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right.

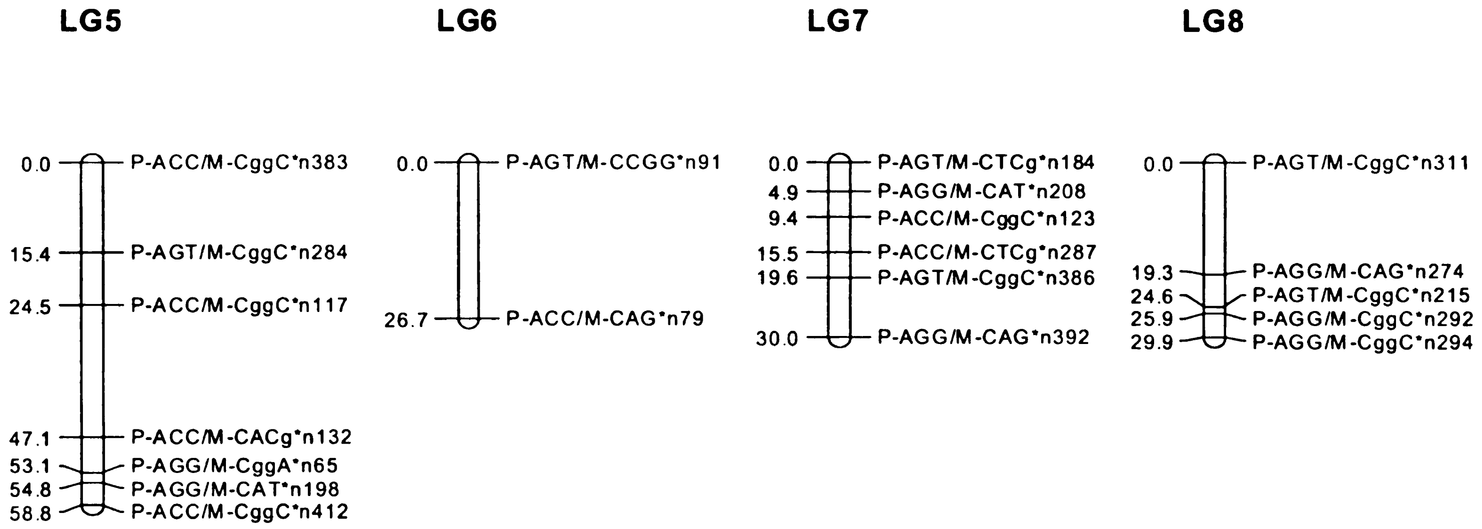

Figure 4.3b A genetic linkage map of the male parent (ASR368) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

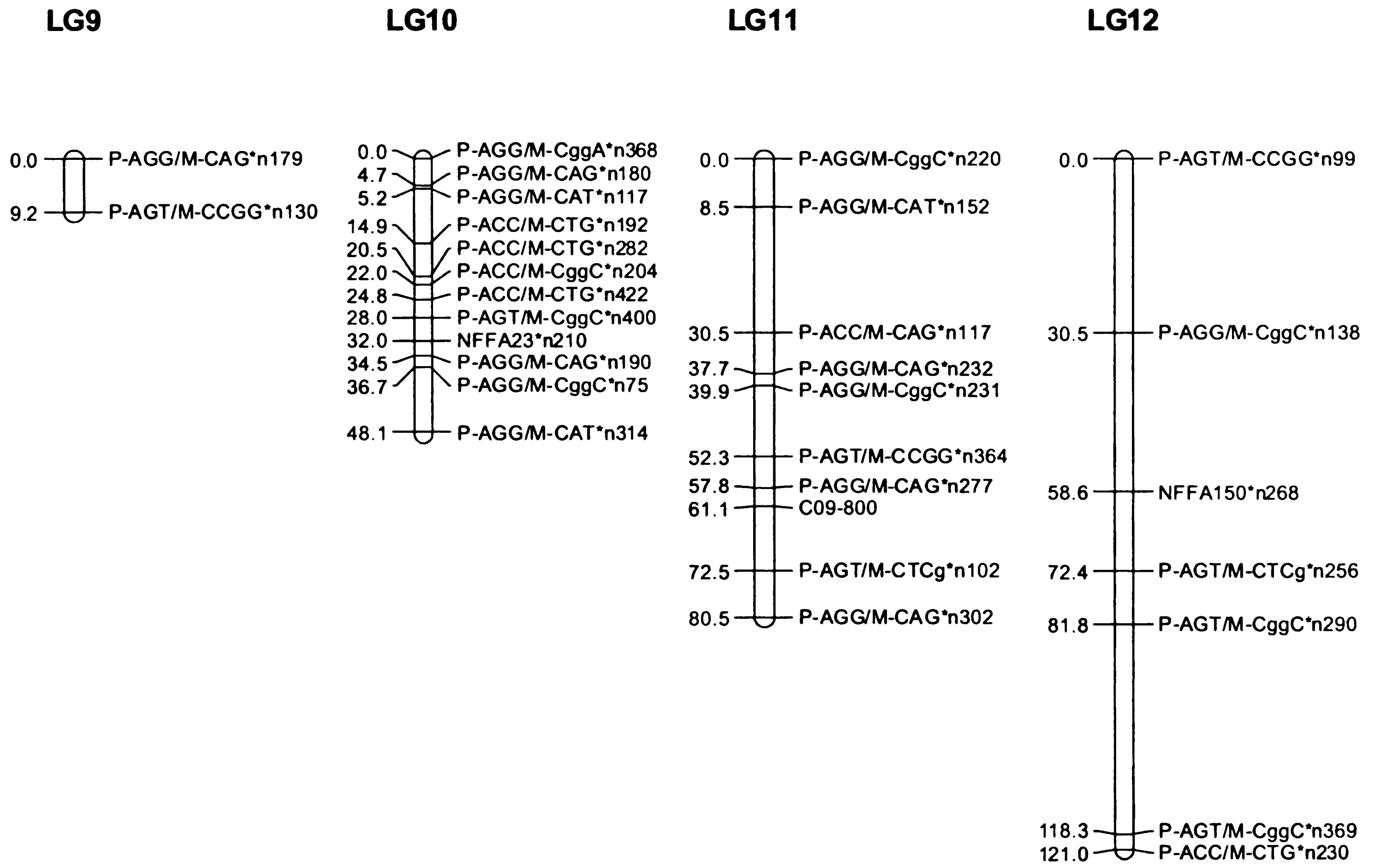

Figure 4.3c A genetic linkage map of the male parent (ASR368) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

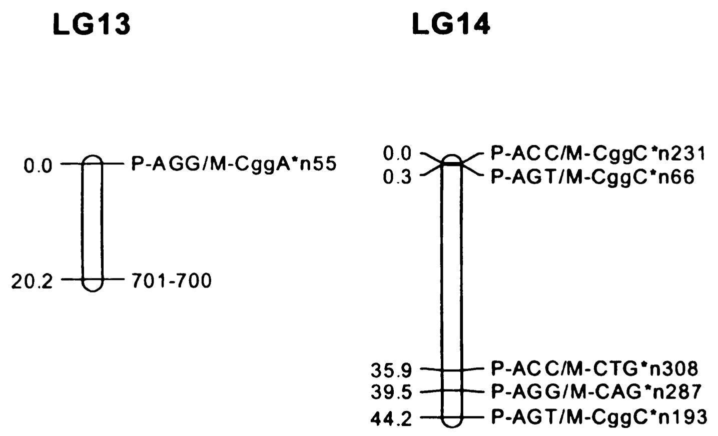

Figure 4.3d A genetic linkage map of the male parent (ASR368) generated from 188 individuals. The *numbers* on the left are the genetic distances in Kosambi centiMorgans (cM) between markers. Marker names are on the right (cont'd).

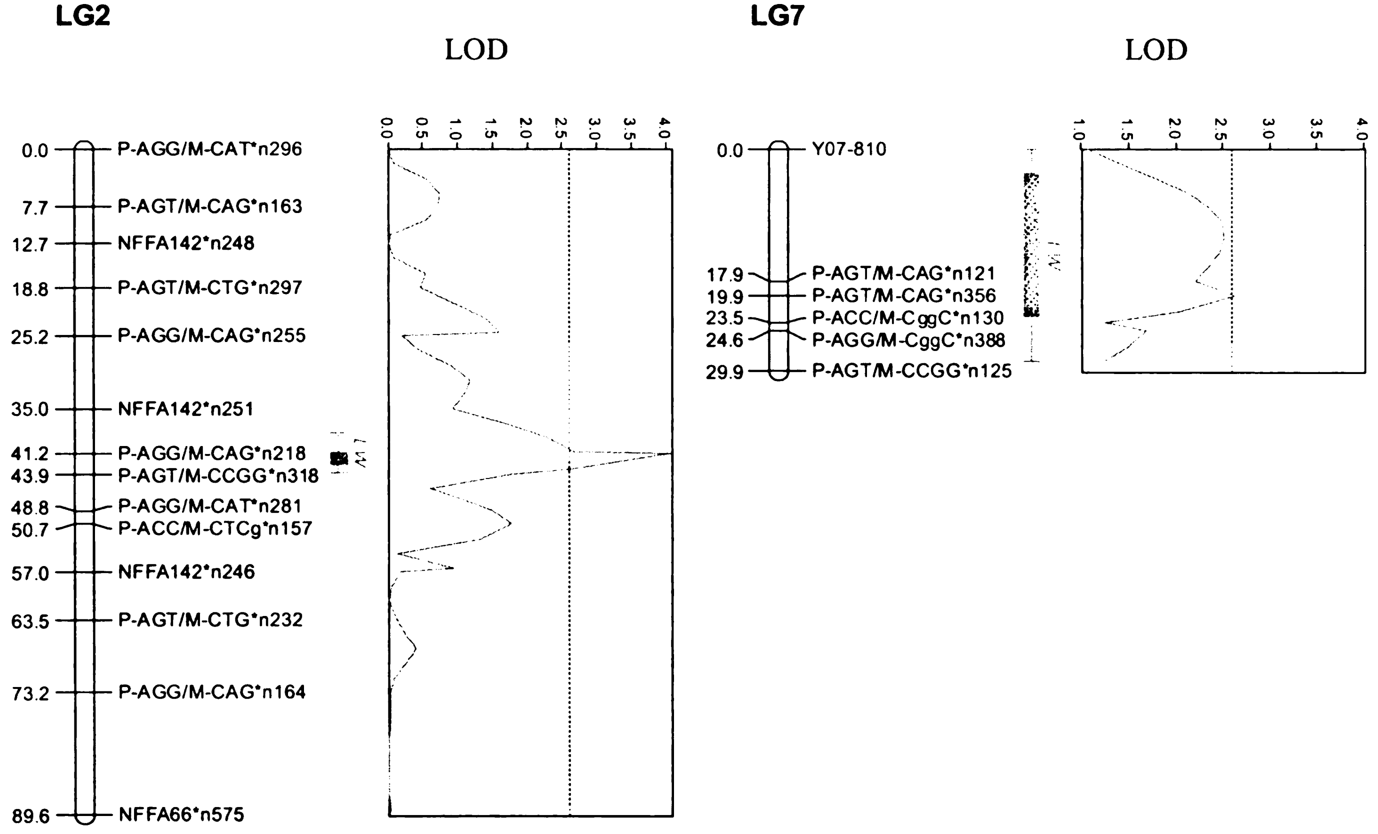

Fig.4. 4a Linkage map and the QTL likelihood plots indicating leaf width QTLs. The vertical dot lines in QTL likelihood plots indicate significant threshold level based on permutation tests, corresponding to LOD score=2.5. LG2 and LG7 are female parent (MSU#8) linkages.

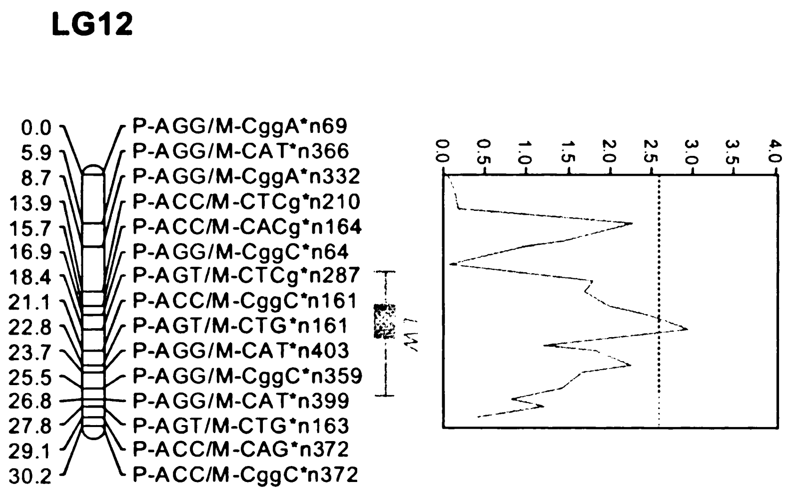

Fig.4. 4a-1 Linkage map and the QTL likelihood plot indicating leaf width QTL. The vertical dot line in QTL likelihood plot indicates significant threshold level based on permutation tests, corresponding to LOD score=2.5. LG12 is female parent (MSU#8) linkage.

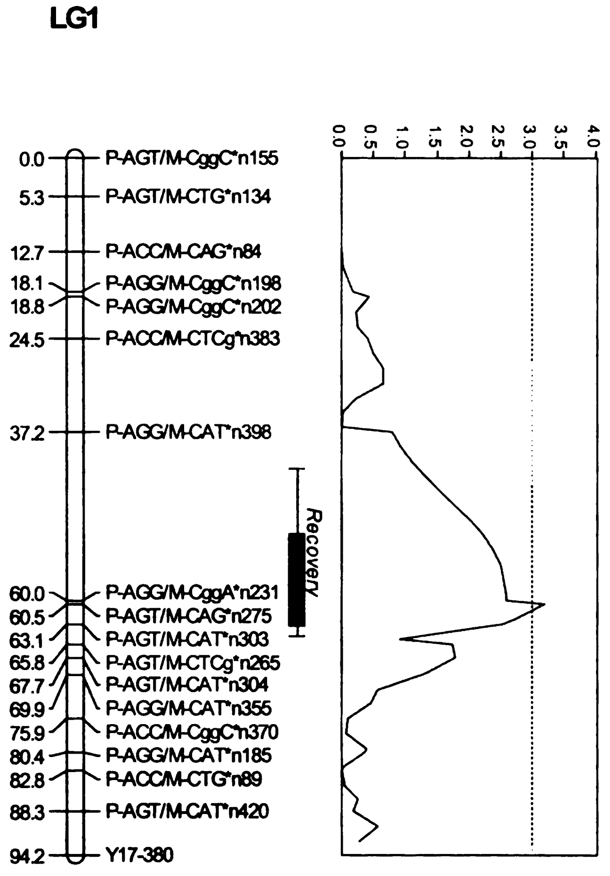

Fig. 4.4b Linkage map and the QTL likelihood plot indicating recovery QTL after *Typhula* infection. The vertical dot lines in QTL likelihood plot indicate significant threshold level based on permutation tests, corresponding to LOD score=3.0. LG1 is female parent (MSU#8) linkage.

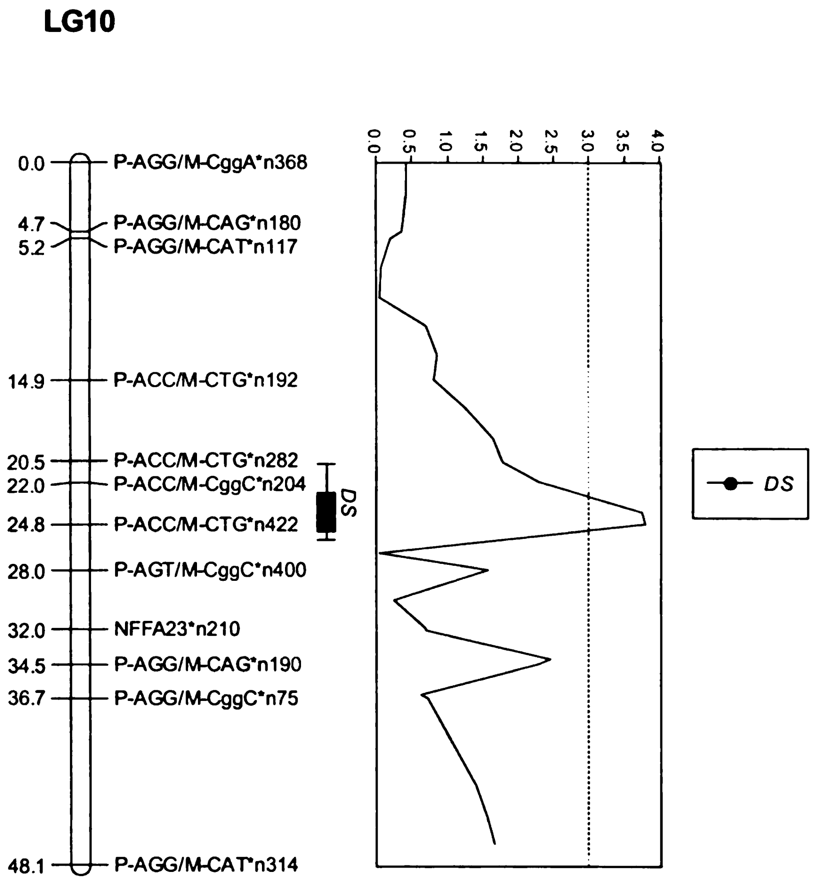

Fig.4. 4c Linkage map and the QTL likelihood plots indicating snow mold disease resistant QTL. The vertical dot lines in QTL likelihood plots indicate significant threshold level based on permutation tests, corresponding to LOD score=3.0. LG10 is male parent (ASR368) linkage.

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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF COLD-REGULATED TRANSCRIPTIONAL ACTIVATOR *LPCBF3* GENE FROM PERENNIAL RYEGRASS (*Lolium perenne* L.)

Abstract

In plants, low temperatures can activate the CBF cold response pathway which plays a prominent role in cold acclimation by triggering a set of cold-related gene expression. We identified and characterized a *CBF* homologous gene, designated as *LpCBF3*, from a cold-tolerant perennial ryegrass (*Lolium perenne* L.) accession, PI598441. *LpCBF3* encodes a protein of 237 amino acids with a molecular mass of 25.5kDa. It carries the sequences for the typical nuclear localization signal (NLS), AP2 DNA-binding domains and an acidic activation present in most of the plant CBF proteins. Monocot *CBF* genes are grouped together and separately clustered from dicot *CBF* genes. Southern analysis indicated the presence of at least three homologs of *LpCBF3* gene in the perennial ryegrass genome. Only one amino acid variation in LpCBF3 protein between cold-tolerant and -sensitive (PI610939) perennial ryegrass accessions was observed. A glycine to glutamine substitution at amino acid residue 190 was found in the cold-tolerant accession. However, in their putative promoter regions, some differential regions were found. Northern blotting and RT-PCR analysis found *LpCBF3* reached the highest expression after 1.5 hours of cold treatment (4°C). A *COR* homologous gene, a downstream gene of *CBF*, can be expressed in the plant stem of cold-tolerant perennial ryegrass accession without cold treatment. Conversely, the *COR* gene can not be activated in cold-sensitive perennial ryegrass accession without cold treatment, although the cold treatment can prompt expression level of *COR* homologous gene in both perennial ryegrass accessions. In transgenic *Arabidopsis*, the over-expression of *LpCBF3* with the 35S promoter resulted in plants with dwarfism, later flowering and greater freezing tolerance. Implications and applications of *LpCBF3* gene for improvement of cold tolerance with other desirable traits in turfgrass breeding program are proposed.

Key Words: *LpCBF3*, Perennial ryegrass, COR, Promoter regions, Cold tolerance

Introduction

Perennial ryegrass (*Lolium perenne* L.) is the most common grass grown in the temperate regions of the world (Asay, 1992). It is primarily used for turf and forage because of its high quality and yield, but low winter hardiness (ability of a plant to survive winter) limits its application in northern continental climates (Humphreys and Eagles, 1988). Improving cold tolerance in perennial ryegrass is one of the most important breeding objectives. However, implementing this objective has been met with limited success by conventional breeding methods because of the quantitative characteristics of the cold tolerance trait (Skinner et al., 2006; Skot et al., 2002). Modern biotechnology provides an opportunity to improve cold tolerance and winter hardiness in perennial ryegrass, as well as other plant species by manipulating cold-tolerant related genes and their expression (Kosmala et al., 2006; Zhang et al., 2006).

Many plant species from temperate regions can increase their cold tolerance in response to low, non-freezing temperatures, a phenomenon called cold acclimation. Cold acclimation undergoes physiological and biochemical adaptations which are regulated by at least two pathways, abscisic acid (ABA) and ABA independent pathways (Knight et al., 2004; Liu et al., 1998). In the ABA-independent process, cold temperatures indirectly trigger the transcription of the CBF family which in turn activates the transcription of *COR* genes encoding a diverse array of proteins with a presumed function in tolerance to the dehydration caused by freezing (Kobayashi et al., 2005). In *Arabidopsis*, the transcription of *COR* genes has been shown to be regulated by cis-acting drought and cold responsive elements (DRE) containing a core CCCGAC (CRT) sequence (Liu et al., 1998; Skinner et al., 2006). The trans-acting factor, CBF which can bind to DRE and activate *COR* genes' expression, was first isolated from *Arabidopsis* (Liu et al., 1998) and then several other plants, such as rice (*Oryza sativa*);(Qin et al., 2005), diploid wheat (*Triticum monococcum.*); (Miller et al., 2006), barley (*Hordeum vulgare*);(Choi et al., 2002). The *CBF* genes, as transcriptional factors, belong to a small multi-gene family and have a potential nuclear localization sequence (NLS), an APETALA2 (AP2) DNA-binding motif and an acidic activation domain (Gilmour et al., 2000; Skinner et al., 2006). In *Arabidopsis*, the genes, *CBF1*, *CBF2* and *CBF3* (also known as *DREB1b*, *DREB1c* and *DREB1a*, respectively), are located in tandem on chromosome 4 (Medina et al., 1999), and are cold and stress-inducible. The transcription of *CBF* genes was positively regulated by an upstream transcription factor, *ICE1* (inducer of CBF expression 1), after the cold treatment (Chinnusamy et al., 2003). While the transcription of *CBF3* and *CBF1* can be negatively regulated by over-expressed *CBF2* (Novillo et al., 2004). *CBF* gene expression may also be controlled by Ca^{2+}- related processes, because the *CBF* gene expression pattern can be altered in mutations of Ca^{2+}/H^{+} transporter CAX1 and Ca^{2+}-sensor protein CBL1 (Albrecht et al., 2003; Catala et al., 2003). Therefore, cold signaling of freezing tolerance requires a cascade of transcriptional regulation events. After cold treatment, the upstream transcriptional factor *ICE1* or other factors can up- or down-regulate the *CBF* transcription which activates or represses *CBF* regulon expression (Xiong et al., 2002). This pathway may be conserved in a variety of plant species at the molecular level (Cook et al., 2004).

Because *CBF* genes are master switches that control the expression of a regulon of CRT/DRE-controlled genes that include some of *COR* gene family (Fowler and Thomashow, 2002), constitutive over-expression of *CBF1* and *CBF3* in the *Arabidopsis* was shown to be able to drive expression of *COR* genes in absence of low temperature stimulation and then increase plant cold tolerance by increasing the sugar and proline content (Gilmour et al., 2000). Also transgenic plants exhibit some traits such as a 'striking dwarf', later flowering and stunted phenotype (Gilmour et al., 2000), which are considered to be side effects due to over-expressed *CBF* gene. However, these morphological characteristics of transgenic plants are desirable in turfgrass breeding since they may reduce mowing frequency in turfgrass and increase cold tolerance.

To screen cold tolerant perennial ryegrass, the germination rate of 300 perennial ryegrass accessions across a range of temperatures was evaluated in the laboratory by using a thermogradient plate to determine if accessions would germinate equally well as temperatures declined. In the field, the accessions were grown as monocultures and as binary mixtures with ladino white clover and rated for winter hardiness at three Michigan field locations (Warnock et al., 2005). Based on the results from the laboratory and field, 30 cold-tolerant accessions of perennial ryegrass were identified.

The objectives of this study were to screen 30 cold-tolerant perennial ryegrass accessions, to isolate and characterize a *CBF*-type gene and its putative promoter sequence from the most cold tolerant and sensitive perennial ryegrass accessions, and to determine the effects of overexpression of the gene in transgenic *Arabidopsis* plants. We also discuss the application of *LpCBF3* in turfgrass breeding.

Materials and Methods

Freezing tolerance evaluation

All the Plant Introductions (PI) used in this study were provided by the USDA Regional Plant Introduction Station (Pullman, Washington). Thirty accessions obtained after cold tolerance evaluation were germinated and grown in 0.9L plastic pots filled with Baccto planting mix (Michigan Peat, Houston) at Michigan State University greenhouse (East Lansing, MI) at 25°C for five weeks. The plants were gently pulled out of the soil and cleaned with tap water. The leaves and partial roots were removed. The plants were transferred to freezing incubator and the temperature controller was set to -1, -3, -5, -7, -9, -11,-13, respectively. After leaving in the incubator for 24 hours at the appropriate temperatures, the plants were moved to cold room (4°C) for 12 hours. Then plants were transferred to pots and grown at greenhouse (25°C) for scoring after 1 week. Every accession has six replications at every appropriate temperature. The temperature at which 50% of the plants were killed (LT_{50}) was determined. The plants which have the lowest LT_{50} were used in gene isolation. The accession, PI 610939 with LT_{50}=-1°C, was used as a control.

DNA extraction and Southern analysis

DNA was isolated from 4 g of perennial ryegrass leaves and purified by chloroform extraction (Sharp et al., 1989). Ten micrograms of DNA that had been pretreated with RNase was digested with restriction enzymes (New England Biolabs, Inc., Beverly, MA, U.S.A.) at 37℃ for 2 hours. Conditions for electrophoresis and blotting were performed according to Sharp et al. (1989). DNA was transferred to Hybond-N extra membranes (Amersham, Buckinghamshire, UK). Pre-hybridization, hybridization and probe labeling were followed the manual of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). The digested DNA were hybridized overnight at 28℃ or 27℃ according to probe's CG content and length, washed in 0.1X SSC/0.5% SDS in room temperature, and exposed to X-ray film (KODAK) for 10 min. The probes were prepared from a full-length cDNA of *LpCBF3* and *LpCBF3* fragment containing AP2 and NIL domains produced by PCR with the primers CD1F TCGCAGAGCAAAACCACTTCA and CD1R TGGTGTCGCTCGAATTCGT at Tm=57℃.

RNA extraction and Northern analysis

RNA from leaves and stems of cold tolerant and sensitive perennial ryegrass accessions were isolated at 0 min, 15 min, 30 min, 1 hr, 2 hours, 4 hours and 24 hours with cold treatment (4℃) respectively by using Trizol reagent (GIBCO, BRL, Rockville, MD) according to manufacturer's protocol. Five micrograms of total RNA from different treatments were fractionated in 1.2% agarose/formaldehyde gel and transferred to Hybond-N extra membranes. Total RNA was pre-hybridized in 50% formamide, 5xSSC, 0.1% SDS, 20mM of sodium phosphate pH6.5, 0.1% Ficoll, 0.1% polyvinylpyrolidone, 1% glycine, 250mg/ml of denatured salmon sperm DNA at 42 C for at least 2hours. Hybridization was performed at 65°C overnight in the pre-hybridized solution with ^{32}P-labeled probes (>107 cpm specific activity, cpm/ g DNA). The filters were washed three times in 2x SSC, 0.1% SDS at room temperature for 10 min and then two washes in 0.1 SSC, 0.1% SDS at 65 C for 30min each. The probes were prepared from *LpCBF3* gene and wheat *COR39* of the entire cDNA insert from pWG1 (Guo et al., 1992; Takumi et al., 2003). Probes were labeled with (α-^{32}P)-dCTP (1,000 Ci/mmol, Amersham, Buckinghamshire, UK) using the Prime-a-Gene Labeling System (Promega, Madison, Wis., USA).

cDNA synthesis

cDNA was generated from RNA samples from a whole plant with 4-hours 4 ℃ treatment by using SuperScriptII First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and using 3 μg RNA and 50-ng random hexamers per reaction. Control reactions without reverse transcriptase were run for all samples to confirm removal of contaminating genomic DNA.

Isolation of full length LpCBF3 gene

A perennial ryegrass genomic DNA fragment encoding a CBF-like polypeptide was isolated by PCR using primerD1: GGCCGGCGGGCGAACCAAGTTCC, D2:AGGCAGAGTCGGCGAAGTTGAGGC and CBF degenerate forward primer CC(AGCT)AA(AG)AA(AG)CC(AGCT)GC(ACGT)GG(ACGT), CBF degenerate reverse primer GG(AGCT)A(AG)(AGCT)A(AG)CAT(AGCT)CC(CT)TC(AGCT)GCC . These primers were designed according to conserved regions of the Rice and Arabidopsis CBF proteins at the beginning of the AP2/EREBP domain and putative activation domain, respectively (Thomashow et al., 2001). 3'-and 5'-ends of cDNA were isolated based on the partial gene sequence obtained above using 5' and 3'RACE (5'/3' cDNA amplification kit, Roche, German). The gene-specific primers are Sp1 CCCGCGGCGAGGGCGAGCATGGCGGC and Sp5R GGCGGGGCGAACCAAGTTCC for amplifying 5' and 3' sequence of *LpCBF3* gene. The full length *LpCBF3* gene was synthesis by PCR. The primers were designed according to 5' and 3' sequence of *LpCBF3* gene and the primers are: Forward-primer Cbf3-4F: ACTGAGGTAGCGCTAGCTCCTATT, Reverse-primer Cbf3-4R: CACAATCACATTACCAGAAACTGC at Tm=60°C.

DNA sequence alignments and annotation

DNA was sequenced by Michigan State University Genetics Facility. Alignments were made by Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Other sequences used in this study were obtained from GenBank. Gene-prediction program used to annotate was GENSCAN 1.0 (http://genes.mit.edu/GENSCAN.html). Predicted polypeptide sequences were defined by results of BLASTp searches against National Center for Biotechnology Information nonredundant database (http://www.ncbi.nlm.nih.gov/blast/).

Real-time PCR conditions and analysis

Polymerase chain reactions were performed with the Mx4000 Multiplex Quantitative PCR System (Stratagene Inc, La Jolla, Ca.), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 5 µl 2x SYBR® Green Master Mix reagent (Applied Biosystems), 2.5 ng cDNA and 200 nM of each gene-specific primer in a final volume of 25 µl. The following standard thermal profile was used for all PCRs: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 65°C for 1 min. The quantity of actin gene expression was measured as a normalization control. Optics graphs showing the fluorescence intensity of each reaction plotted against PCR cycles were generated for each run. Melt curves and first derivative melt curves were run immediately after the last PCR cycle. Melt curves were produced by plotting the fluorescence intensity against temperature as the temperature was increased from 65 to 95°C at 0.2°C/s. Data was collected and viewed using the software and graphics programs provided with the Mx4000 Multiplex Quantitative PCR System. For confirmation of amplicon presence and purity, the RT-PCR product was run on a 2% agrose gel, stained with ethidium bromide, and photographed.

PCR Reaction and Electrophoresis

PCR reactions were performed in 25-μL volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 μM MgCl2, μ200 M of each deoxynucleotide, 50 ng of each primer (totally 6 primers), about 50 ng template DNA, and 1 U Taq DNA polymerase. The amplification was performed in an Thermcycler using the following program: 94°C for 2 min followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and 50 s, with a final extension at 72°C for 5 min. PCR products were separated on 1.4% (w/v) agarose gels and visualized under UV light after ethidium bromide staining.

Vector Construction and Arabidopsis Transformation

A 890-bp *Bam*HI/*Hin*dIII fragment from a cDNA clone containing the whole coding region of *LpCBF3* was inserted into the *Asc*I and *Bam*HI sites of the binary transformation vector pFGC5941. The resulting plasmid, which contains the *LpCBF3* coding sequence under control of the CaMV 35*S* promoter, was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Arabidopsis* plants (Columbia) were transformed with the construct or the transformation vector pFGC5941 using the floral dip method (Clough and Bent, 1998). Transformed plants were selected on the basis of bialaphos herbicide resistance. Homozygous T3 plants were used in all experiments.

Freeze test for transgenic Arabidopsis

Under sterile conditions in Petri dishes, Columbia (wild type) and transgenic plants were grown on Gamborg's B-5 medium. The plants were tested for freezing tolerance by first placing the plates at -2°C in the dark for 3 hours followed by ice nucleation with sterile ice chips. The plates were incubated an additional 21 hours at -2°C, then the temperature of the freezer was turned down to −6°C, and the plates were left at this temperature for an additional 24 hours. The plates were taken from the freezer and placed at 4°C in the dark for 18 hours, followed by 2 days at 25°C under cool-white fluorescent lights (40-50 µmol $m^{-2}s^{-1}$) with an 18-hour photoperiod. The plates were then scored for freezing damage as either dead or alive. The freeze test was repeated three times.

Results

Screening perennial ryegrass accessions for freezing tolerance

The lethal temperature (LT_{50}) of most ryegrass accessions spans a range of 9℃, from -1℃ to -9℃, except for PI598441accession. The PI598441 is the most freezing tolerance accession with a corresponding LT_{50} = -11℃. PI598441 was originally collected from Switzerland at a 46.583(Latitude), 6.9333(Longitude) and 860m (Elevation). In the greenhouse, PI598441 has a high-tiller number and dark green leaves. It shows a delay in flowering after vernalization compared to other perennial ryegrass accessions.

Isolation of the CBF conserved regions

The degenerated primers based on the *Arabidopsis* and rice CBF conserved nuclear NLS and AP2 regions amplified a 240bp fragment from perennial ryegrass genomic DNA of PI598441. The deduced amino acid sequence of the fragments consists of 77 amino acids with most significant similarity with rice *RCBF3* responding to the NLS and AP2 regions (blast score=58.2 bits, expect=6e-08).

Isolation of and sequencing of perennial ryegrass LpCBF3 gene

To obtain the full length perennial ryegrass *cbf* gene, 5'and 3' Rapid Amplification cDNA Ends (RACE) were used to amplify two flanking sequences. A set of primers was designed according to 240bp fragment from perennial ryegrass genomic DNA for amplifying the 5' and 3' flanking regions. A 180 bp fragment was produced by 5' RACE and an 800 bp fragment was produced by 3' RACE. We deduced that the full length of *CBF* homolog was close to 1000bp (including 5' and 3' untranslated regions, UTR). The primers for the 5' UTR region and 3' polyA anchor sequence were designed for amplifying the full length *CBF* homolog from cDNA. An approximate 1000bp fragment was obtained and sequenced. Sequence data revealed that it consists of 974 bp. The 711bp ORF of the gene encodes a mass of 25.5-kD polypeptide consisting 237 amino acids (aa); (pI 5.04), which was quite similar to rice CBF3 and barley CBF3. This gene was designed as *LpCBF3*. Sequence comparison between *LpCBF*3 genes from cDNA and genomic DNA indicated that there is no intron present in the perennial ryegrass *LpCBF*3 gene. Genomic DNA was digested with *EcoR*V that cannot cut within the *LpCBF3* coding region, and was hybridized by the labeled *LpCBF*3 probe in low stringency hybridization condition. The results revealed that three hybridization bands were present, which showed *LpCBF*3 has at least three homologs in the perennial ryegrass genome. Induced LpCBF3 protein carries the sequences for the typical NLS, AP2-DNA binding motifs and an acidic domain which are present in most plant CBF proteins. A TMpred (http://www.ch.embnet.org) analysis predicted a helix domain between 199 a.a. and 217 a.a. with significant high scores (775). Although the LpCBF3 amino acid sequence shares conserved NLS, AP2 domains with other species CBF protein family, there is little similarity in the regions corresponding to the activation domain and other regions (Fig.5.1) (Image in this dissertation is presented in color). A glycine to glutamine substitution at amino acid residue 190 was found in the cold-tolerant accession. The amino acid sequence of the LpCBF3 was compared with that of several CBF3-related proteins to understand the evolutionary relationship of the plant *CBF* homologs. A phylogenetic analysis revealed that these CBF3-related proteins were clustered into two distinct groups based on sequence similarity. The first group consists of monocot plants with two most closely related sequences between rice and perennial ryegrass, whereas, the second group consisted of dicot plants (Fig.5.2).

Analysis of LpCBF3 and COR gene expression

The expression pattern of the *LpCBF3* was determined by Northern blotting by using the full-length *LpCBF3* gene as the probe. Total RNA was isolated separately from leaves and stems of the cold-tolerant and sensitive perennial ryegrass accessions after exposure to different cold treatment (4℃) time. The results showed that the expression of *LpCBF*3 is up-regulated for the first 4 hours of cold treatment. At 24 hours, gene expression was below detection in whole plant (Fig.5.5). Due to the presence of at least three potential homologs of *LpCBF*3 gene in the perennial ryegrass genome, the cross-hybridization homologs may lead to obscure specific *LpCBF*3 expression by using Northern analysis. Real time PCR was applied for specifically monitoring the *LpCBF*3 expression in the most cold-tolerant perennial ryegrass accession. The results showed that the *LpCBF3* gene is up-regulated by cold treatment (Fig.5.6). *LpCBF3* transcripts began to increase after 15 minutes of cold treatment and reached a maximum level at 1.5 hours. Later, *LpCBF3* transcripts declined until they were below detection after 24 hours. These results are consistent to the *CBF* gene expression pattern in other plants (Tar'an et al., 2005). To determine expression patterns of *COR* genes which represent a *cbf* downstream gene family, a *COR* gene (*COR39* from wheat) was used as the probe. The highest expression level of the *COR* homologous gene was detected after 24 hours in the leaves and stems of perennial ryegrass accessions. But in the most cold-tolerant perennial ryegrass accessions, the *COR* gene was expressed well without cold treatment and the expression level in the stem was much stronger than the expression in the cold-sensitive perennial ryegrass at all time points tested (Fig.5.7). Cold treatment can prompt expression level of the *COR* homologous gene in both perennial ryegrass accessions.

Analysis of putative promoter of LpCBF3

The *LpCBF3* was further defined by using a primer extension to position the transcription start site with cDNA as template and the putative promoter sequence by using genomic DNA. After analysis of the two putative promoter regions from cold-tolerant and sensitive perennial ryegrass accessions, a conserved motif LTR that can sense the upstream cold signal in barley was found in both accessions. The putative promoter region of cold-tolerant perennial ryegrass accession has three potential conserved boxes, boxIV, boxV and boxVI, which play a critical role in *Arabidopsis CBF* gene expression (Fig.5.3 and Fig.5.4) (Image in this dissertation is presented in color). In *Arabidopsis,* these three boxes are located in a 155-bp upstream region of *CBF2* gene (Zarka et al., 2003). However, in the cold-sensitive ryegrass accession, only two boxes (Box V and BoxVI) were found and BoxIV was missing. The BoxIV contains the MYB motif responsible for cold signal reception.

Tolerance of overexpressed LpCBF3 transgeneic Arabidopsis plants to the freeze test

To determine the function of the *LpCBF3* gene, six transgenic *Arabidopsis* plants having *LpCBF3* gene fused to a cauliflower mosaic virus 35S (CaMV35S) promoter were generated. Three transgenic plants with constitutive expression of *LpCBF3* were found by real time PCR using *LpCBF3*-specifical primers (Fig5.8). Growth characteristics of the 35S:*LpCBF3* transgenic *Arabidopsis* were compared with wild type plants. After the same number of days of vegetative growth at normal temperature (25°C), the transgenic plants showed a "dwarf" phenotype. The size of the leaves and overall dimensions of transgenic plants were considerably less than those of the control plants. Also, there was a substantial difference in time to flowering between the wild type and *LpCBF3*-expressing plants. After nine-week growth, wild type plants bolted and formed flowers well ahead of the transgenic plants period (Fig 5.9) (Image in this dissertation is presented in color). The overexpression of *CBF1* and *CBF3* of *Arabidopsis* has been reported to increase the freezing tolerance of non-acclimated plants (Gilmour et al., 2000; Liu et al., 1998).

Similarly, most the non-acclimated wild type *Arabidopsis* plants were killed by freezing at -6°C after 24 hours. After the freeze test, 5% of the wild type plants survived. However, the transgenic plants showed a high freeze tolerance with 95% plants surviving (Fig 5.10) (Image in this dissertation is presented in color). These results indicated that the over-expressed *LpCBF3* transgenic plants had tolerance to freezing.

Discussion

To improve the winter hardiness of perennial ryegrass or other warm season turfgrass species, a perennial ryegrass *CBF* homolog from a most cold-tolerant perennial ryegrass accession (PI598441) was isolated, sequenced, characterized and overexpressed in *Arabidopsis* (Bughrara et al., 2005). Recently, another *LpCBF*3 gene from perennial ryegrass cultivar Caddyshack was isolated (Xiong and Fei, 2006). The 2 genes share more than 74% identities at the amino acid levels. Considerable differences can be observed in the non-AP2 regions.

In this study, we found that the deduced amino acid sequence of LpCBF3 shared high similarities to the other plant species CBF families. In *Arabidopsis*, CBF3 polypeptides contain a 66-amino acid motif, an AP2 domain, which is evolutionarily conserved in plants (Xue, 2003) and has been described as a DNA-binding domain. The CBF3 gene has potential nuclear localization sequences in its N-terminal regions, and a putative acidic activation domain that is less conserved. Differences in N- and C-terminal acidic regions among plant *CBF* gene families may contribute to the *CBF* gene species specificity. Perennial ryegrass LpCBF3 has a LWSY motif at the end of C-terminal region which was observed in several CBF3-related proteins. In addition, the potential protein kinase phosphorylation sites, Arg-rich and Ser-rich sites were found in LpCBF3.

The *LpCBF*3 allele from cold-sensitive perennial ryegrass accession was isolated, sequenced and compared with *LpCBF*3 of cold-tolerant plant. Surprisingly, only one amino acid difference in the putative acidic activation domain was detected. A glycine to glutamine substitution at amino acid residue 190 was found in the cold-tolerant accession. Without further association between genotypic and phenotypic variations in segregation population derived from cold-resistant & sensitive perennial ryegrass accessions, no definitive conclusions can be made that these variations of *LpCBF3* alleles result in the expression changes of their downstream genes, which in turn affect freeze tolerance. The eco-TILLING (Targeting Induced Local Lesions In Genomes)(Comai et al., 2004) provides a method for further studying allelic variation of *LpCBF3* gene from of different perennial ryegrass accessions that exhibit different lethal temperatures.

The variations in promoters may suggest the possibility that genes might differ slightly in their expression patterns and levels, which can further be amplified and lead to phenotypic changes by a cascade of transcriptional regulation events. A 500bp putative promoter region of *LpCBF3* was isolated. Some cold response-related motifs were found in this promoter region. At -160bp, a cis-acting element involved in low-temperature responsiveness (LTR) is sharing 100% similarities with barley promoter. There are three key conserved motifs in *Arabidopsis CBF3* gene promoter which is extended about 150bp. These motifs also were found in *LpCBF*3 gene promoter. After comparing tolerant and sensitive perennial ryegrass accessions, we found considerable differences in their putative promoter regions, including three key motifs mentioned above. From the evolution perspective, adjusting gene expression levels or patterns by tuning *cis*- and *trans*-elements interactions is the most efficient and accurate method to affect gene function in different ecosystems. Studying the expression diversity of promoters may provide more information on evolution than gene coding sequence in some cases. Wang and his colleagues (1999) provide an excellent example of alterations in the regulation of *tb1* promoter brought about the change from teosinte to maize plant architecture.

Expression analyses revealed that *LpCBF*3 is positively regulated by cold treatment. The accumulation of *LpCBF*3 transcripts increases drastically after cold treatment, and reaches the highest level after a couple of hours of low temperature (4°C) exposure, followed by decreases to below detection after 24 hours. This indicates that the responsiveness of *LpCBF*3 to low temperature is an early event that is consistent with the temporal induction of other plant *CBF* genes. The Northern analysis shows the expression of *CBF*-induced perennial ryegrass *COR* homologous gene(s) continues to increase during the 24-hour cold treatment. At 24-hour after cold treatment, the highest expression was detected in the leaves and stems from both tolerant & sensitive perennial ryegrass accessions. The *COR* gene from stem of cold-tolerant perennial ryegrass' stem can strongly express even without cold induction (0-hour), although it still increases its expression level during cold treatment period. *COR* gene has a role in freezing tolerance which encodes a polypeptide target to chloroplasts (Artus et al., 1996). The expression level and pattern of *COR* gene in the stem of cold-tolerant perennial ryegrass accession may contribute to its cold tolerance resulting from the *COR* encoded protein stabilizing membranes against injury. A slight expression level of *LpCBF3,* which cannot be detected by Northern blotting, was detected by RT-PCR even without cold treatment and mechanical agitation. We deduce that the *COR* gene of cold-tolerant perennial ryegrass can be activated by CBF at a very low concentration level and this scenario would not happen in cold-sensitive material. Other factor(s) besides CBF can regulate the *COR* homolog expression (Chinnusamy et al., 2003). The discovery of a link between *COR* gene expression and CBF is of potential agronomic importance for improvement of cold tolerance (Mastrangelo et al., 2000).

Determining the molecular mechanisms that plants have evolved to survive freezing would not only increase our fundamental knowledge of how plants adapt to changes in the environment, but would also contribute to the development of new strategies to improve the tolerance of crop species to adverse cold. A large number of genes are involved in cold-response physiology process, but only a few of them, as transcriptional factors, can produce a global effects on cold tolerance. To compare the function of perennial ryegrass *LpCBF3* with that of *Arabidopsis CBF3* gene, transgenic plants were generated in which the *LpCBF3* cDNA was over-expressed under the control of the CaMV 35S promoter. Constitutive expression of *LpCBF3* gene in transgenic *Arabidopsis* may result in multiple biochemical changes associated with cold acclimation. In addition to an increase of cold tolerance, the transgenic plant displayed some ‘side-effects’ such as a dwarf, delay flowering, and stunting. However, these phenotypes are desired in turfgrass breeding program, which will directly lead to improved turfgrass with low maintenance requirements and cold-tolerant characteristics.

Figure 5.1. Comparison of CBF3 amino acid sequence from ryegrass, rice, barley, maize and Arabidopsis. Amino acids are designed in single letter code. The red color letter indicates identical, the blue color indicates closely related. Black bold line indicates the NLS and red bold line indicates the AP2. This figure was created by Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Ryegrass (*LpCBF3*), Rice (AAN02486), Barley (AAG59618), Maize (AAN76804) (Image in this dissertation is presented in color).

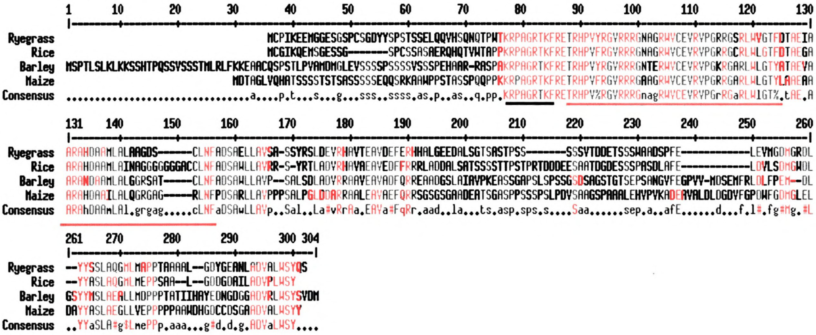


Figure 5.2 Comparison of amino acid sequences of the CBF gene family by using CLUSTALW. Scale indicates branch lengths.

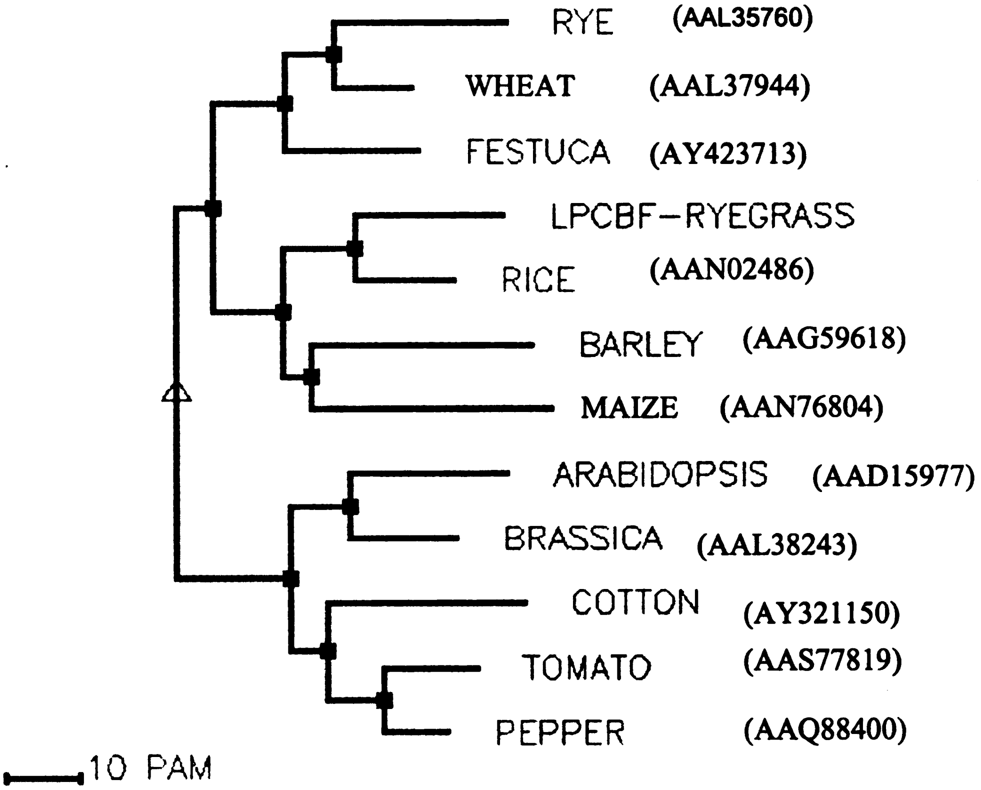


Figure 5.3 Putative *LpCBF3* gene promoter sequence compared with that of Arabidopsis. BoxIV, BoxV and BoxVI are critical motifs for Arabidopsis cbf2 expression after cold treatment. ICEr1 and ICEr2 are ICE gene binding sites.

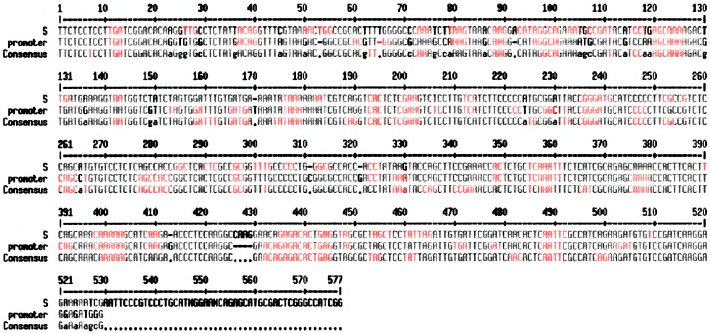
```
          1       10        20        30        40        50        60        70        80        90       100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
 Promoter TTCTCCTCCTTGATCGGACACAGGTGTGGCTCTATGACAGGTTTAGTAAGACGGCCGCACGTTGGGGCGCAAAGCCAAAGTAAGCAAGGCATAGGCAGAAAATGCGATACGTCCAAAGCAAAAGACGTGA
    BoxIV                GGACACATGTCAGA
     BoxV                                                                                    ACGTGGCATT
     TATA                  BoxIV
    BoxVI                  ICEr1                                                              BoxV
Consensus ..................................................................................................................................

          131    140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
 Promoter TGGAAGGTAATGGTCGTTCTAGTGGATTTGTGATGATAAATATAAAAAAATCGTCAGGTCACTCTCGAAGTCTCCTTGTCATCTTCCCCCTTGCGGCTTACCGGGATGCATCCCCCTTCGCCGTCTCCAG
    BoxIV
     BoxV
     TATA
    BoxVI
Consensus ..................................................................................................................................

          261    270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
 Promoter CCTGTGTCCTCTCAGCCACCGGCTCACTCGCCGCGGTTTGCCCCCTGCGGCGCCACCGACCTATAAATACCAGCTTCCGAAACCACTCTGCTCAAATTTCTCATCGCAGAGCAAAACCACTTCACTTCAG
    BoxIV               BoxVI
     BoxV               ICEr2
     TATA                                                              TATAAAT
    BoxVI               ACTCCG
Consensus ..................................................................................................................................

          391    400       410       420       430       440       450       460       470       480       490      499
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+--------|
 Promoter CAAACAAAAAGCATCAAGAGACCCTCCAAGGCGAACAGAGACACTGAGGTAGCGCTAGCTCCTATTAGATTGTGATTCGGATCAACACTCAATTCGCCATCAGAAGATG
    BoxIV
     BoxV
     TATA
    BoxVI
Consensus .............................................................................................................
```

Figure 5.4 Promoter sequence comparison between that from cold-tolerant and cold-sensitive ryegrass accessions. S means cold-sensitive promoter and promoter means cold-tolerant promoter. The red color letter indicates identical nucleotide; the blue color indicates different nucleotide (Image in this dissertation is presented in color).



```
          1       10        20        30        40        50        60        70        80        90        100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
        S TTCTCCTCCTTGATCGGACACAAGGTTGCCTCTATTACAGGTTTCGTAAAACTGGCCGCACTTTTGGGGCCCAAATCTTAAGTAAACAAGGACATAGGCAGAAATGCCGATACATCCTGAGCAAAAGACT
 promoter TTCTCCTCCTTGATCGGACACAGGTGTGGCTCTATGACAGGTTTAGTAAGAC-GGCCGCACGTT-GGGGCGCAAAGCCAAAGTAAGCAAGGG-CATAGGCAGAAAATGCGATACGTCCAAAGCAAAAGACG
Consensus TTCTCCTCCTTGATCGGACACAaGggTGcCTCTATgACAGGTTTaGTAAaAC.GGCCGCACgTT.GGGGCcCAAAgCcaAAGTAAaCAAGG.CATAGGCAGAAAagcCGATACaTCCaaAGCAAAAGACg

          131     140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
        S TGATGAAAGGTAATGGTCTATCTAGTGGATTTGTGATGA-AAATATAAAAAAATCGTCAGGTCACTCTCGAAGTCTCCTTGTCATCTTCCCCCATGCGGATTACCGGGATGCATCCCCCTTCGCCGTCTC
 promoter TGATGGAAGGTAATGGTCGTTCTAGTGGATTTGTGATGATAAATATAAAAAAATCGTCAGGTCACTCTCGAAGTCTCCTTGTCATCTTCCCCCTTGCGGCTTACCGGGATGCATCCCCCTTCGCCGTCTC
Consensus TGATGaAAGGTAATGGTCgaTCTAGTGGATTTGTGATGA.AAATATAAAAAAATCGTCAGGTCACTCTCGAAGTCTCCTTGTCATCTTCCCCCaTGCGGaTTACCGGGATGCATCCCCCTTCGCCGTCTC

          261     270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
        S CAGCATGTGTCCTCTCAGCCACCGGCTCACTCGCCGCGGTTTGCCCCCTG-GGCGCCACC-ACCTATAAGTACCAGCTTCCGAAACCACTCTGCTCAAATTTCTCATCGCAGAGCAAAACCACTTCACTT
 promoter CAGCCTGTGTCCTCTCAGCCACCGGCTCACTCGCCGCGGTTTGCCCCCTGCGGCGCCACCGACCTATAAATACCAGCTTCCGAAACCACTCTGCTCAAATTTCTCATCGCAGAGCAAAACCACTTCACTT
Consensus CAGCaTGTGTCCTCTCAGCCACCGGCTCACTCGCCGCGGTTTGCCCCCTG.GGCGCCACC.ACCTATAAaTACCAGCTTCCGAAACCACTCTGCTCAAATTTCTCATCGCAGAGCAAAACCACTTCACTT

          391     400       410       420       430       440       450       460       470       480       490       500       510       520
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
        S CAGCAAACAAAAAGCATCAAGA-ACCCTCCAAGGCCAAGGAACAGAGACACTGAGGTAGCGCTAGCTCCTATTAGATTGTGATTCGGATCAACACTCAATTCGCCATCAGAAGATGTGTCCGATCAAGGA
 promoter CAGCAAACAAAAAGCATCAAGAGACCCTCCAAGGC----GAACAGAGACACTGAGGTAGCGCTAGCTCCTATTAGATTGTGATTCGGATCAACACTCAATTCGCCATCAGAAGATGTGTCCGATCAAGGA
Consensus CAGCAAACAAAAAGCATCAAGA.ACCCTCCAAGGC....GAACAGAGACACTGAGGTAGCGCTAGCTCCTATTAGATTGTGATTCGGATCAACACTCAATTCGCCATCAGAAGATGTGTCCGATCAAGGA

          521     530       540       550       560       570    577
          |--------+---------+---------+---------+---------+------|
        S GAAAAATCGAATTCCCGTCCCTGCATNGGAANCAGAGCATGCGACTCGGGCCATCGG
 promoter GGAGATGGG
Consensus GaAaAagcG................................................
```

Figure5.5. Expression of *CBF* gene(s) in perennial ryegrass by northern blotting. Plant materials were 2 months old grown in a 16-h photoperiod at 25°C. The total RNAs were isolated from stems and leaves at different times (0, 1, 4, 24 hr) of cold treatment (4°C). The bottom is total RNA stained with ethidium bromide.

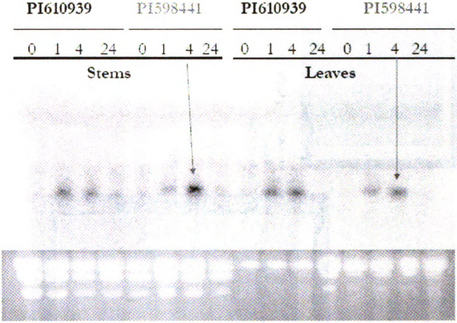


Figure5.6 Expression pattern of *LpCBF3* in perennial ryegrass by RT-PCR analysis. Plants (PI598441) are 2 months old growing with 16-h photoperiod at 25°C. The total RNAs were isolated from whole plants of different times at cold treatment (4°C). The perennial ryegrass actin expressions act as the control.

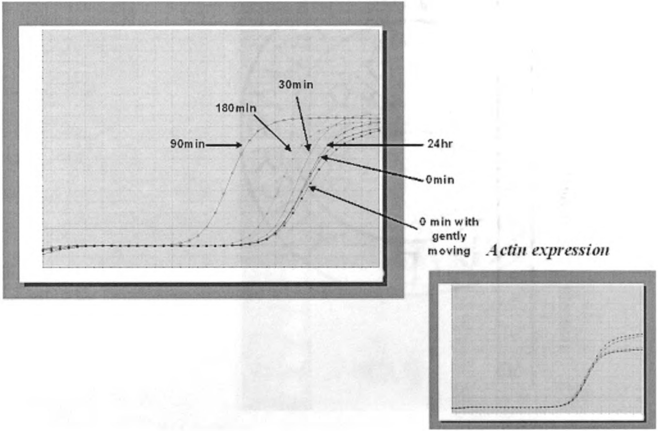


Figure5.7. Expression of *COR39* orthologue gene in perennial ryegrass. Plant materials were 2-month old growing with 16-h photoperiod at 25°C. The total RNAs were isolated from stems and leaves at different times (0, 1, 4, 24 hr) of cold treatment (4°C). The bottom is total RNA stained with ethidium bromide.

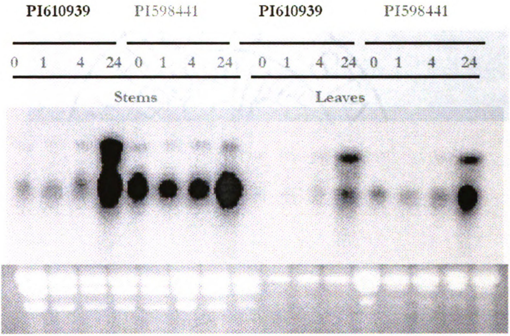


Figure5.8 Overexpressing *LpCBF3* gene in *Arabidopsis* detected by RT-PCR with specific primers. WT is Columbia, A1-A6 are T3 transgenic *Arabidopsis*. Only transgenic *Arabidopsis*, A1, A3 and A4, can constitutively express *LpCBF3*.

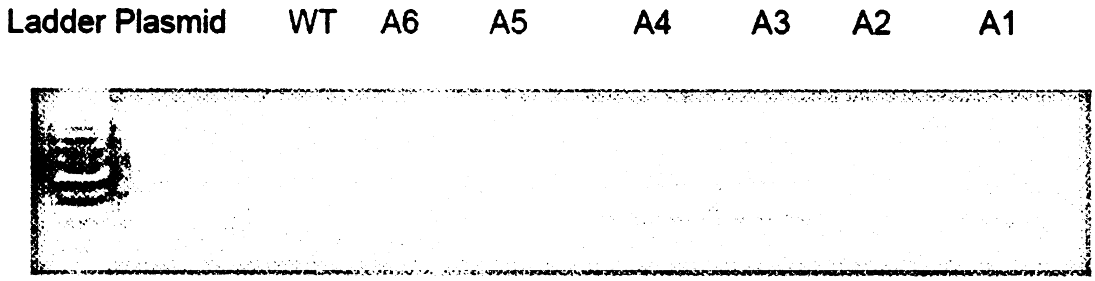


Figure5.9 Growth characteristics of *LpCBF3*-expressing transgenic *Arabidopsis* A1-3 (T3) with wild type (Columbia).

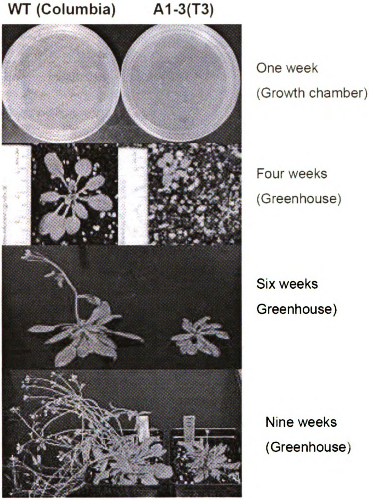
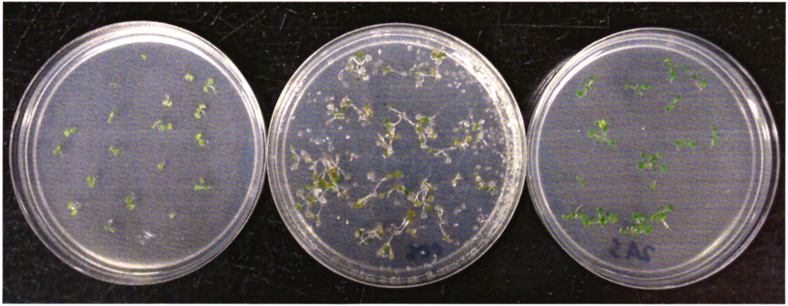


Figure5.10 Effect of *LpCBF3* overexpression on plant freezing tolerance. Seedlings of control Arabidopsis Columbia (Center, 12 days old), *LpCBF3*-expressing A1 (Left, 12 days old) and *LpCBF3*-expressing A1 (Right, 20 days old) were grown at 20°C on solid medium and then frozen at -2°C for 24 h followed by 24 hr at -6°C(Image in this dissertation is presented in color).



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APPENDIX A. GENETIC RELATIONSHIPS AMONG SNOW MOLD RESISTANT CLONES OF CREEPING BENTGRASS

Abstract

Creeping bentgrass (*Agrostis stolonifera*) is the most important turfgrass species for golf courses in temperate climate regions with prevalent gray snow mold disease in winter. The aim of this study was undertaken to evaluate the genetic diversity among 18 snow mold resistant clones and 4 creeping bentgrass commercial cultivars. Meanwhile, the phylogenetic relatedness of creeping bentgrass and other three bentgrass species, colonial bentgrass (*A. capillaris*), dryland bentgrass (*A. castellana*), and velvet bentgrass (*A. canina*) was elucidated. A total of 199 polymorphic AFLP markers were amplified from six selected primer combinations. Both principal component analysis (PCA) and unweighted pair group method with arithmetic mean (UPGMA) dendrogram distinguished three groups, which are consistent with genome compositions of these bentgrass species. Cluster analysis of genetic similarity estimates revealed a high level of diversity among bentgrass species ranged from 0.43 to 0.90 with an average of 0.55. In 18 disease-resistant creeping bentgrass clones, genetic similarities ranged from 0.55 to 0.90 with an average of 0.61. The mean of genetic similarity among the four creeping bentgrass commercial cultivars is 0.67.

Key words: Creeping bentgrass, Bentgrass species, Snow mold resistance, Diversity, AFLP

Introduction

Bentgrass species (*Agrostis* spp.), native to Western Europe, are cool season turfgrasses. Distribution of the species spans shade to open habitats, low to high lands and cool to extremely arctic areas (Harlan, 1992). The bentgrass species are primarily deployed in putting green, golf courses, parks and forage. There are over 200 species of bentgrass with five species predominately used commercially in the United States (Hitchcock, 1951). These include creeping bentgrass (2n=4x=28, A_2A_2SS), colonial bentgrass (2n=4x=28, $A_1A_1A_2A_2$), Dryland bentgrass (2n=4x=28, $A_1A_1A_2A_2$), velvet bentgrass (2n=2x=14, A_1A_1) and redtop bentgrass (2x=6x=42, $A_1A_1A_2A_2SS$) (Jones, 1956a; Jones, 1956b; Jones, 1956c). Creeping bentgrass has high commercial value due to its fine texture and adaptation to mowing heights as low as 3mm, and commonly used in golf course, putting green, tees and fair ways. It has high commercial value. However, all commercial creeping bentgrass cultivars are susceptible to gray snow mold, caused by *Typhula incarnata* (Wu and Hsiang, 1998). Gray snow mold is prevalent in cold, humid conditions, especially under snow cover. This disease is particularly important in northern regions of the United States where it causes significant economical loss (Wang et al., 2005). Fungicides are traditionally used to inhibit this pathogen on the golf courses. However, fungicides are expensive, limited efficacy and adversely affect to the environment. In addition, some fungi pathogens have developed resistance to fungicides after years of repeated applications (Reicher and Throssell, 1997). The deployment of disease resistant cultivars is economically and ecologically attractive. Exploring the resistant resources in creeping bentgrass species is the first step to develop the desirable and disease-resistant cultivars. To identify such resistant resources, we collected one thousand of creeping bentgrass clones with potential gray snow mold resistance from old Northern Michigan golf courses that have not been sprayed with fungicide or over-seeded for the last 10 years. Because creeping bentgrass populations of old golf courses were under a high degree selection of disease (Casler et al., 2003), some clones which may contain the resistant genes derived from natural mutations would be proving to survive and thrive. After two-year repeat artificial snow mold disease screening, eighteen gray snow mold resistant clones were identified. Exploiting and utilizing resistance genes from these clones could be practiced for resistance improvement in bentgrass cultivars, which eliminates the extraordinary efforts of interspecific hybridization for utilizing resistance from related species.

A comprehensive study to clarify the phylogenetic relationship, and reveal genetic diversity among creeping bentgrass resistance clones would be desirable to facilitate the use of resistance resources for improvement in this species by eliminating the duplication of germplasm collection. Estimates of genetic diversity based on molecular markers have proven a useful method for describing phylogenetic relationship and distinguishing heterotic groups within plant species because of their abundant polymorphism and the fact they are independent of environmental effect (Hall et al., 2002) (Bai et al., 2003) (Kress et al., 2005). Two basic types of molecular markers are applied for evaluating the extent of genetic diversity in plant. They are (1) hybridization-based, restriction fragment length polymorphism (RFLP) (Dijkhuizen et al., 1996), and (2) amplification-based, microsatellite or simple sequence repeat (SSR) (Santacruz-Varela et al., 2004), random amplified polymorphic DNA (RAPD) (Hollman et al., 2005), amplified fragment length polymorphism (AFLP) (Miklas et al., 2001) (Spooner et al., 2005). AFLP combines the advantages of RFLP and PCR to provide greatly enhanced performance in terms of reproducibility, resolution, and polymorphism detection on the whole genome level. AFLP technique permits analysis of a subset of restriction fragments from a complete digest of genomic DNA, followed by amplification of a subset of the restriction fragments using PCR with specific primers. It has been applied increasingly for genetic diversity research, and is especially suitable for evaluating genetic diversity among turfgrass species in which no prior DNA sequence information is available (Vergara et al., 2004) (Mian et al., 2002).

The objectives of this study were to assess the genetic diversity within snow mold resistant clones and four commercial creeping bentgrass cultivars, and to quantify the genetic relatedness among creeping bentgrass and other three bentgrass species based on AFLP marker profiling.

Materials and Methods

Plant materials

Eighteen gray snow mold resistant clones (designed to MSU#) of creeping bentgrass were identified by disease evaluation from 1000 candidate clones collected from four Michigan golf courses. These clones were grown in the greenhouse of Michigan State University (East Lansing, Michigan). Four disease susceptible clones designed as S# were included in this experiment. Creeping bentgrass PIs (PI204390 and PI221960), colonial bentgrass PIs (PI234685 and PI171470), dryland bentgrass PIs (PI240131 and

PI240132) and velvet bentgrass (PI194697 and PI230233) were obtained from the USDA Regional Plant Introduction Station (Pullman, Washington). Four cultivars 'ASR368', 'PENN A4', 'PENN G2', and 'Providence' were included in this study. 'ASR368' is a transgenic glyphosate-resistant creeping bentgrass plant provided by Scotts Company (Marysville, OH). The creeping bentgrass plants were resistant to glyphosate via the expression of a gene *cp4-epsps,* which encodes a glyphosate-resistant form of EPSP from the CP4 strain of *Agrobacterium* species. The procedure used for generating the transgenic plants is described by Hartman et al (1994) and Lee (1996).

AFLP analysis

Total genomic DNA was extracted from fresh leaves of clones or 25 plants per PI using the protocol of Sharp et al. (Sharp et al., 1989), and was quantified by DYNA Quant 200 Fluorometer (Pharmacia Biotech, San Francisco, CA). Approximately 200 ng of DNA was digested with *Eco*RI and *Mse*I. The AFLP protocol applied in this research was as described by Vos et al. (Vos et al., 1995) with some modifications. Preamplification was conducted on PTC-100 thermal cycler (MJ Research, Waltham, MA) using 30 cycles of 94°C ,45s, 54°C ,45s, 72°C, 1min, followed by elongation at 72°C ,10min. The condition of selective amplification PCR was 1 cycle for 94°C, 30 sec; 65 °C, 30 sec; 72 °C, 1 min; followed by 11 cycles for 94 °C, 30sec; 65 °C, 30 sec; 72 °C, 1 min, with decreasing temperature of the second step by 0.7 °C for each cycle; 23 cycles for 94 °C, 30 sec; 56 °C, 30 sec; 72 °C, 1 min, followed by 1 cycle for 72 °C, 15 min. Adapters and pre-primers used include *Mse*I adapter, *M00* (universal primer), *Eco*RI adapter, *E00* (universal primer), as listed in Table 2. 2. Selective primers are E-ACA/M-

CAT, E-ACA/M-CAG, E-ACA/M-CGG, E-ACA/M-CGA, E-AGC/M-CAT, E-AGC/M-CGG. The PCR products from the other *Eco*RI /*Mse*I primer combinations were separated on 5% denaturing polyacrylamide gels at a constant 2000V for 3h at 60°C and were visualized with silver staining following the protocol of the Promega silver DNA sequencing system™ (Promega Inc., Madison, WI).

Data Analysis

Bands that showed clear polymorphisms were scored visually as present ('1') or absent ('0'). Bands of different electrophoretic mobilities were assumed to be non-allelic, while bands of the same mobility were assumed to be allelic. Some ambiguous bands were ignored. Genetic diversity analyses were conducted by using Numerical Taxonomy and the Multivariate Analysis System, NTSYSpc v.2.2 (Rohlf 1993). Genetic similarities based on Jaccard's coefficients were computated among all possible pairs with the SIMQUAL option and ordered in a similarity matrix. The similarity matrix was run by Sequential, Agglomerative, Hierarchical, Nested clustering (SAHN) (Sneath and Sokal 1973) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as an option (Sokal and Michener 1958). The dendrogram and cluster groupings were constructed by the UPGMA clustering algorithm from the SAHN option of NTSYSpc v2.2. Also, cluster analysis was based on similarity matrices using the UPGMA program in the WIN BOOT software package with a sample number of 100. Cophenetic correlation was calculated to measure goodness of fit between the similarity and cophenetic matrices. Principle coordinate analysis was run with the Common Principal Components Analysis (CPCA) option (NTSYS) to identify the number of groups based on eigenvectors. The principal coordinates' analysis result was displayed by the Mod3D plot module. Correlation between the two matrices obtained with two marker sets was estimated by means of the Mantel matrix correspondence test (Mantel 1967).

Results

Genetic relationship among the bentgrass species

The six AFLP primer combinations produced 199 polymorphism bands ranging in size from 150 to 500bp. The 35 genotypes were clustered into three major groups on the basis of the UPGMA dendrogram of similarity coefficients with the high cophenetic correlation coefficient value (r=0.96) (Fig. A. 1). Principal coordinate analysis with the same data set supports the above cluster analysis. These three groups closely corresponded with the genome compositions of these species (A_2A_2SS, $A_1A_1A_2A_2$, and A_1A_1). Group A had creeping bentgrass species with the genome composition, A_2A_2SS. In this group, twenty-three creeping bentgrass genotypes, four cultivars and two PIs were included. Group B consisted of two different bentgrass species, colonial bentgrass and dryland bentgrass, which have the same genome compositions ($A_1A_1A_2A_2$). In this group, two colonial bentgrass PIs were separated from two dryland bentgrass PIs, which leads to form two subgroups. The group C included velvet bentgrass PIs with genome composition (A_1A_1). From broader aspect, the group B and group C can be clustered because they share a common genome of A_1 as shown in the Jone's research (Jones, 1956a). However, groupA and groupC were well separated due to no common genome shared each other.

Genetic relationship within the creeping bentgrass species

Group A consisted of the creeping bentgrass species. Genetic similarities coefficients (Jaccard) for pair-wise comparisons ranged from 0.47 to 0.90 with a mean of 0.59. Group A was separated to two subgroups. The first subgroup included the clones from old golf courses and four commonly-used commercial cultivars. The genetic similarities of four cultivars, 'ASR368', 'PENN A4', 'PENN G2', and 'Providence', ranged from 0.68 between 'Providence' and 'PENN G2' to 0.66 between 'ASR368' and 'Providence' with a mean of 0.67. 'ASR368' is separated from other three recent cultivars, 'PENN A4' (released in 1995), 'PENN G2' (released in 1995), and 'Providence' (released in 1987), which grouped together. Four susceptible controls, S39, S13, S21 and S7 were randomly distributed in this subgroup, No obvious genetic classification based on gray snow mold resistance and susceptibility was established. The resistant clones shown a broader diversity (average genetic similarities coefficient=0.61) comparing to the selected cultivars in this study. The MSU#8 showed the greater diversity to commercial cultivars comparing to other resistant clones. The grouping of these clones did not reflect their geographic distribution of the golf courses. The second subgroup consisted of two PIs, PI 204390 from Turkey and PI 221960 from Afghanistan.

Heterogeneous of the creeping bentgrass clone

Creeping bentgrass populations in old golf courses may contain considerable amounts of genetic variation because of their perennial growth habits and stoloniferous growth. To test the heterogeneous of single selected creeping bentgrass clone, one resistant clone, MSU# 247, was divided into two copies designed as MSU#247A and MSU#247B. These two copies were grown, isolated DNA, and conducted AFLP analysis, separately.

Thirteen different bands were detected between MSU#247A and MSU#247B representing 10% of the total number of bands (132) amplified by the six AFLP primer combinations.

Discussion

The bentgrass species has been described as one of the most difficult and complicated of the grass genera from the taxonomic of view (Casler, 2002). The observations of chromosome paring in pollen mother cell and genomic *in situ* hybridization (GISH) can determine their genome composition (Chen et al., 1998). Jones (1956a, 1956b, 1956c) conducted the cytogenetic research of some bentgrass species for elucidating their genome compositions by meiotic chromosome pairing, and designed their genomes. Also, in our previous study, seven bivalents, some univalents and multivalents were observed in the interspecific hybrids between creeping and colonial bentgrass, which shown a common genome shared between these two species. Determination of genome composition by observing the chromosome pairing and GISH is based on the similarity of chromosome composition. However, both methods are time-consuming and require extensive work. The strong hybridization barriers that exist between two species prevent the development of F_1 plants. GISH can be carried out to provide genome composition information. However, interference of homeologous chromosomes can produce the ambiguous results. Diversity analysis of AFLP provides one of promising approaches to elucidate genome composition relationship in the not well-studied and complex genus. In this study, the three groups derived from AFLP markers were consistent with the genome compositions, which offer the alternative method to determine genome relationship. The species lack of genome composition information can be initially clustered into the certain groups accompanying other species with known genome compositions. The cytogenetic research can be applied to further confirm the cluster results.

The dryland and colonial bentgrass species share the same genome composition, and can not be easily differentiate by traditional cytogenetic methods. By using AFLP, these two species were clustered into the different groups. A better understanding of genome relationship between these two species may facilitate the exploitation and incorporation of the desirable characteristics in colonial and dryland bentgrass breeding.

Four commonly-used commercial cultivars were evaluated. The 'ASR368' shared least genetic similarity with new released creeping bentgrass cultivars ('PENN A4', 'PENN G2', and 'Providence'). The newer cultivars were found having narrow genetic diversity, because they have selected as being unique. The low level genetic variation in new released cultivars may result in a bottleneck in future breeding work. The AFLP data showed 18 resistant clones with broader genetic diversity than cultivars, and can severe as a source to improve the cultivar resistance and genetic bases. However, all of the clones were not immune to disease. Inter-pollination between these clones based on the classification results may be deployed to develop higher resistance by pyramiding different sources of resistance and optimizing the resistant gene combinations. Above strategy is successfully applied in the wheat resistance breeding. Wheat scab resistant cultivar, Sumai3, is derived from the two moderate resistance lines, Funo and Taiwan wheat (Bai and Shaner, 1994). This is a successful example of integration of diverse genetic sources to broaden genetic diversity, and enhance disease resistance. One of 18 resistant clones, MSU# 8, was found to be separated from other clones based on genetic distance. It will be used as the parent to construct the mapping population aiming to uncover the QTL linked to gray snow mold resistance in creeping bentgrass species. QTL mapping of different source genes could further facilitate accurate classification of these resistant genes, and improve the breeding efficiency to develop a resistant cultivar to snow mold in near future.

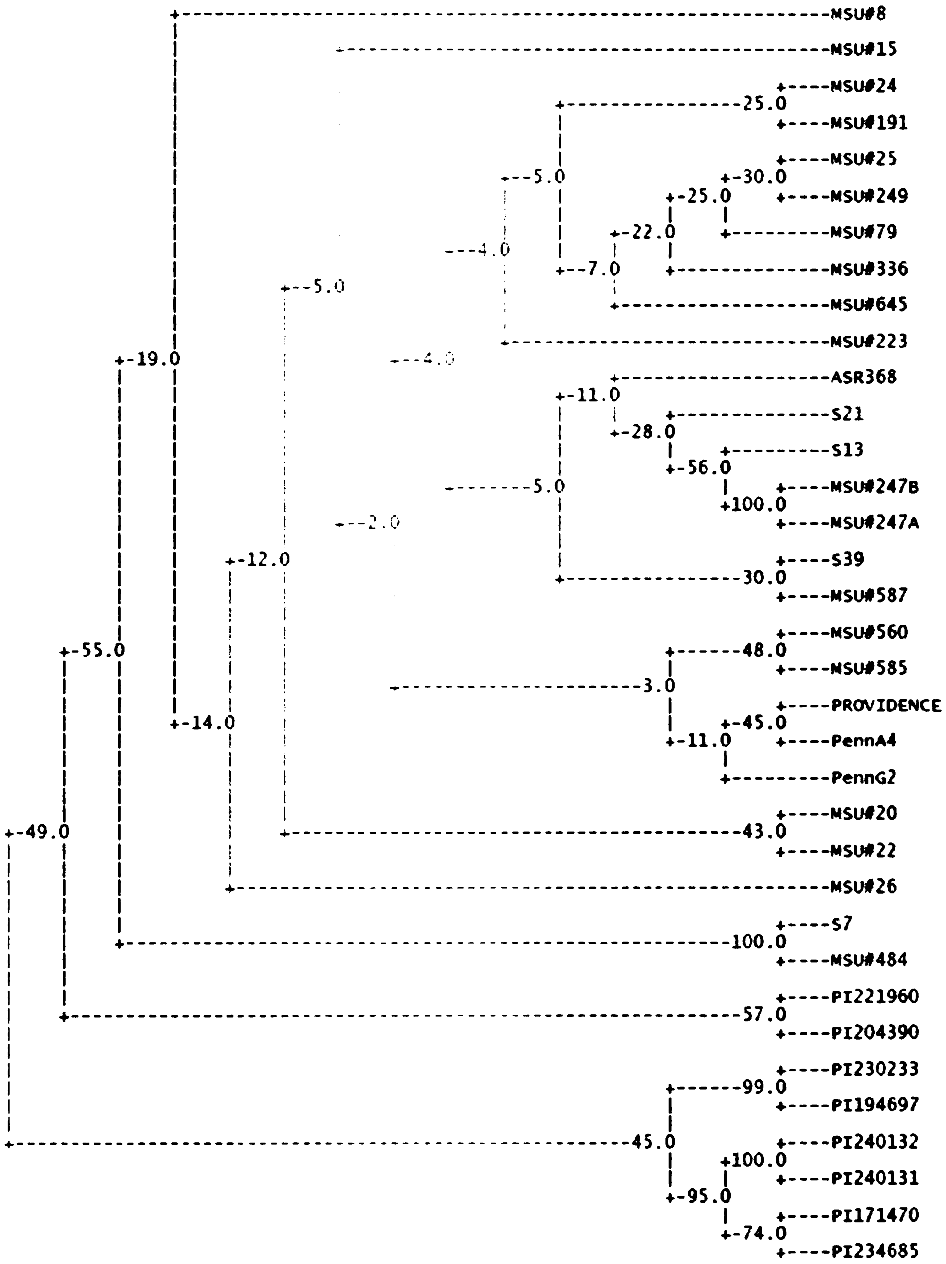

Fig A. 1 The unweighted pair group method with the arithmetic mean dendrogram of 35 genotypes from 199 amplified fragment length polymorphism markers. Bootstrap values obtained from 1000 replicate analyses.

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