Hikmet Budak · German Spangenberg
Editors

Molecular Breeding of Forage and Turf

The Proceedings of the 8th International Symposium on the Molecular Breeding of Forage and Turf

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Preface

Grasslands in the form of both forage and turf are the life support of the planet providing sustenance to wildlife, livestock, and thus to humans. They anchor the soil preventing erosion, harnessing the freshwater resources, and creating an environment for outdoor sports, recreation, and entertainment. They also serve to beautify our environment along with their physical and nutritional value. Bioenergy is also obtained from the grasslands and they have recently become of all the more importance for providing biofuel alongside food. In recent years however this useful resource has been afflicted with environmental insults such as increasing drought spells globally and it is important to further manipulate this resilient and versatile resource for human benefit.

Previously, the 6th MBFT Symposium was held in Buenos Aires, Argentina and the 7th MBFT Symposium was held in Salt Lake City, USA. The 8th International Symposium on Molecular Breeding of Forage and Turf was held in Sabanci University, Istanbul, Turkey. From amongst the attendees there were scientists from 15 countries from all fields of plant biology including geneticists, and molecular biologists as well as breeders and agronomists. The meeting encompassed oral presentations from leading scientists on molecular plant breeding, a surmounting number of diverse poster presentations and also tours of the historic Old Istanbul City and a boat tour of the Bosphorus Strait.

This book features papers from oral presentations of the symposium. It extensively covers the various themes discussed along with definitive reports shedding light on recent developments in systems biology, functional genomics, and application of molecular breeding in forage and turf.

The 8th MBFT Symposium and the publication of all its proceedings in this book, *Molecular Breeding of Forage and Turf*, have been supported by the Bioengineering and Biological Sciences Program, Faculty of Engineering and Natural Sciences, Sabanci University; the Samuel Roberts Noble Foundation, and the Scientific and the Technological Research Council of Turkey.

We thank once again the International and Local Organizing Committees for their efforts in making the symposium such a great success.

Lastly we also thank our authors for their relentless effort and work contributing to the publication of the *Molecular Breeding of Forage and Turf.*

September 2014

Prof. Dr. Hikmet Budak
Prof. Dr. German Spangenberg

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Chapter 1
Deciphering Drought Tolerance in Tall Fescue [*Lolium arundinaceum* (Schreb.) Darbysh.]

Malay C. Saha, S.K. Talukder, P. Azhaguvel, S. Mukhergee and K. Chekhovskiy

1.1 Introduction

Drought is the single most important constraint to crop productivity causing yield loss up to 50 % or more (Boyer 1982). Recently, severe droughts have been prevailed in the Southern Great Plains of the USA. During the unprecedented drought in 2011, Oklahoma experienced driest 4 months since 1921 and accounted for $ 1.6 billion losses in the drought-related agriculture. Agricultural losses in Texas were estimated $ 7.62 billion that made 2011 the costliest drought to date. Tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] is an important cool season hay and pasture grass grown in over 14 million ha in the USA (Sleper and West 1996). The persistence of this perennial grass largely depends on their ability to tolerate drought stress. Tall fescue cultivars do not generally persist for more than 2–4 years in the south central USA due to drought stress (Hopkins 2005). Thus, developing drought-tolerant cultivar is the key strategy for improving productivity and persistence of the Continental tall fescue in the region.

Drought tolerance is difficult to select for because of low heritability occasioned by nonuniform testing conditions and large genotype-by-environment interactions. Simple, reliable, and repeatable measures of drought tolerance can facilitate rapid screening of large number of genotypes to identify superior types. Relative water content (RWC) is a measure of plant water status in terms of physiological cellular water deficit (Barrs and Weatherly 1962). Increased RWC under water deficit is

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associated with the increased drought tolerance. Increased osmotic potential (OP) under water stress has been linked to tissue survival and regrowth. Both RWC and OP could be used as assays to identify drought-tolerant tall fescue genotypes from natural populations (Elmi and West 1995).

Various physiological, metabolic, and defense mechanisms are activated during drought stresses, which make a plant to survive and/or maintain growth and reproduction (Valliyodan and Nguyen 2006). Though various genes and signal transduction involved with drought tolerance have been studied in different species (Chinnusamy et al. 2004; Li et al. 2012), no information is available in tall fescue. Currently, there have been only 63,853 tall fescue ESTs available in the gene bank database. Thus, the objectives of our projects are to: (i) develop a rapid and reliable drought screening protocol for tall fescue germplasms, (ii) genotype and phenotype a mapping population to identify QTL associated with important drought-related traits, and (iii) identify drought-tolerant key genes and genetic factors in tall fescue through transcriptome profiling.

1.2 Materials and Methods

A total of 1000 genotypes of the Noble Foundation Continental tall fescue breeding population PDF584 (released as "Texoma MaxQ II") were evaluated under well watered and drought stressed conditions in the Foundation's greenhouse. These plants were clonally propagated into two replicates. The temperature was set at 24 °C and the photoperiod was maintained 16 h. For each genotype, three fully developed collared leaves were collected from three tillers in each replicate. Data for RWC and OP were gathered for all samples. RWC was determined using 2-cm long-leaf samples from each of the genotypes and replicates (Barrs and Weatherly 1962). RWC was calculated as: RWC = (FW-DW)/(TW-DW)*100. The OP was determined using a vapor pressure osmometer (Wescor's VAPRO Vapor pressure osmometer 5520, Westcor, Logan, Utah). The osmolality units (mmol kg^{-1}) were converted to MPa using the Van't Hoff relation; $\psi_S = -CiRT$, where C is the osmolality value in mol kg^{-1}, i is an ionizing constant assumed equal to unity; R is the ideal gas constant (0.0083143 kg MPa $mol^{-1}K^{-1}$), and T is absolute temperature (K = °C + 273) (Nobel 1983).

Tall fescue genotypes, B400 with low OP and high RWC during drought and W279 with high OP and low RWC were grown in growth chamber. Pots were vernalized for 40 days at 4 °C followed by 15 days of acclimation under optimum light (10 h), temperature (24 °C), and water (frequently irrigated) conditions. Plants were then subjected under water stress for 15 days by providing minimum amount of water (stopped watering till wilting sign appeared and watered again just for the survivability of the plants). Samples of leaf, stem, root, and inflorescence from all the plants were collected. RNA was isolated using TriReagent and purified with RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA quality was substantiated by 2100 Bioanalyzer RNA Nanochip (Agilent, Santa Clara, CA) and quantified us-

ing NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). A total of 20 µg RNA was pooled from the four tissue types of three replications for cDNA library preparation. Double-stranded cDNA was obtained using SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Camarillo, CA). Sequencing was performed at the National Center for Genome Resources (NCGR), Santa Fe, New Mexico, USA using Illumina Genome Analyzer IIx system.

Greenhouse data analysis was performed using the PROC MIXED procedure of SAS. The sequenced reads were processed with a custom R script based on short-read package (Morgan et al. 2009). The high quality filtered reads were then assembled by Trinityrnaseq_r2013-02-25 with strand specific option "–SS_lib_type" set to "F" and "min_kmer_cov" set to 2 (Grabherr et al. 2011). The resulting contigs were clustered and further assembled to reference transcripts by Trinityrnaseq_r2013-02-25. Read mapping and quantification of the reference transcripts were done using Tuxedo suite (Tophat, Bowtie, Cufflinks). Assembled transcriptome data were evaluated by using blastn in tall fescue EST database and *Brachypodium* genome database. All the sequences were used for blast search against our transcriptome database using an E-value threshold of e^{-5} (0.00001). The search for sequence homology was performed by using BLAST v2.2.25+(http://blast.ncbi.nlm.nih.gov/Blast.cgi) with an E-value cut-off of e^{-5} (0.00001). SSRs were detected using MIcroSAtellite Identification Tool (MISA v1.0).

1.3 Results and Discussions

The 1000 tall fescue genotypes differed widely for RWC (range 33.7–97.3%, mean 79.7%), OP (range−0.5–−2.4 MPa, mean −1.2 MPa) and chlorophyll content (range 25.8–62.8, mean 41.0). Only nine genotypes were identified which had RWC, OP, and chlorophyll content 10% above the mean (Fig. 1.1). There were

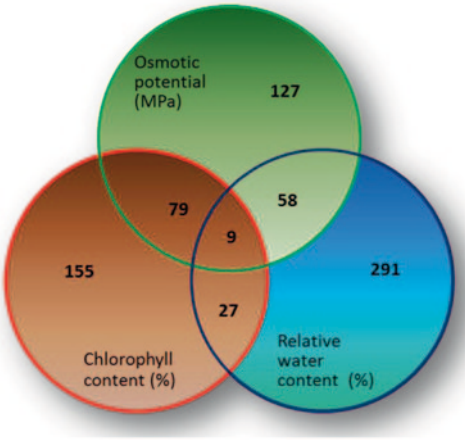

Fig. 1.1 A Venn diagram showing the number of tall fescue genotypes with mean scores 10% above the mean of the population

Table 1.1 Tall fescue genotypes with relative water content (RWC) and osmotic potential (OP) below or above the LSD units when evaluated in greenhouse experiments

Trait	LSD units below			LSD units above			
	−2	−1	0	0	1	2	3
OP	16	168	357	273	152	27	6
RWC	2	136	310	471	80	0	0

27, 58, and 79 genotypes which had chlorophyll content and RWC, RWC and OP, and OP and chlorophyll content 10% above the mean, respectively. RWC has been proposed as the most integrative measure of drought tolerance (Blum 1999). Osmotic adjustment is an important process of plant adaptation to drought because it conserves cellular hydration under stress. Increased OP under water stress has been linked to tissue survival and regrowth (Elmi and West 1995). Both RWC and OP could be used as assays to identify genotypes with high drought tolerance. Most of the genotypes had RWC and OP within one LSD unit of the mean, but some genotypes had OP values over two LSD units above and below the mean. Only two genotypes had RWC values below two LSD units (Table 1.1). There were six genotypes with OP values above three LSD units. The results indicated that the genotypes we evaluated had different physiologies and differed widely for RWC and OP.

The RWC and OP were negatively correlated ($r = -0.68$, $P < 0.0001$) for all genotypes. This implicates that overall observed changes in RWC might be influenced by differences in OP, but not necessarily by cell water volume change. Based on RWC and OP, the 25 most tolerant and 25 most susceptible genotypes were selected for further evaluations. After two sets of field and greenhouse experiments, we finally selected the most contrasting genotypes for drought tolerance. Genotypes NFTD348 and NFTD400 were identified the most tolerant, while NFTD279 and NFTD947 were identified as the most susceptible in the population. Across all studies, NFTD400 and NFTD348 were 85.7 and 76.2% times above the LSmeans, while genotypes NFTD279 and NFTD947 were 81.0 and 85.7% times below the mean, respectively. NFTD400 and NFTD279 were crossed and a mapping population of 252 genotypes has been developed.

The mapping population has been evaluated in field experiments under irrigated and rainfed conditions. Data on various morphological and physiological traits, e.g., chlorophyll content, RWC, OP, heading date, plant height, recovery time, and biomass yield, have been collected. The two parents were very distinct for most of the traits. Transgressive segregation was observed in the population (data not shown). All the 252 progenies of the population and the parents have been genotyped following the genotyping-by-sequencing protocol. Mapping and QTL analyses are in progress.

Transcriptome profiling between NFTD400 and NFTD279 genotypes was carried out to unravel crucial genetic regulatory mechanism of water stress responses in tall fescue. A brief summary of the transcriptome assembly is presented in Table 1.2. A total of 39.4 M and 58.6 M pair end reads were obtained from the NFTD400 and NFTD279, respectively. After assembly, 199,399 reference transcripts were recovered with an average read length of 585 bp (Table 1.2). A total of 2986 transcripts

Table 1.2 Summary of transcriptome assembly data obtained from two tall fescue genotypes contrasting for drought tolerance characteristics

Item	Measurements
Number of paired end reads of B400	39395238*2
Number of paired end reads of W279	58648268*2
Total number of reference transcript	199,399
Maximum transcript length	9671
Minimum transcript length	201
Average transcript length	585
Total transcript length (bases)	116,688,388
Actual read length	54 × 2
Quality trimmed read length	33 × 2
N50	752
E-value cutoff	0.00001

were significantly differentially expressed between the two genotypes. Thousand of them were found to be annotated and associated with metabolic pathways and enzyme coding genes.

MIcroSAtellite Identification Tool (MISA v1.0) was used to identify SSRs in the transcriptomes. The minimum number of repeats used to report a dinucleotide SSR was eight, six for a tri-nucleotide repeat, and four for tetra-nucleotide and above. A total of 8788 SSRs were identified in 8490 sequences of which, 243 sequences contained multiple SSRs. Primer-3 software was used to call primer pairs (PPs) and a total of 6348 PPs were developed. Among these potential SSRs, 313 had di-nucleotide, 1207 had tri-nucleotide, 826 had tetra-nucleotide, 1727 penta-nucleotide, 2082 hexa-nucleotide repeat motifs, and rest 193 had compound repeats. On an average, one SSR was found in every 13.36 kb with a frequency of 4.38 %. All the SSR containing sequences were then aligned and mapped into *Brachypodium* chromosomes sequence using blastn. In general, the SSRs were evenly distributed throughout all five *Brachypodium* chromosomes (Fig. 1.2). As expected, majority of the SSRs were concentrated in the distal part of each chromosomes and the centromeric region was lacking any SSR. Similar distribution of EST-SSR markers were observed in tall fescue genetic map (Saha et al. 2005).

1.4 Conclusion

We are in the process to develop genetic and genomic resources for deciphering drought tolerance in tall fescue. Thousand genotypes of a tall fescue population were screened for chlorophyll content, RWC and OP. Most contrasting genotypes were selected through a series of greenhouse and field experiments. Two most contrasting genotypes were used to construct a mapping population. Genotyping and phenotyping of the population is in progress. Transcript profiling of the genotypes identified differentially expressed transcripts at drought-stressed conditions. We identified a set of SSR markers well distributed in the genome. All these resources

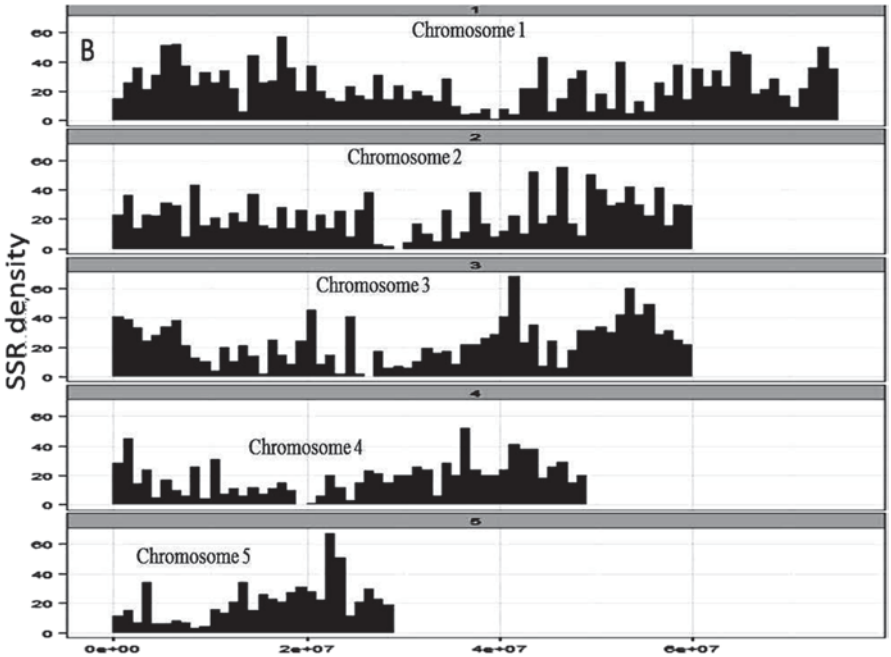

Fig. 1.2 Distribution of tall fescue EST-SSRs on *Brachypodium* chromosomes

can be used to expedite the cultivar development process in tall fescue, an outcrossing polyploid grass species.

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Chapter 2
Evaluation of Perennial Ryegrass Association Mapping Population for Freezing Tolerance Traits

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2.1 Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important agricultural cool-season grass species in temperate climate areas throughout the world, grown for forage with a high nutritive value. It is also widely used as a turf grass species with rapid establishment rate and excellent tolerance to traffic. These properties make the perennial ryegrass economically the most important species within the genus *Lolium* (Humphreys et al. 2006). Despite its many superior properties perennial ryegrass is sensitive to abiotic stresses. It exhibits poor winter survival under harsh and cold winters (Hulke et al. 2008), thus limiting its cultivation in certain areas. It was shown that perennial ryegrass responds to cold-acclimation, which increases its freezing tolerance (Ebdon et al. 2002). Cold acclimation is a multigenic quantitative trait associated with many physiological and biochemical changes within the plant cell (Hannah et al. 2005). Increase of water soluble sugar and proline concentration, as well as alteration of lipid composition and significant differentiation in profiles of protein accumulation is observed during cold acclimation in perennial ryegrass (Hoffman et al. 2010; Bocian et al. 2011). Proline acts as a free radical scavenger (Kaul et al. 2008), as osmoprotectant (Yoshiba et al. 1997), or as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995). The assumption was made that proline accumulation in plants under stress conditions has a protective function; however, correlation between proline accumulation and abiotic stress tolerance in plants is not always apparent (Szabados and Savouré 2010). Some data indicate that overproduction of proline in plants stabilises the cell membrane thereby preventing electrolyte leakage (Hayat et al. 2012). Moreover, low temperature can induce cell membrane lipid peroxidation (Campos et al. 2003), thus proline, as a free radical scavenger, could have an effect on membrane stability

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