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MOLECULAR AND MORPHO-PHYSIOLOGICAL STUDY ON DROUGHT TOLERANCE IN TURFGRASSES

By

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ABSTRACT

MOLECULAR AND MORPHO-PHYSIOLOGICAL STUDY ON DROUGHT TOLERANCE IN TURFGRASSES

By

Jianping Wang

Drought stress is a major limiting factor for the growth of cool season grasses particularly in the transitional and warm climatic regions. Selecting grasses with improved drought tolerance is the best strategy to increase survival and growth of grass during time of drought. An Atlas fescue (*Festuca mairei*, Fm) selection and three tall fescue (*F. arundinacea* Schreb.) cultivars were subjected to drought treatment. The drought stress had a significant negative effect on leaf elongation, leaf water content, and leaf water potential for all grasses. Fm maintained leaf growth, leaf water content, and leaf water potential longer than the tall fescue grass species and had an exceptional ability to accumulate water in leaf tissue when under severe drought stress, suggesting the better drought tolerance of Fm and its potential value for grass drought tolerance improvement.

Intergeneric hybridization between *Festuca* and *Lolium* can generate improved cultivars by combining stress tolerance of *Festuca* and rapid establishment of *Lolium*. However, wide-distance hybridizations usually result in the wild genome being eliminated from the hybrid. RAPD and SSR markers were used to detect the parental genome composition of hybrids and backcross derivatives from crosses between Fm and perennial ryegrass (*Lolium perenne*). Each progeny exhibited integration of Fm and

perennial ryegrass genomes with varying levels of genome ratios. The non-coinheritance of the linked markers suggested chromosome crossover between two parents. Cluster and principle component analyses of the progeny consistently revealed four groups. These results would be useful to guide the breeding program.

Increasing knowledge of genes induced by drought in Fm would be essential to understand the molecular mechanism of drought tolerance in grasses and to facilitate gene manipulation for grass breeding programs. In order to apply cDNA amplified fragment length polymorphism (cDNA-AFLP) technique to identify genes involved in drought tolerance in Fm, we empirically evaluated the experimental conditions of this technique in *Festuca* species. Results showed that NspI coupled TaqI were a pair of efficient enzymes for transcript derived fragment (TDF) discovery in Fm. The number of repeatable bands was not affected by magnesium concentration and dilution of pre-amplification products, suggesting the high reproducibility of this technique. The chimeric fragments derived from ligation between digested fragments were not eliminated by increasing adapter concentration. The application of the cDNA-AFLP technique to identify genes responding to drought stress in Fm revealed a total of 464 (4.1% of 11,346 TDFs) differentially expressed fragments (DEFs). The differential expression pattern for 171 (42.1% of 406) DEFs were confirmed by macroarray hybridization analysis. Functional analysis of confirmed DEFs using BLASTX revealed 17 functional categories. Some novel genes were identified in Fm during the drought response procedure. The combination of data from studies on the genetic model plant and on diverse plant species will provide a better understand the underlying mechanism of drought tolerance in plants.

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INTRODUCTION

Importance of turfgrass and drought stress problem

Turfgrass is used extensively on sports fields, golf courses, parks, lawns, and roadsides. The turfgrass contributes considerably to our environment by adding pleasant beauty to the surroundings, providing a safe playing surface for sports and recreation, controlling dust and pollen in the air, absorbing gaseous pollutants, and preventing erosion. However, a healthy, vigorous turf requires abundant water. In some sections of the country, water use for turf irrigation accounts for 50 percent or more of the consumption of city water supplies during the summer (Duble, 2005).

It has been estimated that over half the world's land surface is exposed to periodic drought (Boyer, 1982). In urban environments, drought stress is exacerbated due to soil factors as well as elevated temperatures (Cregg, 1995). Drought stress is a major limiting factor for the growth of cool season grasses in dry time of the year. Because of less than optimal water supply, the turfgrass quality often declines. Problematically, as regions experiencing drought conditions increase, and cities and municipalities declare water emergencies, water for turfgrass irrigation will be severely restricted. Turfgrass managers have put significant effort into cultural modifications in lawn management to reduce water consumption, but the effects were not significant. Using more drought tolerant varieties or cultivars will be a promising approach to alleviate stress in current lawns, fairways, and parks.

Two important grass genera and their drought tolerance

Turfgrasses are comprised of highly diverse grass genera and species. These species differ in their drought stress adaptation, as some can survive much greater drought stress than others. *Lolium* and *Festuca* are two important turfgrass genera (Table 1). Both belong to the tribe Poeae, subfamily Pooideae in which the basic chromosome number (x) is 7 (Jauhar, 1993). *Lolium* contains eight species (Clayton and Renvoize, 1986). The two most important species used extensively as turfgrass are *L. perenne*, commonly known as perennial ryegrass and *L. multiflorum* known as Italian ryegrass. Ryegrass (2n=2x=14, LL) is widely distributed cool-season grass throughout the United States, Europe, and in the temperate regions of the world. It has become a very popular turfgrass for over-seeding athletic fields, golf courses and lawns. The improved turf-type ryegrass varieties have better turf characteristics: finer texture, greater density, darker color, and better establishment. However, ryegrass is the least drought tolerant turfgrass species and needs frequent irrigation in the spring and early summer (Morrison et al 1980; Turgeon, 1991). *Festuca* is a large diverse genus comprising about 450 species (Clayton and Renvoize, 1986) and is widely distributed across the cool regions of the world. The most useful turfgrass species are *F. arundinacea* var. *genuina* – tall fescue (hexaploid, 2n=6x=42) and *F. pratensis*- meadow fescue (diploid, 2n=14), *F. rubra*- creeping red fescue, *F. ovina*- sheep fescue and *F. longifolia*- hard fescue. All of the fescues have been recognized for their exceptional drought tolerance (Aronson et al., 1987; Fry and Butler, 1989; Humphreys and Thomas, 1993; Turgeon, 1991). *Festuca mairei* St. Yves is a xerophytic tetraploid (2n=4x=28, $M_1M_1M_2M_2$) species, commonly

known as atlas fescue. It tolerates high temperature and drought, and has a high photosynthetic rate, but lacks turf quality (Borill et al, 1971; Marlatt et al, 1997).

Table 1. Proposed genomic formula of *Festuca* species (Sleper, 1985)

Species	Chromosome number (2n)	Proposed genomic formula
F. pratensis	14	PP
F. arundinacea var. *glaucescens*	28	$G_1G_1G_2G_2$
F. mairei	28	$M_1M_1M_2M_2$
F. arundinacea var. *genuina*	42	PP $G_1G_1G_2G_2$
F. arundinacea var. *atlantigene*	56	$G_1G_1G_2G_2$ $M_1M_1M_2M_2$

Drought tolerance mechanism in plant

Drought resistant mechanisms can be classified into three primary categories (Jones et al., 1981): *Drought escape mechanisms* are related to rapid phenological development. The plant completes its life cycle before a serious plant water deficit develops as evidenced by desert ephemerals. Such a mechanism would not be useful for turfgrass. *Drought avoidance* is the mechanism of drought tolerance where the plant maintains high tissue water potential through the ability to maintain water uptake or reduce water loss. Large root systems, which increase water uptake efficiency, and adapted leaf morphology such as lower specific leaf areas and lower stomatal density, that reduce water loss, are the most recognized morphological adaptations of drought avoidance. The physiological adaptations in increasing drought tolerance at high tissue water potential are related to

water conservation through low stomatal conductance (rapid stomatal closure) and low transpiration rate, which contribute to reduce water loss. *Drought resistance mechanism* is drought tolerance at low tissue water potential indicated by maintenance of stomatal conductance, transpiration rate and other physiological processes. Drought tolerance is usually achieved by osmotic adjustment. Under water deficit conditions, plant growth is substantially reduced, partly because lower turgor pressure in the cells results in a lower cell expansion rate (Pattanagul and Madore, 1999). The osmotic adjustment in the plant maintains cell elongation. The production and partitioning of metabolically important non-structural carbohydrates (sugars, starch, and sugar alcohols) have been found to be altered by drought in a number of different ways (Vyas et al., 1985; Jacomini et al., 1988; Keller and Ludlow, 1993; Volaire and Thomas, 1995). Sugars may serve as compatible solutes permitting osmotic adjustment to maintain the water potential during mild drought (Bohnert, 1995). Enzymes of sugar metabolism are probably critical in desiccation tolerance. In addition to sugars, other compatible solutes also contribute to osmotic adjustment. Enzymes involved in the synthesis of proline and glycine betaine are clearly up-regulated during drought (Bohnert, 1995).

The genotypes with a relatively high capacity to osmotically adjust (i.e., decrease osmotic potential in response to drought stress) may be better able to maintain photosynthesis and other physiological processes during drought than those that lack the ability to osmotically adjust. Therefore, it is possible that the ability to osmotically adjust during drought may serve as a criterion for the drought tolerance selection program (Cregg, 1993).

Genetic approaches for drought tolerance improvement

Genetic improvement is one of the most effective ways to increase drought tolerance of turfgrass. Classical breeding through sexual hybridization has been the principal approach for turfgrass improvement over the past half century. Intergeneric hybridization between *Lolium* and some *Festuca* species has been a long time goal to combine the complementary traits of these species since they allow certain levels of intergeneric chromosome pairing, recombination, and gene exchange (Morgan and Thomas, 1991; Crowder, 1953), and cultivars derived from crosses between *Lolium* and *Festuca* have been released (Buckner et al, 1977; 1983). However, progress in breeding turfgrass for drought resistance has still been very slow, primarily because of the genetic complexity of drought stress responses and lack of knowledge of the major genetic components underlying drought tolerance of plants.

Successful breeding program depends on a broad understanding of the genetic architecture of the relevant trait (Humphreys et al., 2004). Due to the biological complexity of grass species and the associated difficulties encountered by traditional breeding methods, the potential of molecular breeding for the development of improved cultivars is evident. Molecular improvement presents both new challenges and clear opportunities for the application of biotechnology. Major genes associated with drought tolerance include both genes with major effects, which are directly involved in the biochemical pathway, and genes contributing to the expression of the major gene, such as transcription factors. Knowledge of gene function and characterization will facilitate the target modification of drought tolerance through transgenic approaches or gene introgression.

Molecular basis study of drought tolerance

A drought stress response is initiated when a plant recognizes the stress, which then activates signal transduction pathways to transmit the information within individual cells and throughout the plants. Ultimately, changes in gene expression will occur and are integrated into plant's adaptive responses to modify growth and development.

Several hundred genes are differentially expressed in response to dehydration in the resurrection plant *Craterostigma plantagineum*, as evidenced by transcript profiling (Bockel et al., 1998). In *Arabidopsis,* genes involved in many different pathways are expressed in response to drought stress (Seki et al., 2002). All these identified genes can be assigned to diverse biological pathways, such as sugar metabolism and biosynthesis (Bohnert, 1995), ion and water channel proteins synthesis (Guerrero et al., 1990) cell wall lignification processes (Peleman et al., 1989) detoxification of active oxygen species (Williams et al., 1994; Mittler et al., 1994) and so on. Although the precise functions of these genes has not yet been demonstrated, Bartels and Salamini (2001) have summarized all the drought inducible genes and grouped them into five main categories, as genes encoding (a) proteins with protective properties, (b) membrane proteins involved in transport processes, (c) enzymes related to carbohydrate metabolism, (d) regulatory molecules, such as transcription factors, kinases, or other putative signaling molecules, and (e) open reading frames that show no homologies to known sequences.

Obviously a network of signal transduction pathways allows the plant to adjust its metabolism to the demands imposed by water deficit (Shinozaki and Yamaguchi-Shinozaki, 2000; Kirch et al., 2001). The complex signal transduction cascade can be divided into three basic steps (Ingram and Bartels, 1996): (a) perception of stimulus, (b)

signal amplification and integration, and (c) response reaction in the form of de novo gene expression. The signaling molecules involved in the signal transmission process and the activation of gene expression in response to stress have been identified. One molecule is the plant hormone abscisic acid (ABA). Endogenous ABA levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction (Chandler 1994; Giraudat 1994). Besides the ABA-mediated gene expression, the investigation of drought-induced genes in *A. thaliana* has also revealed ABA-independent signal transduction pathways (Yamaguchi and Shinozaki, 1994). Both ABA-dependent and -independent stress signaling first modifies constitutively expressed transcription factors, leading to the expression of early response transcriptional activators, which then activate downstream stress tolerance effective genes (Zhu, 2001).

Even though a large number of drought induced genes have been identified in a wide range of species and impressive progress has been made in gene annotation, the molecular basis still remains far from being completely understood (Ingram and Bartels, 1996). It is important to identify more drought inducible genes and analyze the functions of the genes. Increasing knowledge of the function of these genes would be essential to understand the molecular mechanism of drought tolerance in plant and to facilitate gene manipulation for breeding programs.

cDNA-AFLP technique

Transcript profiling is playing a substantial role in annotating and determining gene functions by revealing gene expression on a genome-wise scale. A variety of high-

throughput transcript profiling techniques have been established including cDNA-amplified restriction length polymorphism (cDNA-AFLP), serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), expressed sequence tag (EST) sequencing, differential display PCR (DD-PCR), cDNA microarray, oligo-chips, and suppression subtractive hybridization (SSH). cDNA-AFLP analysis is an mRNA fingerprinting technique that demonstrates high reproducibility and sensitivity, good correlation with northern blot analysis and low set-up cost, even though it requires a comprehensive reference database (Donson, et al., 2002). Recently, the rapidly expanding field of genomics, the creation of a large-scale EST database from various species, and the complete sequencing of *Arabidopsis* (Arabidopsis genome initiative [AGI], 2000) and rice genome (Yu et al., 2002) were made public. This genomic information source can provide a vast reference database for evaluating the coordinated function and expression of genes identified by using the cDNA-AFLP approach. cDNA-AFLP has been successfully used to identify differentially expressed transcript derived fragments (TDFs) from almond (*Prunus amygdalus*) treated with abscisic acid (ABA) (Campalans et al., 2001); tissue-specific TDFs during potato (*Solanum tuberosum L.*) tuber development (Bachem et al., 2001); and TDFs associated with putative pathogenicity factors during infection of tuber by potato cyst nematode (*Globodera rostochiensis*) (Qin, et al., 2000). cDNA-AFLP technique was found to be an efficient method of isolating differentially expressed genes or TDFs.

Marker assisted selection

The genetic improvement for drought tolerance is slow and complicated, especially by conventional breeding approaches. The main reason is the low heritability of traits associated with drought tolerance. Marker assisted selection (MAS) provides breeders with valuable tools to develop newer germplasm with improved drought tolerance (Quarrie et al., 1999; Hoisington et al, 1996). Drought tolerance involves a cascade of events and is controlled by multiple genes. To clarify the genetic network involved, key agronomic traits need to be clarified into individual components to reduce complex analysis (Modarres et al., 1998; Tollenaar and Wu, 1999). After specific components of the genes corresponding to drought tolerance are isolated and cloned, they can be used for transgenic breeding or be converted into PCR-based markers to assist in selection, which is more effective as their expressions are independent of environment. For PCR-based marker development, sequence data is used to design PCR primers specific to the differential genes or fragments, and use PCR to produce specific fragments from genomic DNA. These markers are suited for identifying the desired form of the gene (allele) from the onset of the selection process and allow us to rapidly identify genetic lines that had the desired allele and discard those without. Revealing the genes function in drought tolerance and converting the cloned genes into PCR-based markers to assist the selection would be more effective and efficient in a drought tolerance breeding program.

Objectives and hypothesis

The main goal of this research is to identify specifically expressed drought tolerant genes in *F. mairei* to understand the molecular genetic basis underlying drought tolerance in grasses and to introgress the drought tolerance of *F. mairei* into perennial ryegrass. This study is based on the hypothesis that a) there must be a genetic code responsible for the drought tolerance and the genetic code would be able to be isolated and defined, and that b) drought tolerance observed in *F. mairei* can be genetically transferred into the hybrids of *F. mairei* X *L. perenne.*

The specific objectives are as follows:

1. To evaluate the morpho-physiological drought tolerance characteristics of *F. mairei*;
2. To assess the genome introgression of *F.mairei* into *L. perenne* hybrids using SSR and RAPD markers.
3. To optimize the experiment conditions of cDNA-AFLP procedure in discovering transcript derived fragments in *F. mairei*;
4. To identify the differentially expressed fragments (DEF) in *F. mairei* during drought stress treatment by using cDNA-AFLP coupled with macroarray hybridization;
5. To functionally analyze the DEFs identified in *F. mairei* during drought stress treatment;

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CHAPTER I

Morpho-physiological Responses of Several Fescue Grasses to Drought Stress

ABSTRACT

An Atlas fescue (*Festuca mairei*) selection and three tall fescue (*F. arundinacea* Schreb.) cultivars, Barolex, Kentucky 31, and Falcon II were subjected to drought stress imposed for a 12-week period. Soil water content (SWC), leaf elongation (LE), leaf water content (LWC), and leaf water potential (Ψw) were measured weekly, and root length (RL) and biomass (RM) were recorded after 12 weeks. The SWC declined progressively during the 12 weeks drought period. The SWC decreasing rates of the three tall fescue cultivars were similar, but declined faster than Atlas fescue indicating that Atlas fescue extracted soil water slower and developed less intensive stress than the three tall fescue cultivars. The imposed drought treatment had a significant negative effect on LE, LWC, and Ψw for all grasses. These three parameters of the treated plants for Atlas fescue remained similar to control plants longer than the three tall fescue cultivars. The relationships between LE and LWC verses SWC and Ψw respectively were fitted to a polynomial function. Results suggested that (1) the LE of Atlas fescue and Falcon II were less sensitive to the imposed drought stress than Barolex and

Kentucky 31 as SWC and Ψw decreased; (2) a mechanism may exist in Falcon II and Atlas fescue to maintain cell turgor necessary for cell expansion as SWC declined and Ψw became more negative; (3) all the grasses conserved water as the drought stress initiated and Atlas fescue maintained water in the leaf tissue longer than the three grasses; (4) Atlas fescue had an exceptional ability to accumulate water in leaf tissue under severe drought stress (Ψw = -1.2 ~ -2.4 Mpa). The long root system (115-132 cm) of the four grasses may help avoid the effect of drought by absorbing more water from soil through extensive root systems. The slower decline of LE, LWC, and Ψw in Atlas fescue during the drought stress period suggested that Atlas fescue possessed drought tolerance and afforded potential to improve drought tolerance in turfgrass breeding program.

Key words: Atlas fescue, tall fescue, drought tolerance, leaf elongation, leaf water content, leaf water potential, root.

INTRODUCTION

It has been estimated that over half the world's land surface is exposed to periodic drought (Boyer, 1982). In urban environments, drought stress is exacerbated due to negative soil factors as well as elevated temperatures (Cregg, 1995). Drought stress is a major limiting factor for the growth of cool season grasses in the transitional and warm climatic regions of the world. Because of less optimal water supply, the turfgrass quality and forages yield often declines. Problematically, as water conservation becomes an important issue, water for landscape and agronomic irrigation is restricted. This

suggested identification and screening of grasses with improved drought tolerance and reduced water use may be the best strategy to increase survival and growth of grass in drought prone areas through plant breeding.

In general, drought resistance is the capacity of a plant to survive or grow during drought stress. The mechanisms of drought resistance have been classified into three primary categories: drought escape, drought avoidance and drought resistance (Jones et al., 1981). Drought escape mechanisms are related to rapid phenological development. The plant completes its life cycle before a serious plant water deficit develops as evidenced by desert ephemerals. Drought avoidance is the mechanism of drought tolerance where plants maintain high water potential in tissues through the ability to maintain water uptake or reduce water loss. Large root systems that increase water uptake efficiency (McCully 1999; Weerathaworn et al., 1992) and adapted leaf morphology such as lower specific leaf areas and lower stomatal density that reduce water loss are the two morphological adaptations that plants use to avoid drought. The physiological adaptations related to water conservation are through low stomatal conductance (rapid stomatal closure) and low transpiration rate to reduce water loss (Jones et al., 1981). Drought resistance mechanism in plants is the drought tolerance at low tissue water potential indicated by maintenance of regular physiological processes. Drought tolerance through resistance mechanism is usually achieved by osmotic adjustment. Under water deficit conditions, plant growth is substantially reduced, partly because lower turgor pressure in cells affected by low water potential results in a lower cell expansion rate (Pattanagul and Madore, 1999). The osmotic adjustment in response to water deficit can results in maintenance of cell elongation or enhancement of turgor

(Begg and Turner, 1976), which may sustain cell expansion and leaf elongation (Hsiao, 1973). A dormancy mechanism can also be related to long-term responses to severe drought in perennial grasses. When perennial grasses are quiescent or dormant, plants temporally suspense visible growth of any structure containing a meristem such as basal buds, to avoid drought damage and allow survival (Mcwilliam, 1968).

Grass genotypes and cultivars vary in their responses to drought stress, which involve changes in various morphological and physiological factors (Wu and Huff, 1983). Knowledge of relative involvement of various morphological and physiological characteristics in drought tolerance is important in selecting grass genotypes that persist during drought stress and facilitate the breeding of drought tolerant cultivars. In the current study, we sought to investigate drought responses of a selection of Atlas fescue (*Fetuca mairei*) grass compared with three tall fescue (*F. arundinacea* Schreb.) cultivars, "Kentucky 31", "Falcon II", and "Barolex". Tall fescue is the most useful turfgrass and forage species in cool and transition zone regions. This species is originated from Europe and has been recognized for its exceptional drought tolerance (Norris and Thomas, 1982; Fry and Butler, 1989). Within tall fescue species, cultivars also vary in drought resistance (White et al., 1993; Carrow, 1996). Kentucky 31 and Falcon II have been identified as good drought tolerant cultivars (Huang and Gao, 1999; Huang, 2001). Barolex is a new tall fescue forage type cultivar, and its drought tolerance is unknown. Atlas fescue species is only found in the Atlas Mountain ranges of northwest Africa. There is no definitive report of drought tolerance of Atlas fescue, although this grass species has a xerophytic adaptation to survive long summers under drought stress (Marlatt et al., 1997).

The objectives of this study are to (i) determine the leaf elongation, leaf water content, leaf water potential, root biomass and length of a selection of Atlas fescue, Barolex, Kentucky 31, and Falcon II during drought stress imposed for 12 weeks; and (ii) investigate the drought responses of Atlas fescue compared with tall fescue cultivars.

MATERIALS AND METHODS

Plant materials and drought treatment application

Four grasses of *Festuca* species were compared. One was an Atlas fescue selection, originally collected from Morocco. The other three grasses were commercial tall fescue cultivars used for turf and forage: "Barolex", "Falcon II", and "Kentucky-31". A single tiller of each grass was used to propagate vegetatively a mature plant in the greenhouse. From each plant, two tillers were transplanted into each of six PVC tubes (100 cm deep x 34 cm in diameter). These tubes were filled with the same weight (11.8 kg) of substrate (recommended soil for athletic fields, 85% sand and 15% field soil). Between the tube and substrate, a heavy duty plastic sleeve was placed inside the tube, to facilitate moving the root system from the tube at the end of the experiment. All the transplanted plants were established for 15 weeks in the greenhouse during fall with regular irrigation, fertilizer and trimming. The greenhouse temperature was 25 ± 3 °C, with average 13 h/day photoperiod. A pre-conditioned drought was applied by withholding water for two weeks. Plants were recovered by irrigation for one week and then trimmed to same height (around three inches). Then, three tubes of each plant were

randomly allocated to a block for drought treatment and the other three tubes were randomly allocated to the other block as treatment control. Drought stress was imposed by withholding water progressively from plants in the drought treatment by supplying 200 ml (up to 100% soil capacity in the tube), 150 ml, 100 ml, and 50 ml water in the first four weeks respectively and stopping water during the remaining 12-week drought period. The plants in the control treatment were irrigated regularly during this period. The PVC tubes were re-randomized weekly during this drought period to minimize effects of possible environmental gradients within the greenhouse.

Soil water content measurement

The PVC tubes were weighed every week at the same time (1:00 p.m.) to determine gravimetric soil water content (SWC) from water loss. The mass of soil mixture was measured for each tube at the beginning of the experiment, which also ensured the same weight of substrate (11.8 kg) in each tube. The moisture of the soil mixture was estimated by weighing 10 fresh, and then 80°C oven dried soil samples.

Leaf elongation measurement

After 15 weeks establishment, three tillers in each tube were randomly chosen and labeled with wires of different color for consistent leaf elongation (LE) measurement. Length of the top two freshly –emerged leaves on the labeled tiller were measured from the tip of each lamina to the ligule of the next oldest leaf (Norris and Thomas, 1982) every week until leaf growth of drought stressed plant ceased.

Leaf water content measurement

A fully-extended leaf of the drought stressed plants was detached weekly for leaf water content (LWC) measurement. Control plants were sampled in 3^{th}, 6^{th}, and 9^{th} week only during the drought period. The fresh weight (FW) (weight of the leaf immediately after detachment), turgor weight (TW) (weight of the leaf after soaked in the miniQ water for 24 hr at room temperature), and dry weight (DW) (weight of the leaf after dry in oven at 80°C for 24 hr) of the leaf were used to calculate the relative LWC described by Slavik (1974) and White et al., (1992): LWC (%) = (FW-DW)/(TW-DW) x 100.

Leaf water potential measurement

All the plants were covered by a black plastic sheet in the evening to imitate a pre-dawn condition (closed stomata and low respiration). The following morning, duplicated fully-emerged, undamaged laminae in each tube were sampled every week and immediately were subjected to leaf water potential (Ψw) measurements by using a pressure chamber (Soil moisture equipment corp., Santa Barbara, CA). All the measurements were conducted at 22-25 °C within 2 hours in the greenhouse. The data was eliminated when the Ψw of the control was greater than -0.6 MPa.

Root length and biomass measurement

At the end of the experiment, the heavy duty plastic sleeve, which contained the root system, were taken out of each tube. The soil substrate was gently removed from the root system by flowing water from. Length of root system (RL) was measured using a ruler. Biomass of the root (RM) was weighed after blotted dry using paper towel and

further evaporated at room temperature for around 6 hr to remove surface moisture of the root.

Statistical analyses

The data of LE, LWC, SWC, and Ψw, were subjected to analysis of variation (ANOVA), using repeated measurements in time by SAS program (SAS Institute Inc. 2003). Comparisons were made within the four grasses by one-way ANOVA and between drought and control treatments by student *t* test at specified week. Mean separations were performed by a least significant difference (LSD) procedure where the *F*-value were significant at the 0.05 probability level. RL and RM data were subjected to one way ANOVA analysis to compare within the four grasses and between stressed and control plants. The relationships between parameters were fitted to appropriate nonlinear regressions model in Microsoft Office Excel (Microsoft Co. 2002).

RESULTS

Soil water content and leaf water potential

When the soil was at full water capacity, the SWC for the grasses included in this experiment was 9.33%. SWC declined significantly ($P < 0.0001$) starting at the second week of the drought treatment (Figure 1.1). The rate of soil water depletion was similar among the grasses except it was higher with Atlas fescue. Specifically, during the four- to eight-week of drought stress treatment, SWC of Atlas fescue was significantly higher,

indicating that Atlas fescue extracted less soil water and developed severe stress status slower than the tall fescue cultivars.

Ψw has been widely accepted as a definitive indicator of plant water status and stress level. The imposed drought stress had a significant effect on Ψw of the grasses we studied. In irrigated plants, Ψw was similar ($P = 0.086$) among the grasses, and remained relatively high across the 12-week drought treatment period. Ψw of stressed plants decreased differently among the four grasses (Figure 1.2). Ψw of stressed plants showed significant difference from the irrigated ones after four (Falcon II), five (Kentucky 31), six (Barolex), and eight (Atlas fescue) weeks respectively. The results indicated that Atlas fescue maintained Ψw at the level of irrigated plants longer than the three cultivars during drought period, suggesting that Atlas fescue developed stress status relatively slower.

The variation of Ψw highly depended on SWC through a power equation (Figure 1.3). The Ψw in response to declining SWC showed a roughly similar trend for the four grasses: Ψw remained constant at a high level (> -1 Mpa) on a wide range of SWC from 9.33 to about 2.8 %, then decreased rapidly. The results reflected that soil water was readily available and kept sufficient for the plants in the SWC range from 9.33 to 2.8 %. The critical SWC of 2.8 % was basically in agreement with the threshold of SWC for initial stomatal closure due to drought stress in tobacco (*Nicotiana tabacum*) (Riga and Vartanian, 1999). For Atlas fescue, Ψw decreased steeply from a SWC of around 1%, while for the three grasses, Ψw declined dramatically from a relatively higher SWC (1.5 ~ 1.8 %), suggesting Atlas fescue was less sensitive to soil water deficits than the tall fescue cultivars.

Leaf elongation

LE of all grasses was negatively affected by the imposed drought stress ($P <$ 0.0001). In irrigated plants, the average LE for Atlas fescue, Barolex, and Kentucky 31 across the 12-week period were similar and significantly greater than that of Falcon II, while Atlas fescue was less than Barolex and Kentucky 31 in the first week, (Figure 1.4). These results revealed that Falcon II grew relatively slower than other grasses at normal condition, and Atlas fescue initially had a low LE and greatly increased in later weeks during the drought stress period (Figure 1.5). Between 8th and 10th week, LE of the irrigated plants was greater than the first seven weeks during the drought treatment period. At 10th week, the leaf elongation of irrigated plants of Kentucky 31 dropped dramatically, when the plants started to bloom and vegetative growth was switched to reproductive growth. In drought treated plants, the average LE for four grasses across the whole drought stress period was not significantly different ($P = 0.5078$). For the three tall fescue cultivars, the LE of stressed plants started to decrease to below the level of irrigated plants at 5th or 6th week stress treatment, whereas for Atlas fescue, LE started to reduce later, at 7th week stress. The LE of drought treated plants in Barolex and Falcon II ceased after nine weeks treatment, while in Atlas fescue and Kentucky LE last longer, up to 10th week.

The relation between LE and SWC was fitted to a second order polynomial function (Figure 1.6). When the SWC was high close to full soil capacity (8-9.33 %), the LE of Barolex and Kentucky 31 were higher than that of Falcon II and Atlas fescue, indicating that Barolex and Kentucky 31 were growing faster at a high SWC. As the SWC was declining, LE decreased differently among the four grasses. Falcon II and

Atlas fescue showed a relatively slow decreasing rate compared with Barolex and Kentucky 31, because the slopes of trend line for Falcon II and Atlas fescue were less steep, suggesting that the growth of Falcon II and Atlas fescue was less sensitive to the declining SWC.

The LE responded to the decreasing Ψw following a polynomial function (Figure 1.7). As Ψw was declining and becoming more negative, the LE decreased for all grasses at different rates. The decreasing rate of LE in Atlas fescue and Falcon II was less than that of Barolex and Kentucky 31, reflecting that LE of Atlas fescue and Falcon II was relatively insensitive to the increasing severity of drought stress.

Leaf water content

Drought stress treatment had a significant ($P < 0.0001$) effect on the LWC of the grasses. In irrigated plants, LWC remained constant at a relatively high level (around 87.7%) during the whole experimental period (Figure 1.8). In plants subjected to drought stress treatment, LWC decreased differently among the four grasses. For the tall fescue cultivars, LWC of stressed plants was at the level of irrigated plants during the first three or four weeks of growth, whereas for Atlas fescue, LWC maintained the same level as irrigated plants much longer, up to eight weeks in the drought treatment period. The LWC of Atlas fescue was significantly higher than that of the tall fescue cultivars between sixth and ninth week in the drought stress treatment. The results implied that Atlas fescue may accumulate or conserve water in leaf tissue as the stress triggered plants to maintain the turgor through adapted leaf and root morphology.

The LWC in response to SWC showed three stages (Figure 1.9). In the first stage, when SWC was high (8-9.33 %), LWC of the grasses maintained at a high level (around 80-90 %). In the second stage, as SWC was decreasing from 8 % to around 4 %, the LWC showed a slightly increasing trend. It was clearer in Atlas fescue, the LWC increased faster than the tall fescue cultivars. In the third stage, when the SWC was decreasing from 4 % to zero, the LWC reduced dramatically for all the grasses. It was notable that when SWC was between 2 and 6 %, a medium drought stress status, LWC of Atlas fescue was higher than that of the other three grasses.

The variation of LWC was dependent on the Ψw through a polynomial function (Figure 1.10). As Ψw was becoming more negative, specifically between -1 and -2.5 Mpa, the LWC of grasses was declining. However, Atlas fescue maintained much higher LWC than the other grasses. In addition, decreasing rate of LWC for Atlas fescue was less than the other grasses under severe drought stress, specifically at Ψw = -1.2 ~ -2.4 Mpa. The results again suggested that Atlas fescue had an exceptional ability to accumulate or conserve water in leaf tissue under severe drought stress.

Root length and biomass

The RL of the grasses ranged from 115 to 132 cm and varied significantly (P = 0.0335) among the four grasses. Barolex had the longest root system, while Kentucky 31 had the shortest one (Figure 1.11. A). RL of Falcon II was negatively affected by the drought treatment, whereas there was no significant difference in RL between irrigated and drought treated plants for Atlas fescue, Barolex, and Kentucky 31. No significant difference was found in RM among these grasses across the irrigated and drought stress

treatments (P=0.0717). However, the drought treatment had an significant (P=0.0003) effect on RM. The stressed plants had significantly less RM than that of irrigated plants for Atlas fescue, Barolex, and Falcon, but not for Kentucky 31 (Figure 1.11. B). The results suggested that Barolex and Atlas fescue with longer roots might be more adaptive to drought stress than Kentucky 31. However, the RM of Kentucky 31 was not reduced by severe drought stress suggested that Kentucky 31 may tolerant the drought stress through maintenance of viable root capable of extracting available water, even though it had a shorter root.

DISCUSSION

Understanding drought tolerance mechanisms in grass species and the genetic variation among genotypes would guide breeding and management programs in improving the drought tolerance in grass species. Several mechanisms have been implicated in causing differences in drought tolerance of plants (Levitt, 1972; Jones, 1981). Unlike annual plants that can escape drought by maturing before stress becomes severe, perennial grasses can not escape drought completely by flowering early. In our study, no drought stress treated plant was flowering, except some control plants, which supported the grasses we studied did not escape drought by earlier mature, but oppositely, reproductive growth was inhibited by the imposed drought stress. In our study, all the four grass maintained leaf elongation until a very low SWC (1.2 %) (Figures 1.1 and 1.5), suggesting they are active rather than dormant during the drought stress period. As we observed, with the SWC decreasing, leaves of all the four grasses rolled initially. As

SWC decreased further, the leaf tip showed firing and lower leaves became bleached. These symptoms suggested that these grasses may employ similar strategy to reduce the transpiration surface area and close stomata to limit plant water loss. Tall fescue relied primarily on an extensive root system for drought tolerance (Qian et al., 1997), because the longer root system had greater volume and surface areas of roots in contact with soil to facilitate water and nutrient uptakes under drought stress. Root system has been chosen as a selection trait in breeding programs to improve drought tolerance of fescue (Torvert, et al., 1990). In one previous study, the root length of 16 tall fescue cultivars, which represented four growth types, dwarf, turf, intermediate, and forage, was 60-75 cm (Kim, et al., 1999). In our study, however, the RL of the four grasses was 115-132 cm, although varied, and was much greater than previously reported values (60-75 cm). The results of this study implied that the four grasses can at least avoid the drought consequences by producing extensive roots to absorb more water from soil. Barolex had a relative longer root, but did not maintain LE and LWC optimally longer than other grasses, suggesting that beside the adapted leaf and root characters, the grasses employ other mechanism to resist drought stress to avoid drought stress. Usually, drought tolerant genotypes will posses more than one mechanism, and many factors can contribute to drought tolerance of plants (Aussenac et al., 1989).

During water stress, numerous physiological functions are affected before the leaves show signs of wilting. However, cell expansion is the most sensitive trait (Boyer, 1988) and is reduced by drought before any other physiological process (Wardlaw, 1969). In our study, LE was measured weekly during the drought stress as a major indicator of the status of plant response to drought. Notably, for Atlas fescue and Kentucky 31, LE

had a significant decline even one week earlier than Ψw, which has been shown to be an effective measurement of the maximum soil water potential available to roots (Tardieu and Simonneau, 1998) (Figures 1.2 and 1.5). The results confirmed the value of LE for its sensitiveness as a parameter for drought tolerance evaluation in plants. In addition, it was not possible to make measurements of Ψw on severely drought stressed leaves due to the limitation of equipment, but LE could be measured at any time and situation. Cell expansion directly contributes to the leaf elongation. A reduced leaf growth is mainly caused by a decrease in turgor pressure of enlarging cells (Matyssek et al., 1988). Osmotic adjustment may enable a leaves to maintain sufficiently high turgor pressure in the growing zone to maintain the leaf elongation. The LE of Falcon II and Atlas fescue declined slower as the SWC and Ψw were decreasing (Figures 1.6 and 1.9). The result suggested that the osmotic adjustment may play a role in maintaining cell pressure necessary for LE, which is the resistance mechanism employed by the grass to resist drought stress.

The LE and LWC data (Figures 1.5 and 1.8) showed that Atlas fescue maintained leaf growth and regular LWC longer than the other three grasses. It can be debated that maintenance of growth and LWC of Atlas fescue may be the result of a relatively low rate of water use by Atlas fescue, because in studies of container-grown plants, dry-down responses are often confounded with plant size (Graves et al., 2002). The small size plant may not evaporate sufficient water to cause severe stress. Therefore, SWC and Ψw may decline slower in large plants, regardless of their relative drought tolerance. However, in our study, plant size was controlled to be the same by establishment for 15 weeks and trimming to same height (Figure 1.12). In order to eliminate any possible effect of plant

size or rapidness of stress development on the leaf water loss and reduced leaf growth, a regression analysis was performed between LE and LWC verses SWC (Figures 1.6 and 1.9) and Ψw (Figures 1.7 and 1.10), respectively. Results of comparison suggested that (1) the LE of Atlas fescue and Falcon II were less sensitive to the drought stress than Barolex and Kentucky 31 as SWC and Ψw were decreasing (Figures 1.6 and 1.9); (2) a marked mechanism may exist in Falcon II and Atlas fescue to maintain the cell turgor necessary for cell expansion as SWC was declining and Ψw was becoming more negative; (3) all the grasses intended to accumulate more water as they sense the drought stress and Atlas fescue was more capable to have an earlier sense and to accrue more water in the leaf tissue than the other three grasses (Figures 1.7 and 1.9); (4) Atlas fescue had an exceptional ability to accumulate water in leaf tissue under severe drought stress (at Ψw = -1.2 ~ -2.4 Mpa) (Figure 1.10). Atlas fescue consumed less volume of soil water between the fourth and eighth week of drought stress (Figure 1.5), but maintained LE and LWC higher (Figures 1.5 and 1.8) further indicating its efficienct water use and expression of drought tolerance.

In summary, drought stress reduced LE, LWC, Ψw, root biomass & length of the grasses. Grasses avoid drought stress through changes in leaf and root morphology and through osmotic adjustment to maintain sufficient high turgor pressure in the growing zone for leaf elongation. The slower decrease in LE, LWC, and Ψw for Atlas fescue during the drought stress period suggested its greater drought tolerance and the potential value for grass drought tolerance enhencement in the breeding program.

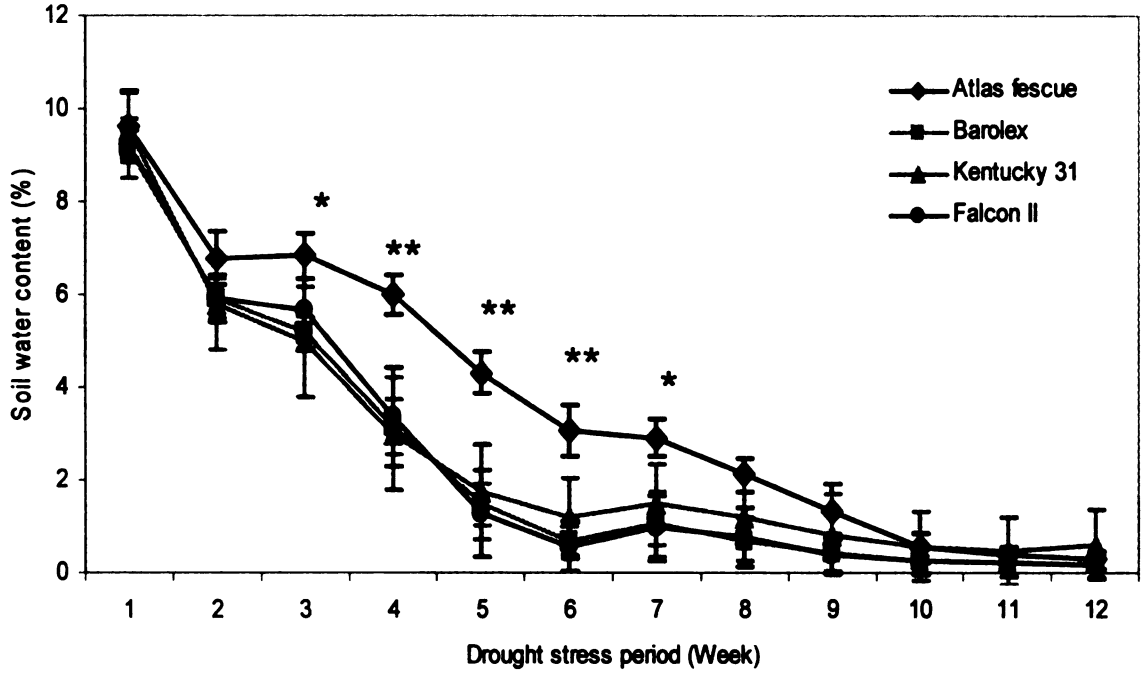

Figure 1.1. Soil water content during the drought stress period. Error bars indicate standard errors. *, ** Significantly different means among the stressed plants of four grasses at certain week at $P \leq 0.05$ and 0.01 respectively.

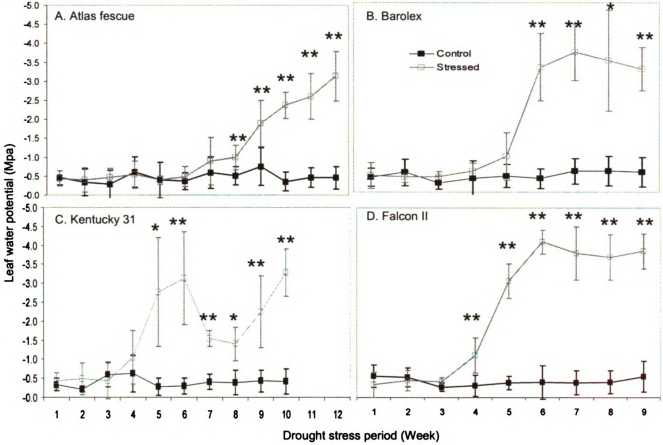

Figure 1.2. Leaf water potential during the drought stress period. Error bars indicate standard errors. *, ** Significantly different means between control and stressed plants at certain week at $P \leq 0.05$ and 0.01 respectively.

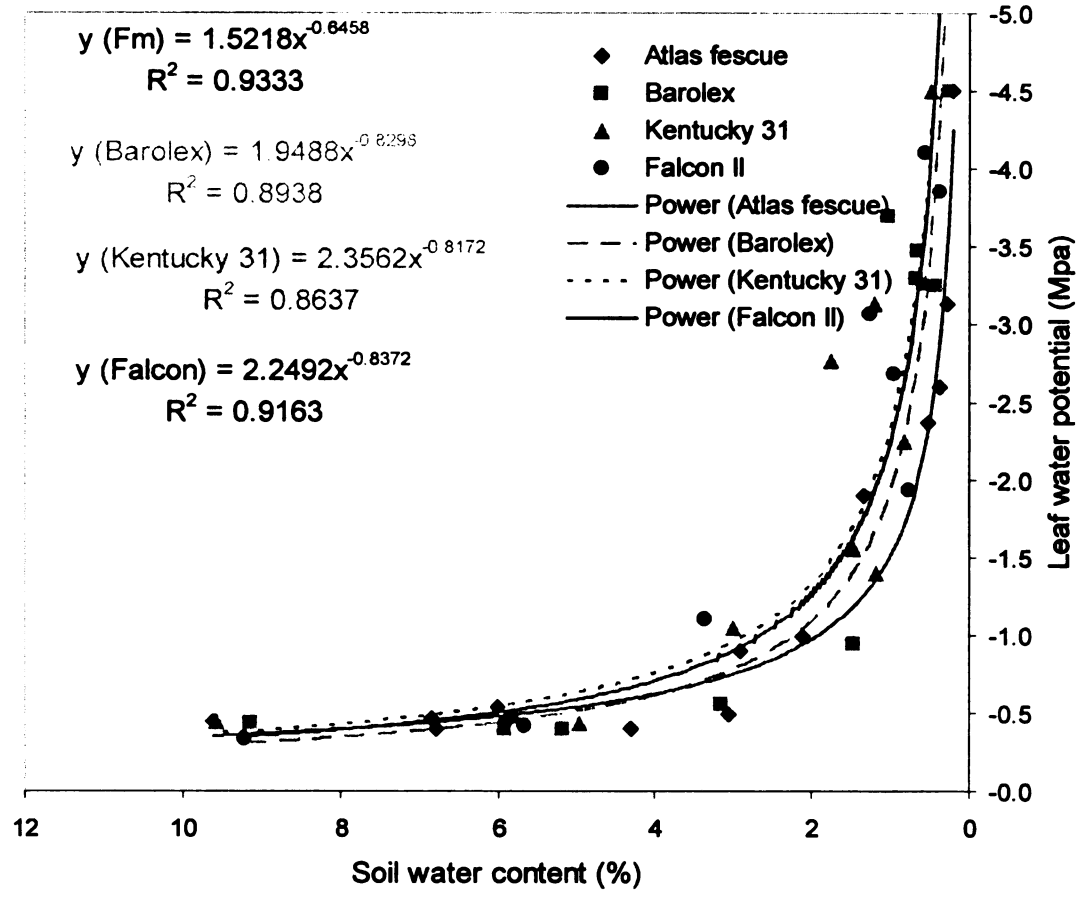

Figure 1.3. The relationship between soil water content and leaf water potential.

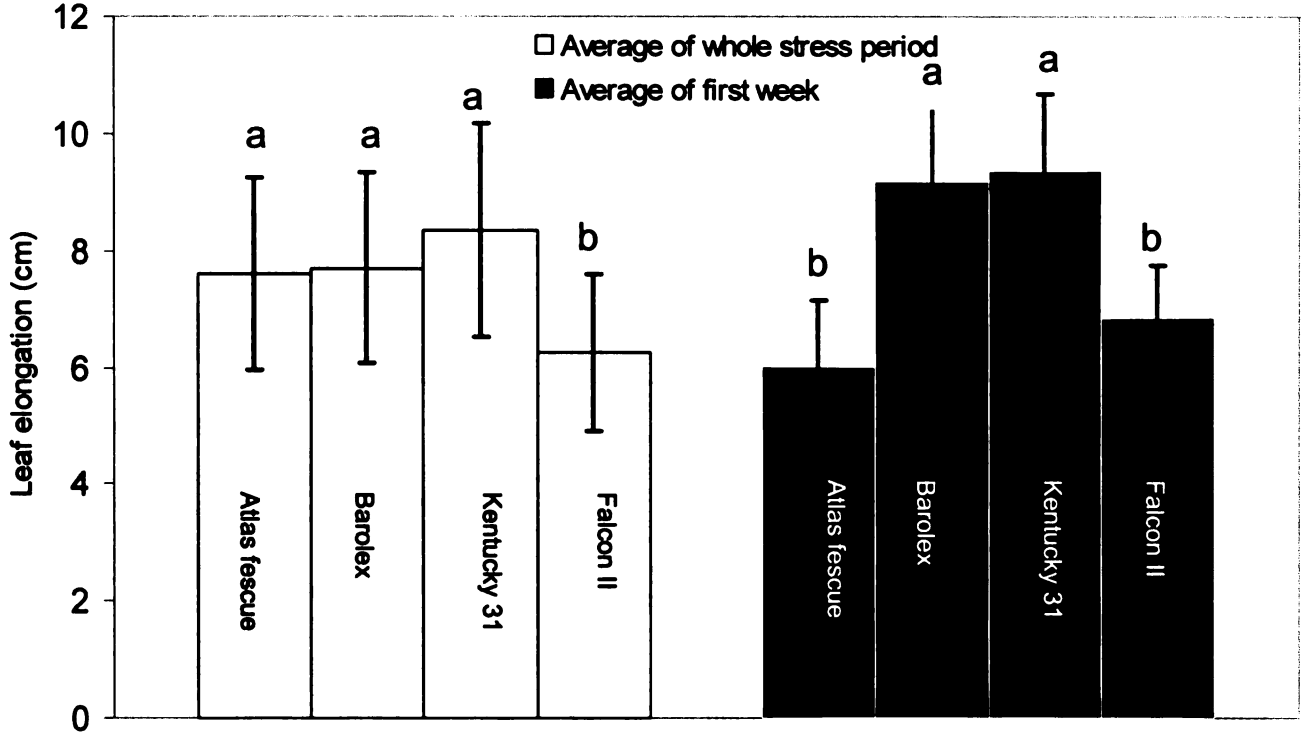

Figure 1.4. Leaf elongation of the treatment control plants during the drought period. Columns with the same letter indicate there are no significant differences among them at $P \leq 0.05$. Error bars indicate standard errors.

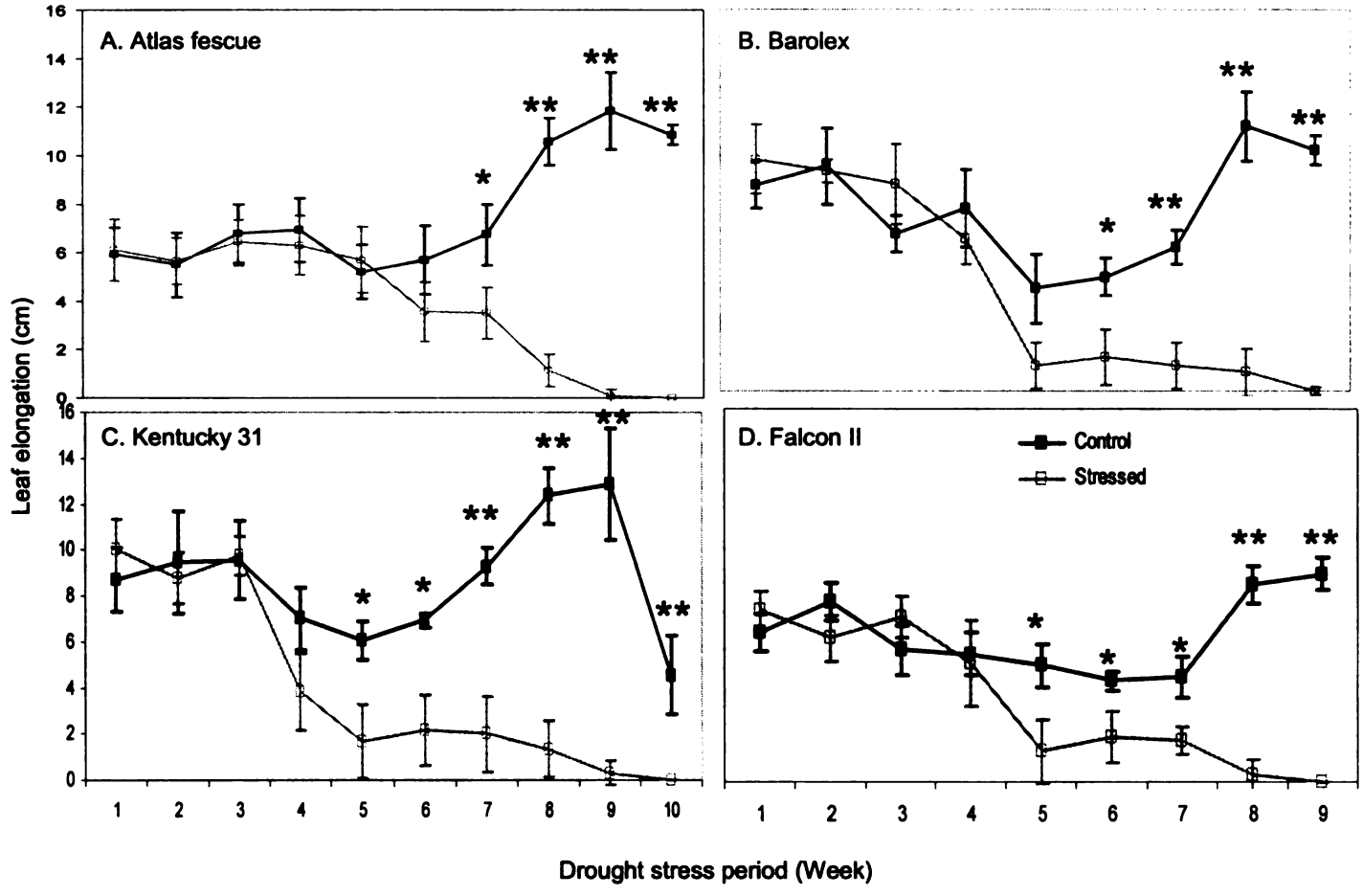

Figure 1.5. Leaf elongation during the drought stress period. Error bars indicate standard errors. *, ** Significantly different means between control and stressed plants at certain week at $P \leq 0.05$ and 0.01 respectively.

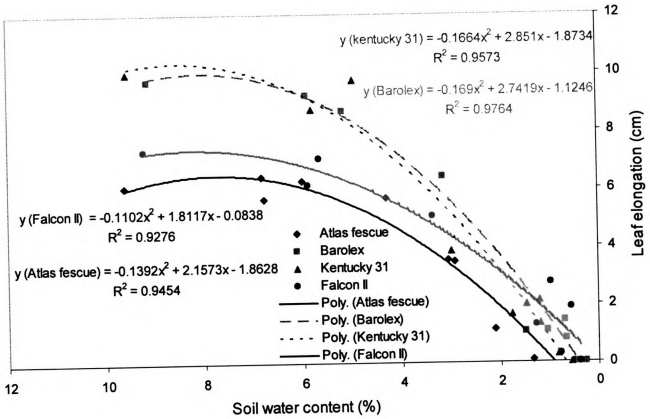

Figure 1.6. The relationship between leaf elongation and soil water content.

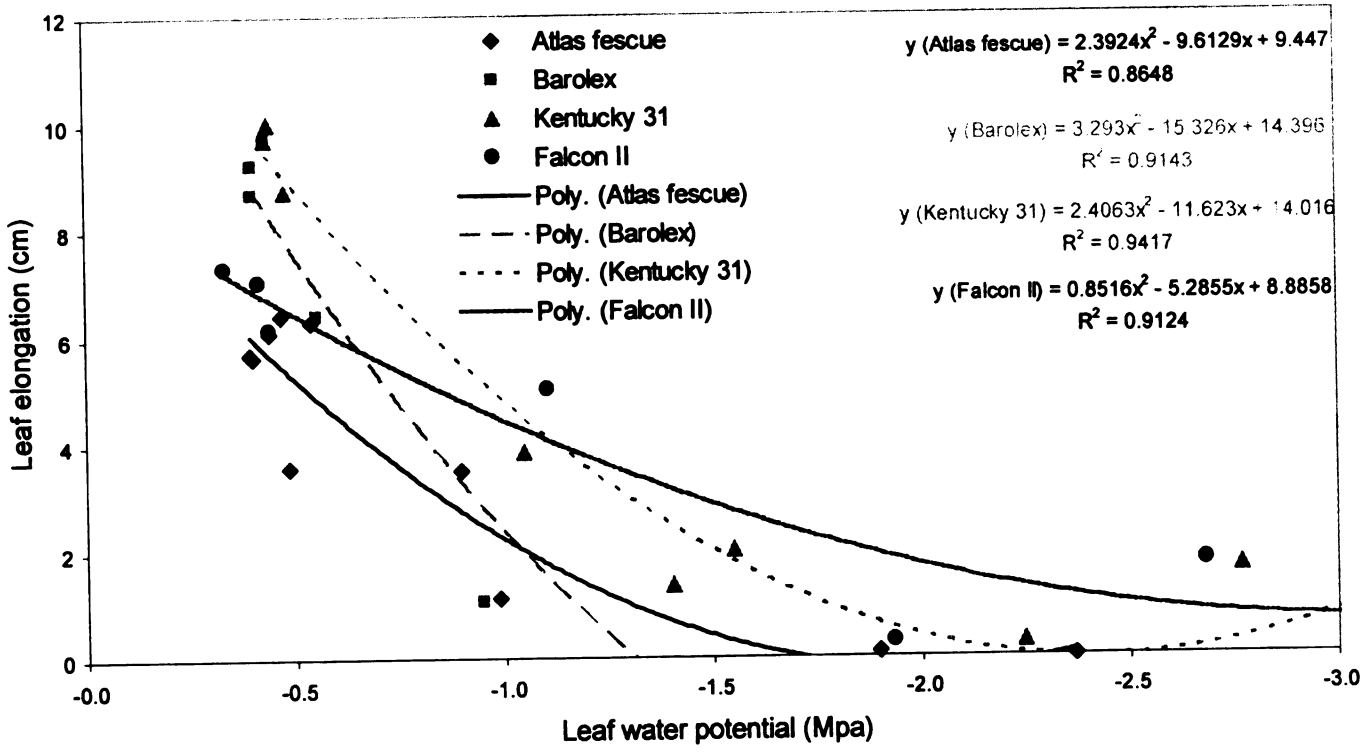

Figure 1.7. The relationship between leaf elongation and leaf water potential.

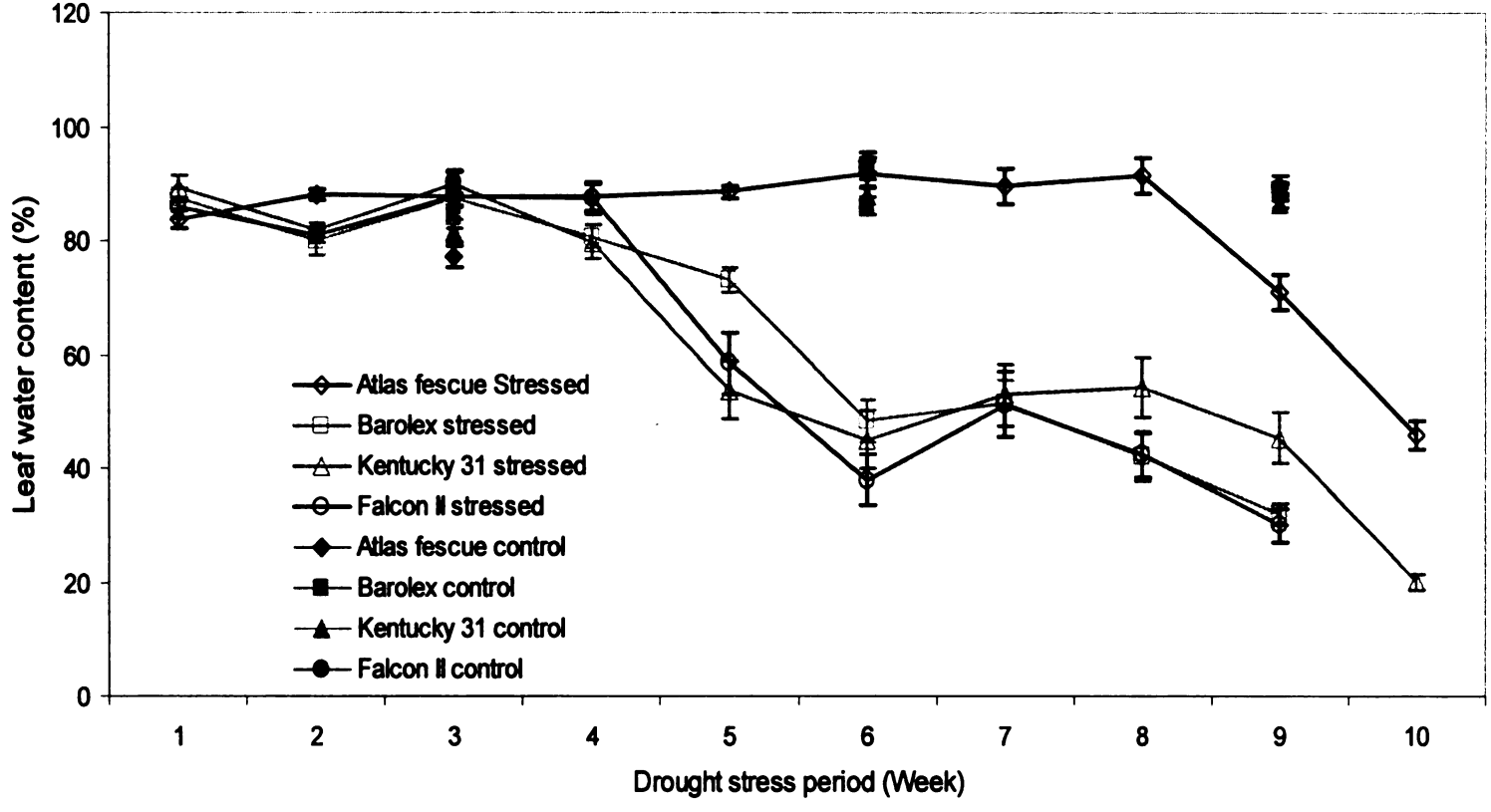

Figure 1.8. Leaf water content during the drought stress period. Error bars indicate standard errors. * Significantly different means among the stressed plants of four grasses at certain week at $P \leq 0.05$.

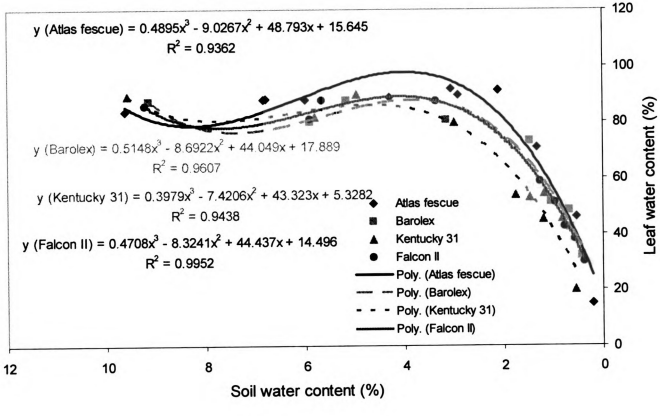

Figure 1.9. The relationship between soil water content and leaf water content.

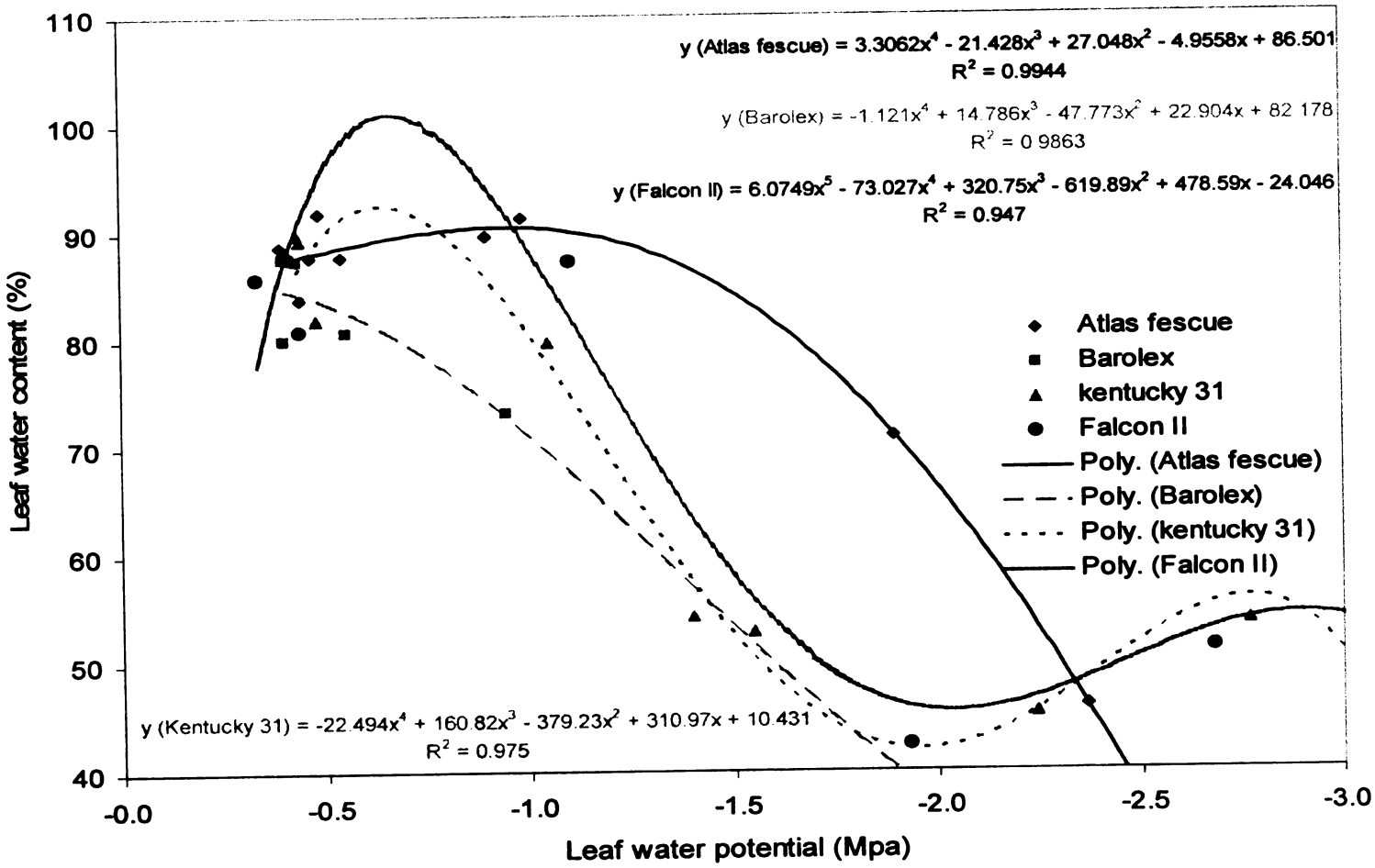

Figure 1.10. The relationship between leaf water potential and leaf water content.

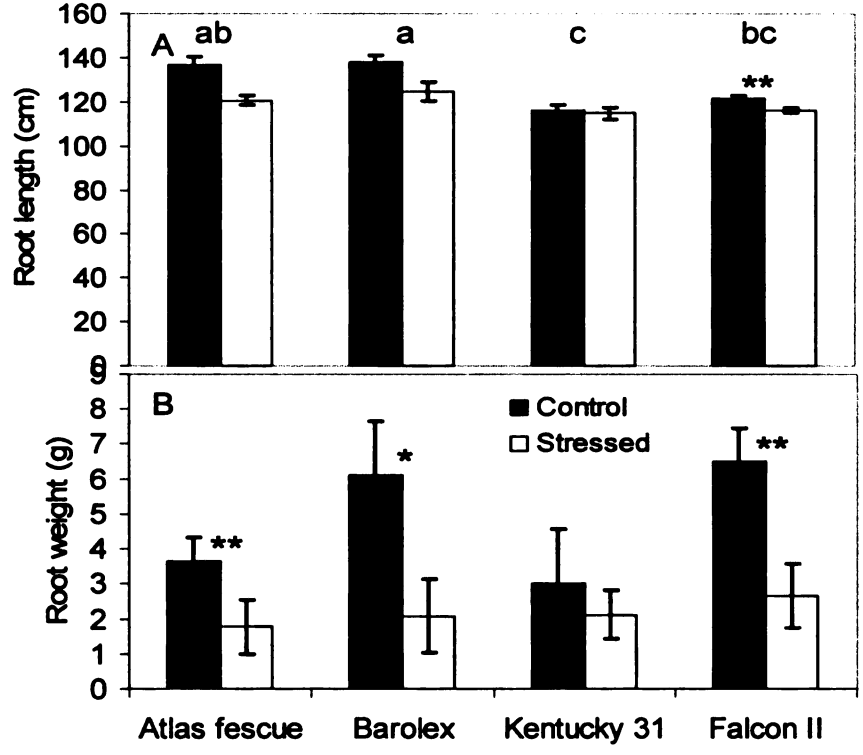

Figure 1.11. Root length and biomass comparisons of treatment control and stress treated plants. Error bars indicate standard errors. Bars with the same letter indicate there are no significant differences between them at $P \leq 0.05$. *, ** Significantly different means between control and stressed plants at certain week at $P \leq 0.01$.

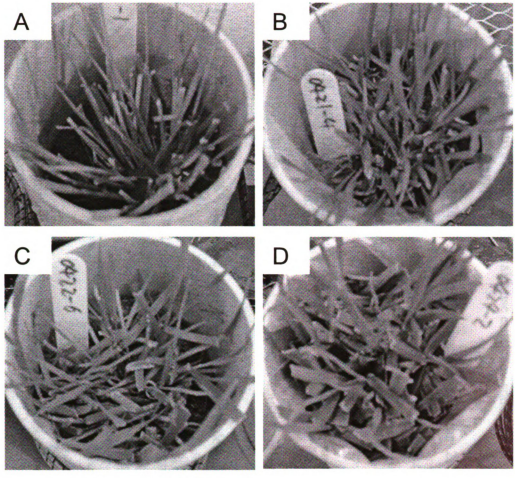

Figure 1.12. The phenotype of the Atlas fescue (A), Barolex (B), Kentucky 31 (C), and Falcon II (D) before imposing drought stress.

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CHAPTER II

Parental Genome Composition and Genetic Classifications of F_1 Hybrids and Backcross Progeny Derived from Intergeneric Crosses of *Festuca mairei* and *Lolium perenne*

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ABSTRACT

Intergeneric hybridization between Festuca and Lolium has been a long-term goal of forage and turfgrass breeders to generate improved cultivars by combining stress tolerance of *Festuca* with rapid establishment of *Lolium*. However, wide-distance hybridizations usually result in one of the genomes being eliminated from the hybrid due to incomplete chromosome pairings and crossovers. In this study, RAPD and SSR markers were used to detect the parental genome composition of hybrids and backcross derivatives generated from crosses between *Festuca mairei* St. Yves (Fm) and *Lolium perenne* L. (Lp). A total of 229 RAPD and 127 SSR polymorphic bands were used to estimate the parental genome composition of two F_1 hybrids, one amphiploid and 13 backcross progeny. Each of the 16 progeny exhibited integration of Fm and Lp genomes with varying levels of Fm/Lp genome ratios. Correlation (r=0.80) between the Fm/Lp genome ratios assessed by SSR and RAPD markers was highly significant ($P = 0.0004$).

The non-coinheritance of the linked markers suggested chromosome crossover between the two parents. Cluster and principle component analyses of the progeny consistently revealed four groups. Group I composed of one backcross progeny that had a distinctly different genetic background from other individuals. Group II included seven progeny that introgressed more of the Fm than Lp genome and clustered with the Fm parent. Group III comprised of six progeny showing similar amounts of genome introgression from both parents. Group IV contained two backcross progeny that introgressed more of the Lp genome and clustered with Lp parents. These results provide information on parental genome composition and classifications of 16 intergeneric progeny that would be useful to forage and turfgrass breeders.

Keyword: *Festuca mairei*, *Lolium perenne*, Simple sequence repeats (SSR), Random amplified polymorphic DNA (RAPD), Parental genome composition.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) (Lp) is a cool-season grass (2n=2x=14, LL) that has been widely used as turf and forage with superior quality and rapid establishment. However, lack of drought tolerance makes Lp less persistent during hot and dry summers. One approach for improvement of drought tolerance in perennial ryegrass is introgression of alien genomes from other drought tolerant genera, such as *Festuca* (Riewe and Mondart 1985). Intergeneric hybridization followed by backcrossing or by chromosome doubling can produce alien chromosome addition, substitution, or translocation progeny (Sharma et al. 1995). Derivatives from

intergeneric hybrids between *Festuca* and *Lolium* combining desirable agronomic attributes, or creation of novel allopolyploids may have great potential in grass breeding.

Festuca mairei St. Yves (Fm), commonly known as Atlas fescue, is a xerophytic tetraploid (2n=4x=28, $M_1M_1M_2M_2$). Fm tolerates high temperature and drought (Borill et al. 1971) and has a high photosynthetic rate (Randall et al. 1985). Combining the genomes of Fm and Lp could be an effective means to produce hybrids of high agronomic potential. Fm and Lp genomes show a distant relationship due to a low level of homeologous chromosome pairing and hence less genetic recombination (Chen et al. 1995). Using genomic in situ hybridization (GISH), homeologous chromosome pairing between L and M genomes has been detected in hybrids from crosses between Fm and Lp (Cao et al. 2000). This finding raises the expectation that chromosome crossover and genetic recombination may occur between the Lp and Fm genomes and the possibility of introgression of desirable genes from Fm into Lp exists. This possibility has also been confirmed by cytogenetic studies of the *Lolium/Festuca* complex (Humphreys et al. 1997).

Molecular markers have been widely used for efficiently detecting alien chromosome segments in many crops and are of increasing importance in distinguishing genomes between plant species. Fluorescence in situ hybridization (FISH), which utilizes chromosome-specific DNA probes, could be a powerful tool to detect alien genome introgression in such hybrids and backcross progeny. Compared to PCR-based molecular markers, the procedure of FISH is more difficult, needs trained personnel, and is relatively expensive. In addition, the amount of information obtained by the FISH procedure is very limited. The PCR based markers such as simple sequence repeat (SSR)

and random amplified polymorphic DNA (RAPD) are important genetic markers for plant genome analysis due to their genome-wide distribution, simple assay by PCR, and high levels of polymorphism. SSR markers are co-dominantly inherited and have been successfully isolated from perennial ryegrass, which constitute a valuable resource of markers for the molecular breeding of ryegrass (Kubik et al. 2001; Jones et al. 2001). In tall fescue (*Festuca arundinacea* Schreb.) (FA), a large number of SSR markers have been generated through mining the FA expressed sequence tag (EST) database, which could be applied in molecular mapping, comparative genomics, and molecular plant breeding across a wide range of turfgrass species (Saha et al. 2004). RAPD is another marker of choice for routine fingerprinting of germplasm and cultivars because of the low cost and random distribution throughout the genome. Even though RAPD markers are dominantly inherited, they are useful for monitoring genome introgressions from wild donor species to cultivated species (Bemabdelmouna et al. 1999; Siffelova et al. 1997).

Assessment of the genome introgression status of the progeny from intergeneric hybridization by using SSR and RAPD markers will be critical in directing breeding programs to develop improved grass cultivar. With the goal of transferring drought tolerance of Fm into Lp, a population comprised of hybrids, amphidiploid, and backcross progeny derived from intergeneric crosses between Fm and Lp were generated (Chen et al. 1995). For breeding application, evaluating or monitoring the Fm and Lp genome compositions in these progeny will assist in identifying individuals with desirable genome combinations and as a result new perennial ryegrass cultivars with improved drought tolerance can be developed.

The objectives of this study were to determine the Fm and Lp genome compositions of hybrids and backcross progeny using PCR-based molecular markers and to estimate relatedness between progeny and parents.

MATERIALS AND METHODS

Plant materials

A single Fm plant (Fm1) was chosen from a population collected in Morocco. This Fm population was adapted to the hot and dry summers of Northwest Africa (Borill et al. 1971). The other single plant of Fm (Fm2) was obtained from plant introduction, PI 283313. Two single Lp plants from turfgrass cultivars 'Citation II' (Lp1) and 'Calypso' (Lp2), respectively, were also chosen. Reciprocal intergeneric crosses between selected Fm and Lp plants were made to introduce drought tolerance of Fm to Lp (Chen et al. 1995). Three 3x F_1 hybrids (2n=3x=21) with reproductive tillers and two 4x F_1 hybrids (2n=4x=28) were generated. The 4x F_1 was generated by 2n gamete production (Chen et al. 1997). The partially fertile 4x F_1 hybrids, used as the female parents, were back-crossed to the diploid Lp (Chen 1996). The BC1 progeny were open-pollinated in isolation and 13 backcross progeny were obtained. A triploid F_1 hybrid from the Fm1 x Lp1 cross was treated with 0.25% colchicine at room temperature for 24 hr and an amphidiploid (2n=6x=42) plant was produced by successful chromosome doubling (Chen 1996). Plant materials used in this study included parental plants (Fm1, Lp1 and Lp2), two F_1 hybrids: a 4x F_1 (Fm1xLp2) and a 3x F_1 (Lp2xFm2), 13 backcross progeny, and the amphidiploid (the Fm2 plant was not included because it died after crossing).

DNA isolation

Total genomic DNA was extracted from young growing leaves. Plant cells were lysed using the extraction buffer (0.1M Tris- HCl, 0.05M EDTA-Na, 0.25M NaCl, PH=8.0 and 0.04M dodecyl sulfate (SDS)). Potassium acetate (5M) was used for deproteinization and recovery of DNA. Nucleic acid was precipitated by isopropanol followed by RNase treatment to degrade the RNA. The DNA concentration was measured by spectrophotometer readings at 260 nm and the purity was determined by the ratio of the absorptions at 260 nm and 280 nm. DNA quality was checked by loading 100 ng DNA in a 1% agarose gel by electrophoresis at 72 V for 2 hr.

RAPD screening Protocol

Forty-one decamer RAPD oligonucleotides (Operon Technologies, Alameda, California) (Table 2.1) were used in screening and detecting maximum polymorphism between the Fm and Lp parents and a F_1 hybrid. These 41 primers were mostly from C and Y kits (Charmet et al. 1997; Siffelova et al. 1997). Genomic DNAs from all the progeny were used as templates for RAPD analyses with these 41 polymorphic primers. The 25 µl RAPD reaction mixture contained 10mM Tris-HCl (pH=8.3), 4 mM MgCl2, 0.24 mM of each dNTP, 1.2 µM of primers, 30 ng of template DNA, and 1 U Taq. Amplification conditions were as follows: 3 pre-amplification cycles (94°C for 1 min, 35°C for 1 min and 72°C for 2 min). After initiation of the reaction, 35 amplification cycles were conducted (94°C for 20 s, 40°C for 20 s, and 72°C for 2 min). The last cycle was followed by 5 min at 72°C to ensure that primer extension reactions proceeded to

completion. RAPD profiles were generated in 2% agarose gel with 0.003% ethidium bromide subjected to electrophoresis at 72 V for 3.5 hr. A 1 Kb ladder was used to mark the size of the fragments. RAPD images were obtained through an Eagle Eye II still video system V3.2 (Stratagene, La Jolla, California).

SSR Screening Protocol

Seventy-six tall fescue EST-SSR primer pairs developed at the Samuel Roberts Noble Foundation (Saha et al. 2004), and 32 SSR primer pairs developed from ryegrass (Kubik et al. 2001; Jones et al. 2001) were tested on the Fm and Lp parents. The primer combinations that produced polymorphic bands between parents (Table 2.2) were then utilized to test all plant materials. The ethidium bromide detection protocol was used for ryegrass and 19 tall fescue EST-SSR primer pairs and the silver staining protocol was used for screening of the remaining primer pairs.

In ethidium bromide detection protocol, 10 µl PCR reaction mixture contained 10mM Tris-HCl (pH=8.3), 3 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of forward and reverse primers, 10 ng of template DNA, and 1 U Taq polymerase (Gibco Invitrogene, Grand Island, New York). PCR amplification was conducted in a PTC-100 programmable thermal controller (MJ Research, Waltham, Massachusetts). Amplification conditions were as follows: initial denaturation at 95°C for 5 min, 40 amplification cycles [95°C for 50 s, 42~60°C (the optimum annealing temperature for each primer pair, Table 2.2) for 50 s, and 72°C for 90 s], and the final extension of the reaction at 72°C for 10 min. SSR profiles were generated by running PCR products in a 6% non-denaturing polyacrylamide gel for 2.5 hrs at 350V. TBE buffer with 0.002%

ethidium bromide filled in the positive node tank was pre-run one hour for visualizing bands under UV light.

In silver staining protocol, 20 ng of DNA was used as a template for each PCR reaction. The PCR reactions consisted of one unit of AmpliTaq Gold® with GeneAmp PCR buffer II (Applied Biosystems/Roche, Branchburg, NJ), 3 mM MgCl2, 0.2 mM of dNTPs, and 0.2 μM of each primer in a 10 μl reaction. PCR amplification conditions were same as in ethidium bromide detection protocol. PCR products were resolved on 6% polyacrylamide denaturing gels (Gel Mix 6, Invitrogen Life Technologies). Gels were silver stained using Silver Sequence Kit (Promega, Madison, WI) for SSR band detection.

Data Analysis

Intense and repeatable bands in RAPD profiles were scored as 0 and 1 for absence and presence, respectively. In SSR profiles, the intense bands within the expected size range were scored as 0 and 1 for absence and presence, respectively. Parental Fm/Lp genome specific band ratios (Fm/Lp genome ratio) of the Fm-Lp hybrids and backcross progeny were calculated as the ratio of the percentage of Fm-specific-bands to that of Lp-specific bands with an assumption that all the markers are randomly dispersed in the whole genome. Dice coefficient (Dice 1945) was used to calculate similarity matrices for both SSR and RAPD data by running similarity for the qualitative data module using the numerical taxonomy and multivariate analysis system (NTSYSpc version 2.1, Exeter software, Setauket, New York). The Dice coefficient similarity matrices from SSR and

RAPD data were applied for cluster analysis independently with the option of sequential agglomerative hierarical nested (SAHN) cluster analysis and the unweighted pair-group method, an arithmetic average (UPGMA). The goodness of fit of each clustering with the distance matrix was tested using cophenetic matrix correlation. Resulting dendrograms from SSR and RAPD data were compared using cophenetic matrices and mantel test (Mantel 1967). Merged data from RAPD and SSR was used for cluster analysis to generate a final dendrogram. Bootstrap analysis was applied to assess the significance of the clusters in the dendrogram using FreeTree software (Pavlicek et al. 1999) with a resampling method of 2000 repetition counts. Principle component analysis was conducted using a correlation matrix from merged RAPD and SSR data and three eigenvectors were extracted. The data was projected onto these three eigenvectors and displayed by the Mod3D plot module.

RESULTS

Polymorphism and fragment segregation

1. Detected by SSR markers

The preliminary screening detected eight out of 32 ryegrass primer pairs and 27 of 76 tall fescue EST-SSR primer pairs that were polymorphic between the parents. Sequences and sources of these polymorphic primers are presented in Table 2.2. Amplification of ryegrass genomic SSRs and tall fescue EST-SSRs in the preliminary screening panel are presented in Figure 2.1.a and 2.1.b, respectively. A total of 127

polymorphic bands were scored from the 35 SSR primer pairs. Among the 127 bands, 23 (18.1%) were present in the Fm-Lp progeny but not in the three parents (Fm, Lp1, and Lp2). Such bands probably were contributed by the lost parent (Fm2). Fifty-one (40.2%) were Fm-specific and 51 were Lp-specific bands (12 Lp1-specific, 22 Lp2-specific and 17 common bands between Lp1 and Lp2). The relatively large number of bands common to Lp1 and Lp2 indicated a close relationship between these two genotypes. Only one common band was found between Fm and Lp1 as well as between Fm and Lp2, which indicate wide genetic distances between the Fm and Lp genomes.

All 127 bands segregated among the Fm-Lp progeny (Table 2.4). More than half of the alleles of both parents (Fm1 and Lp1/Lp2) were combined in the 4x F1 hybrid (Fm1 x Lp2) and the amphiploid derived from a 3x F1 (Fm1 x Lp1) crosses, indicating successful wide crosses. In all backcross progeny, different levels of alleles of both Fm and Lp parents were present in each individual (Table 2.4), which suggested segregation of alleles from both parents during backcrossing.

Table 2.5 showed the Fm/Lp genome ratio of the Fm-Lp progeny. The higher Fm/Lp genome ratio basically indicated more Fm genome introgression into the progeny. Results revealed all the progeny had an Fm/Lp genome ratio above zero indicating that the Fm genome was successfully introgressed into these individuals. However, the ratios ranged from 0.09 (G14) to 1.95 (Fm1 x Lp2) indicating that the Fm genome had been retained in these progeny at various extents.

Of the 27 polymorphic EST-SSR loci, 15 have been mapped to ryegrass linkage groups (LGs) (Warnke et al. 2004) (Table 2.3). Three groups of these marker loci were uniquely mapped on both male and female maps. NFFA031 and NFFA75 were mapped

on LG 1 with an interval of 19 cM. NFFA015, 036, and 048 were mapped on LG 6 with the interval of 29 and 17 cM, respectively. NFFA019 and NFFA069 were tightly linked on LG 7 with an interval of 6 cM. To investigate the event of chromosome crossover between genome M and L, the co-segregation of markers on each of the three LGs was assessed among the Fm-Lp hybrids and backcross individuals. The results indicated that all the linked markers, including the tightly linked NFFA019 and NFFA069, were not co-inherited into the hybrids or backcross individuals. The separations of the linked markers suggested the crossover of homeologous chromosomes and M and L genome recombination in the progeny from intergeneric hybridization.

2. Detected by RAPD markers

Amplification of RAPD primers in the preliminary screening panel is presented in Figure 2.1.c. In total, 229 polymorphic bands were generated from 41 RAPD primers. The number of polymorphic bands scored for each primer ranged from 1 to 11. Distribution of the 222 RAPD bands among the parents was similar to that of the SSR markers. Thirty-six bands (15.7%) were present in the progeny but not in the three parents indicating the contribution of the Fm2 genome. Ninety-six (41.9%) were Fm-specific and 87 (38.0%) were Lp-specific bands including the Lp1- and Lp2-specific bands and the bands common to both parents. Similar to the SSR results, a higher number of common bands between Lp1 and Lp2 (41, 17.9%) suggested a relatively close relationship between Lp1 and Lp2, and a lower number of common bands between Fm and Lp (Lp1, 4.5%; Lp2, 3.0%) suggested a distant relationship between Fm and Lp.

RAPD results were consistent with SSR results as both parent- specific bands were inherited in the F_1 hybrids and amphiploid, and various levels of segregation

occurred in the backcross progeny (Table 2.4). Fm/Lp genome ratios (Table 2.5) of these Fm-Lp progeny ranged from 0.08 (G11a) to 1.79 (Fm1 x Lp2). This result confirmed that all progeny retained the Fm genome at different levels. The correlation coefficient (r=0.80) of the Fm/Lp genome ratios assessed by SSR and RAPD markers was highly significant (P = 0.0004), which reflected the reliability of the two marker systems in assessing genome introgression.

Genetic classification analysis

Cluster analysis of RAPD and SSR data generated two similar dendrograms. The goodness of fit of the clustering with the similarity matrix was tested using cophenetic matrix correlation. Both dendrograms were fitted with the corresponding similarity matrices by showing a correlation value of 0.778 with SSR data and 0.884 with RAPD data. The two dendrograms were compared using cophenetic matrices and mantel tests. The correlation between the two dendrograms was significant (r=0.723, *Prob. Random Z< obs. Z: P=1.000*), and therefore SSR and RAPD data were merged to yield one pairwise similarity matrix. The similarity coefficients ranged from 0.063 (between Fm1 and Lp2) to 0.868 (between G6 and G27a), demonstrating a wide distance between parents and a varied range of genetic distances among the progeny. Cluster analysis based on this similarity matrix generated a dendrogram (Figure 2.2) with a high goodness of fit (r=0.864).

The cluster analysis revealed four groups (Figure 2.2). Group I contained only one backcross progeny individual, G8. Group II consisted of the parent Fm1, 4x F_1

hybrid of Fm1 x Lp2, the amphiploid derivative from the chromosome-doubled 3x F_1 of Fm1 x Lp1 and 5 backcross progeny: G6, G16, G26, G27a, and G30b. Group III included a 3x F_1 hybrid of Lp2 x Fm2, and 5 backcross progeny: G11b, G15, G24, G27b, and G30a. Group IV had two Lp parents and two backcross progeny, G11a and G14. Results suggested that: (1) G8 in group I has a distinct genetic background and differentiated from the Fm-Lp genetic basis by showing novel fragments in both RAPD and SSR analysis, because all the other progeny except G8 formed one distinct branch from group I with a bootstrapping value of 99% (Figure 2.2), (2) progeny within group II introgressed more of the Fm genomes, because of clustering with Fm1, (3) group III was between the two parental groups, the progeny in this group showed similar amount of genomes from both Fm and Lp, and (4) in group IV the two backcross progeny had retained very little of the Fm genomes and therefore were highly Lp-like progeny. The reliability of the clusters was evident by relatively high bootstrapping values at all the branches (Figure 2.2).

The principle component analysis, which is based on the original data from SSR and RAPD, rather than a similarity matrix, was performed for further confirmation of the genetic differences of the progeny (Figure 2.3). The three-dimensional scatter plot distribution of the Fm-Lp progeny consistently revealed the four groups derived from cluster analysis. Group I with only G8 was distinct from the other individuals in the analysis by showing a high R3 value. This result suggested a big genome change or rearrangement in G8, which may have happened during hybridization of the wide cross between Fm and Lp, or G8 might have been mislabeled in the greenhouse. Fm-Lp progeny in group II were relatively more sparsely scattered between the parents, Fm and

Lp, particularly G16, which is consistent with the cluster analysis showing a relatively lower bootstrapping value (47%). Group III was located further away on the edge of the graph along the R1 and R2 axes, which suggested genetic differences between Fm1 and Fm2 plants. Group IV was tightly clustered indicating the close relationship among the two backcross progeny and two Lp parents and suggested that this two backcross progeny had less Fm genome and more Lp genome, which is consistent with the Fm/Lp genome ratio results (Table 2.5).

DISCUSSION

Molecular marker application for genome composition detection

Reproducibility of some PCR-based markers (e.g., RAPD) can be a source of concern. In this study, we took an approach involving two steps for screening the molecular markers. First, the markers were tested only on the two parents for PCR amplification and the sizes of high intensity clean bands were recorded. Second, the parents were included along with the progeny for recording the segregation and inheritance of only the bands that were previously detected in the parents. This approach improved the reproducibility of the markers and increased the reliability of the analysis. Significant correlation between the Fm/Lp genome ratios assessed by SSR and RAPD markers verified the value of the PCR-based markers in genome introgression assessments, which is in agreement with previous studies (Charmet et al. 1997; Prakash et al. 2002).

The mapped EST-SSR markers on ryegrass LGs showed a great applicable value in assessment of the homeologous chromosome cross-over and genome recombination in the intergeneric hybrid, which is normally tested by sophisticated cytogenetic studies. In addition, the map location of EST-SSRs derived from transcripts with known functions, may provide functional genetic markers for direct characterization of the QTLs for putatively correlated traits (Saha et al. 2005). Genomic SSRs are highly variable because they are mostly in the non-coding sequence and are less conserved, which limits their uses across different species. EST-SSR markers are derived from transcribed regions of the DNA, and are generally more conserved and have a higher rate of transferability when compared with genomic SSR markers (Scott et al. 2000). Gupta (2002) found 95% of the EST-SSR primer pairs exhibited 100% similarity between *Hordeum* and *Triticum*, which indicated that the flanking sequences of SSRs were not only conserved across species but also across related genera within Poaceae (Triticeae EST-SSR Coordination). In this study, a total of 32 SSR markers developed from the perennial ryegrass genome were screened against Fm and Lp and 8 (25%) displayed distinct and polymorphic amplification from both genomes, whereas 27 of 76 EST-SSR markers (35.5%) successfully discriminated between Fm and Lp. The relatively higher rate of cross genera amplification might reflect the usefulness of EST-derived microsatellite markers for molecular genetic analysis for wide distance hybridization.

Fm-Lp genome recombination in the 4x F_1

In the 4 x F_1 hybrids derived from Fm x Lp1, 84.3 and 90.6% of Fm-specific SSR and RAPD bands were inherited, respectively (Table 2.4). Theoretically, at these loci, the

genotype of Fm, as an autotetraploid, could be Aaaa, AAaa, AAAa or AAAA and the Lp genotype is aa. As a result, the band ratios of F_1 should be 1:1 for Aaaa x aa, 5:1 for AAaa x aa, 1:0 for AAAa x aa and AAAA x aa. If the four Fm genotypes among these loci have the same ratio, then on average, the F_1 could have 83.2% Fm-specific bands. The $\chi 2$ test was used to test the significance of consistency of observed Fm-specific bands presented in the 4x F_1 with the theoretical expectation. The analysis revealed that, for both SSR and RAPD data, the observations were consistent with the expectation (P=0.8 and P=0.05 for SSR and RAPD data, respectively). Fm was considered an autotetraploid or at least a partial allotetraploid because the genomes of M1 and M2 are closely related and readily paired in the F_1s of Fm and Lp (Chen et al. 1995). Our results supported the autotetraploidy of Fm. The genome of the other parent, Lp1 was transferred into the 4x F_1 by 75% and 53% dominant alleles detected by SSR and RAPD, respectively. Lp1 was transferred to the F_1 through 2n pollen and the relatively high rate of dominant alleles transfer suggests that the 2n pollen were produced through first division restitution (FDR) (Chen et al. 1997).

Fm-Lp genome recombination in the progeny

Exploitation of available variability for improvement of any crop depends on the ability to introgress the desirable genome of source plants to cultivated varieties (Prakash et al, 2002). This strategy is largely facilitated by precise monitoring of alien and cultivated genome combinations at a molecular level. In backcross progeny detected in this study, dominant alleles from both Fm and Lp parents were present in each individual at various levels (Table 2.4), suggesting segregation of alleles of both parents during the

backcrossing. Estimating allelic segregation ratios was not useful because of the involvement of two genotypes of Lp, the interpollination for the generation of backcross progeny and the limited population size. In general, the Fm/Lp genome ratios (Table 2.5) could reflect recombination of the two genomes from different genera in backcross individuals. Fm/Lp genome ratios estimated for the two F_1 hybrids and amphiploid (Table 2.5) did not indicate the ratio of the parents' genome involvement. The reason is that for the estimation of genome ratios, the number of Lp-specific bands including both Lp1- and Lp2-specific bands was used, but for generation of F_1 hybrids, only one genotype of Lp (either Lp1 or Lp2) was utilized for crossing. For example, in 4x F_1 of Fm x Lp1, the Fm/Lp genome ratio from the SSR marker is 1.95, which is derived from the ratio of the percentage of Fm-specific to Lp-specific bands. However, the Fm/Lp genome ratio should be the percentage of Fm-specific bands to Lp1-specific bands and should equal 1.12 (84.3/75).

In backcross progeny G6 and G27a, the Fm/Lp genome ratios assessed by SSR and RAPD markers were not highly consistent (Table 2.5) because of the lower ratios obtained by SSR markers and much higher ratios by RAPD markers. This result could be due to limited number of markers applied.

Classification of the progeny

Weak grouping and low bootstrapping value typically associate with a flow of genetic information among the individuals (Prakash et al, 2002). In this study, grouping from cluster analysis and bootstrap (Figure 2.2) was highly consistent with the grouping from principle component analysis (Figure 2.3), and the bootstrapping values at all the

branches were greater or at least equal to 47%. This result might suggest the process of genome stabilization in the Fm-Lp progeny.

Most backcross individuals contained large amounts of the Fm genome, and therefore, either clustered with Fm or in the middle of parental groups, except G11a and G14, which closely clustered with Lp. Also, the 4x F_1 of Fm x Lp was clustered with Fm and the amphiploid was closer to Fm than to Lp (Figure 2.2, Figure 2.3, and Table 2.5). The Fm-biased classification might be due to larger amounts of Fm-specific bands that were detected and used in this study. Even though, more backcross generations are necessary to recover more of the Lp genome and at the same time maintain the desired introgressed Fm characteristics. In general, cluster analysis and the principal component analysis consistently provided visualized information on the introgression status of these Fm-Lp progeny, which could be used as a guide in breeding programs, such as which progeny retained the Fm alien genome, and which progeny should be backcrossed to the cultivated Lp to recover good turf quality.

Application of the Fm-Lp progeny for turfgrass breeding

The partially fertile 4x F_1 hybrid could be very useful in a backcross-breeding program to develop a diploid perennial ryegrass, which hopefully would inherit a certain level of drought tolerance from Fm. The 3x F_1 hybrid was sterile, however, fertility could largely be restored through chromosome doubling and therefore has potential use in developing new cultivars. With several generations of backcrossing and selection for meiotic stability and turf quality, a drought tolerant cultivar could be developed. In this study, progeny G11a and G14 recovered most of the Lp genome with only one generation

of backcrossing to Lp (Table 2.5). They could be tested for improved drought tolerance and meiotic stability to evaluate the potential for new cultivar release. The other backcross progeny need more generations of backcrossing to Lp to recover more perennial ryegrass attributes.

The ability of molecular marker to discriminate between Lolium and Festuca DNA in hybrids and backcross progeny enables introgression maps to be created if these markers have been localized on a linkage map. By combining the genetic mapping approach and physiological complex trait dissection, it should be possible to identify and localize the importance of trait components that contribute to drought tolerance (Humphreys et al, 1997). Markers associated with trait components can be applied to assist in drought tolerant progeny selection and speed up the breeding process. It is worth noting that in our study a number of Fm-Lp progeny successfully combined both genomes from Fm and Lp and some of those progeny showed desirable agronomical traits in our initial greenhouse evaluation (unpublished data, 2005). The results provide information that the Fm-Lp progeny could be used not only as the basis for new cultivar release but also for drought tolerance associated marker development.

Table 2.1. The name and sequence of 41 RAPD polymorphic primers and the number of fragments amplified. All the RAPD oligonucleotides are from Operon Technologies.

Primer	Sequence	Number of polymorphic fragments scored
OPA-04	5'-AATCGGGCTG-3'	7
OPA-05	5'-AGGGGTCTTG-3'	4
OPA-07	5'-GAAACGGGTG-3'	6
OPA-08	5'-GTGACGTAGG-3'	6
OPA-20	5'-GTTGCGATCC-3'	4
OPB-12	5'-CCTTGACGCA-3'	9
OPC-01	5'-TTCGAGCCAG-3'	9
OPC-02	5'-GTGAGGCGTC-3'	5
OPC-04	5'-CCGCATCTAC-3'	2
OPC-05	5'-GATGACCGCC-3'	11
OPC-06	5'-GAACGGACTC-3'	3
OPC-07	5'-GTCCCGACGA-3'	5
OPC-08	5'-TGGACCGGTG-3'	11
OPC-09	5'-CTCACCGTCC-3'	9
OPC-10	5'-TGTCTGGGTG-3'	5
OPC-11	5'-AAAGCTGCGG-3'	7
OPC-13	5'-AAGCCTCGTC-3'	7
OPC-15	5'-GACGGATCAG-3'	7
OPC-16	5'-CACACTCCAG-3'	4
OPC-19	5'-GTTGCCAGCC-3'	3
OPC-20	5'-ACTTCGCCAC-3'	6
OPE-09	5'-CTTCACCCGA-3'	4
OPY-01	5'-GTGGCATCTC-3'	5
OPY-02	5'-CATCGCCGCA-3'	10
OPY-03	5'-ACAGCCTGCT-3'	6
OPY-05	5'-GGCTGCGACA-3'	7
OPY-06	5'-AAGGCTCACC-3'	5
OPY-07	5'-AGAGCCGTCA-3'	4
OPY-09	5'-AGCAGCGCAC-3'	5
OPY-10	5'-CAAACGTGGG-3'	4
OPY-13	5'-GGGTCTCGGT-3'	1
OPY-14	5'-GGTCGATCTG-3'	5
OPY-15	5'-AGTCGCCCTT-3'	3
OPY-16	5'-GGGCCAATGT-3'	4
OPY-17	5'-GACGTGGTGA-3'	6
OPY-18	5'-GTGGAGTCAG-3'	2
OPY-19	5'-TGAGGGTCCC-3'	5
OPY-20	5'-AGCCGTGGAA-3'	5
OPX-01	5'-CTGGGCACGA-3'	11
OPX-06	5'-ACGCCAGAGG-3'	4
OPX-13	5'-ACGGGAGCAA-3'	3
Total		229

Table 2.2. The sequences, annealing temperatures and sources for both ryegrass genomic- and tall fescue EST-SSR primer pairs.

No.[a]	Locus	Source or Sequence ID	Forward primer sequence	Reverse primer sequence	Tm[b] (°C)
1.	PR14	Kubik et al. 2001	agg gtt cgt ctg cat tc	agc aga acc gag ccg tc	47
2.	PRG	Kubik et al. 2001	gcc gag tgt cat caa ggt	cct ttt cgc ctt cgt a	42
3.	LP8	Kubik et al. 2001	tga ctt ctc tcg atc ct	atg tga cta caa aac ca	40
4.	M4 213	Kubik et al. 2001	cac ctc ccg ctg cat ggc atg t	tac aac gac atg tca agg	45
5.	H01E10	Jones et al. 2001	cgc agc tta att tag tc	gct ttg agt atg taa agt t	40
6.	H02C11	Jones et al. 2001	tgg aat aac gat gaa aag	cat cac gaa tta aca aga g	40
7.	K01A03	Jones et al. 2001	gga cga act gcc gag aca	cgg gca tgg tga gaa gga	52
8.	H01H06	Jones et al. 2001	att gac tgg ctt ccg tgt t	cgc gat tgc aga ttc ttg	47
9.	NFFA001	FA42E11LF087	ctg ctg ctg cca aga aag t	taa ggg gag cga gct aca ga	60
10.	NFFA013	FA10F10LF090	tca ttg tgt tcg ctc tcc tg	cct tcg tcg cca tgg tag	62
11.	NFFA015	FA03G10RT072	gcg tcc act aac aac acc aa	agc aag gcc agc aaa aat ta	60
12.	NFFA019	FA01E03ST023	tgg att tgc aat tag cct ca	gct cgt gta tgg cct tca at	60
13.	NFFA021	FA29F08DS075	cac agc tcg tat agg cgt ca	ctt gtc gaa gag cgg gaa c	62
14.	NFFA022	FA02B04RT032	atg atg tcc gag gag gag aa	cat cat gat cca gtg cct tg	60
15.	NFFA024	FA37F03LF031	tgc cca cga ggt cta tct tc	agc ttc ccc ttc att cca ct	60
16.	NFFA027	FA12H05RT048	cga ggt ctc aat cct cca tt	gac aga gac gac gac gac at	62
17.	NFFA030	FA05D05RT042	agt cgg tgg tga agc tga ag	aca act agg ggg ctg gtc a	62
18.	NFFA031	FA46C01LF006	acg gtc tgt acc gtg gat gt	gct gta gac tca gcc gaa cc	64
19.	NFFA032	FA28D02LF026	acg gtc tgt acc gtg gat gt	gct gta gac tca gcc gaa cc	64

Table 2.2. The sequences, annealing temperatures and sources for both ryegrass genomic- and tall fescue EST-SSR primer pairs (cont'd).

No. [a]	Locus	Source or Sequence ID	Forward primer sequence	Reverse primer sequence	Tm [b] (°C)
20.	NFFA035	FA31F07DS063	tgc tag cag ggg tct aag ga	cac acg tac cac gtc tcc at	62
21.	NFFA036	FA18B10RT089	aga gga aga gcg aaa gag ca	ccc tgg tac tcg tgg atg tt	60
22.	NFFA038	FA22B04RT041	gtg gtg gtg gtg tgt tgt tg	gca gat tta cca gcc aag ga	62
23.	NFFA039	FA51A08LF065	gtc tgc acc cct ctc ctc tc	ctc ctt atc ttg gcg atg ga	64
24.	NFFA042	FA25B06DS057	ctg tcg tgg acg agg aga a	cac gat acc cag ttc aag ca	60
25.	NFFA043	FA08D07RT060	tcc agg ttc cac tcc cac t	agc cga aac cag att gga c	60
26.	NFFA045	FA55E03DS023	acg agg gaa agg tag ggt tt	gat gaa gcc aat ttc ctt gg	60
27.	NFFA048	FA05E11LF087	cag gct gtt aac ggt gtc ct	cct tct tct tgg gag gga aa	60
28.	NFFA051	FA39D12LF102	ttt gca ctc tcg gac cta gc	cgg tac acc ttc tgc acc tt	62
29.	NFFA056	FA20E05DS039	gca cga ggc tct ttc ctc ta	ggt gct tgg cct tct tcc	62
30.	NFFA061	FA16B03DS029	tgg att tgc aat tag cct ca	gct cgt gta tgg cct tca at	60
31.	NFFA064	FA11E11DS087	tca ttt gac gcc act tga ac	gtc tta gcg cct tcc ttg gt	60
32.	NFFA065	FA34A01RT005	gga tgg atc ctc aca atg ct	ctc ctc ctc tcc tcc agc tc	64
33.	NFFA069	FA32F09LF079	ccc aag aag aag acg acc aa	acg acc gaa tgg aca gag ac	62
34.	NFFA071	FA29A02RT017	tcc taa gca gag ctc gat cc	gag gtt ggc gaa ctt cct c	62
35.	NFFA075	FA34E06DS049	ctc tgc cct tcc ttc ctc tt	atg gtc tcc ctc tgc tcg ta	60

[a] Loci numbered 1-8 were from Lp genomic SSR (1-4 from Kubik et al. 2001 and 5-8 from Jones et al. 2001). Loci numbered 9-35 were from fall fescue EST-SSR (Saha et al. 2004).

[b] Annealing temperature.

Table 2.3. Linkage location of the EST-SSR markers on ryegrass linkage groups.

Locus	Linkage location [a]
NFA015	A6, B6
NFA019	A7, B7
NFA021	A2, A7, B7
NFA024	B7
NFA027	A5, B5
NFA030	B2
NFA031	A1, B1
NFA036	A6, B6
NFA039	A2, B2
NFA045	A4, B4
NFA048	A6, B6
NFA061	B7
NFA064	A5
NFA069	A7, B7
NFA075	A1, B1

[a] Refer to Warnke et al. 2004.

Table 2.4. The segregation of polymorphic bands among the Fm-Lp progeny based on the parent bands pattern from SSR and RAPD screening.

Genotype [a]	000 [b]		001		010		011		100		101		110		111	
	SSR (N=23)(%)	RAPD (N=36)(%)	SSR (N=22)(%)	RAPD (N=29)(%)	SSR (N=12)(%)	RAPD (N=17)(%)	SSR (N=17)(%)	RAPD (N=41)(%)	SSR (N=51)(%)	RAPD (N=96)(%)	SSR (N=1)	RAPD (N=3)	SSR (N=1)	RAPD (N=2)	SSR (N=0)	RAPD (N=5)
Fm1XLp2	2 (8.7)	0 (0.0)	1 (4.5)	2 (6.9)	9 (75.0)	9 (52.9)	12 (70.6)	33 (80.5)	43 (84.3)	87 (90.6)	1	3	1	2	0	5
Lp2XFm2	17 (73.9)	20 (55.6)	6 (27.3)	14 (48.3)	5 (41.7)	5 (29.4)	11 (64.7)	17 (41.5)	24 (47.1)	35 (36.5)	0	2	1	1	0	3
D1	3 (13.0)	1 (2.8)	4 (18.2)	2 (6.9)	9 (75.0)	7 (41.2)	11 (64.7)	27 (65.9)	33 (64.7)	68 (70.8)	1	3	1	1	0	5
G6	3 (13.0)	1 (2.8)	12 (54.5)	12 (41.4)	5 (41.7)	4 (23.5)	12 (70.6)	29 (70.7)	16 (31.4)	62 (64.6)	0	0	1	0	0	0
G8	8 (34.8)	15 (41.7)	4(18.2)	6 (20.7)	1 (8.3)	5 (29.4)	6 (35.5)	8 (19.5)	12 (23.5)	16 (16.7)	0	1	0	0	0	1
G11a	0 (0.0)	3 (8.3)	13 (59.1)	18 (62.1)	6 (50.0)	9 (52.9)	17 (100.0)	27 (65.9)	5 (9.8)	5 (5.2)	1	2	1	1	0	5
G11b	11 (47.8)	22 (61.1)	8 (36.4)	13 (44.8)	6 (50.0)	7 (41.2)	11 (64.7)	21 (51.2)	20 (39.2)	40 (41.7)	0	1	1	2	0	2
G14	1 (4.3)	1 (2.8)	10 (45.5)	12 (41.4)	9 (75.0)	3 (17.6)	13 (76.5)	30 (73.2)	3 (5.9)	7 (7.3)	0	2	1	1	0	5
G15	14 (60.9)	23 (63.9)	4 (18.2)	13 (44.8)	6 (50.0)	5 (29.4)	9 (52.9)	18 (43.9)	22 (43.1)	44 (45.8)	0	3	1	1	0	5
G16	3 (13.0)	3 (8.3)	10 (45.5)	12 (41.4)	8 (66.7)	4 (23.5)	13 (76.5)	35 (85.4)	22 (43.1)	37 (38.5)	0	2	1	1	0	5
G24	14 (60.9)	23 (63.9)	9 (40.9)	9 (31.0)	5 (41.7)	7 (41.2)	13 (76.5)	19 (46.3)	29 (56.9)	45 (46.9)	0	2	0	2	0	5
G26	2 (8.7)	2 (5.6)	8 (36.4)	13 (44.8)	6 (50.0)	7 (41.2)	13 (76.5)	32 (78.0)	16 (31.4)	56 (58.3)	0	3	1	1	0	4
G27a	3 (13.0)	2 (5.6)	10 (45.5)	10 (34.5)	6 (50.0)	4 (23.5)	14 (82.4)	30 (73.2)	14 (27.5)	59 (61.5)	0	3	1	0	0	5
G27b	12 (52.2)	17 (47.2)	10 (45.5)	13 (44.8)	2 (16.7)	4 (23.5)	10 (58.8)	15 (36.6)	24 (47.1)	40 (41.7)	0	2	1	1	0	3
G30a	10 (43.5)	16 (44.4)	9 (40.9)	8 (27.6)	6 (50.0)	6 (35.3)	12 (70.6)	18 (43.9)	25 (49.0)	48 (50.0)	0	2	1	0	0	3
G30b	1 (4.3)	3 (8.3)	7 (31.8)	10 (34.5)	7 (58.3)	5 (29.4)	12 (70.6)	33 (80.5)	20 (39.2)	53 (55.2)	1	2	1	1	0	5

[a] Fm1 , (*Festuca mairei*); Lp1, (*Lolium perenne,* Citation II); Lp2 (*Lolium perenne, Calypso*); D1, amphidiploid; G6 ~ G30b, backcross lines.

[b] 000, Bands from neither of the three parents, presumably from Fm2; 001, Lp1-specific bands; 010, Lp2-specific bands; 011, Common bands between Lp1 and Lp2; 100, Fm-specific bands; 101, Common bands between Fm and Lp2; 110, Common bands between Fm and Lp1; 111, Common bands among Fm, Lp1 and Lp3; N, The total band number in the category.

Table 2.5. The Fm/Lp genome specific band ratios of the Fm-Lp complexes based on SSR and RAPD marker screening.

Genotype [a]	SSR			RAPD		
	Lp-specific bands (N=51)(%)	Fm-specific bands (N=51)(%)	Fm/Lp genome specific band ratios [b]	Lp-specific bands (N=87)(%)	Fm-specific bands (N=96)(%)	Fm/Lp genome specific band ratios
Fm1 x Lp2	22 (43.1)	43 (84.3)	1.95	44 (50.6)	87 (90.6)	1.79
Lp2 x Fm2	22 (43.1)	24 (47.1)	1.09	36 (41.4)	35 (36.5)	0.88
D1	24 (47.1)	33 (64.7)	1.38	36 (41.4)	68 (70.8)	1.71
G6	29 (56.9)	16 (31.4)	0.55	45 (51.7)	62 (64.6)	1.25
G8	11 (21.6)	12 (23.5)	1.09	19 (21.8)	16 (16.7)	0.76
G11a	36 (70.6)	5 (9.8)	0.14	54 (62.1)	5 (5.2)	0.08
G11b	25 (49.0)	20 (39.2)	0.80	41 (47.1)	40 (41.7)	0.88
G14	32 (62.8)	3 (5.9)	0.09	45 (51.7)	7 (7.3)	0.14
G15	19 (37.3)	22 (43.1)	1.16	36 (41.4)	44 (45.8)	1.11
G16	31 (60.8)	22 (43.1)	0.71	51 (58.6)	37 (38.5)	0.66
G24	27 (52.9)	29 (56.9)	1.07	35 (40.2)	45 (46.9)	1.17
G26	27 (52.9)	16 (31.4)	0.59	52 (59.8)	56 (58.3)	0.98
G27a	30 (58.8)	14 (27.5)	0.47	44 (50.6)	59 (61.5)	1.22
G27b	22 (43.1)	24 (47.1)	1.09	32 (36.8)	40 (41.7)	1.13
G30a	27 (52.9)	25 (49.0)	0.93	32 (36.8)	48 (50.0)	1.36
G30b	26 (51.0)	20 (39.2)	0.77	48 (55.2)	53 (55.2)	1.00

[a] Fm, (*Festuca mairei*); Lp1, (*Lolium perenne,* Citation II); Lp2 (*Lolium perenne, Calypso*); D1, amphidiploid; G6 ~ G30b, backcross lines.
[b] Fm/Lp genome specific band ratios = percentage of Fm-specific bands/ percentage of Lp-specific bands.

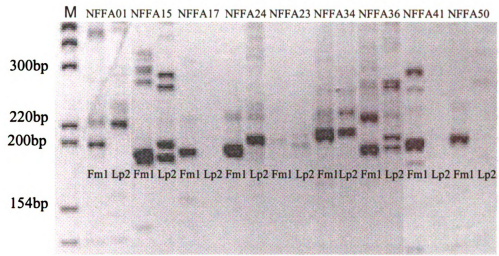

Figure 2.1.a. The image of SSR primer screening with ethidium bromide staining. M is a 1000bp ladder. Size standards are labeled on the left-side of the panel. Every two lanes are a profile of one primer pair screened against Fm (*Festuca mairei*) and Lp (*Lolium perenne*). Primer names are on the top of the panel.

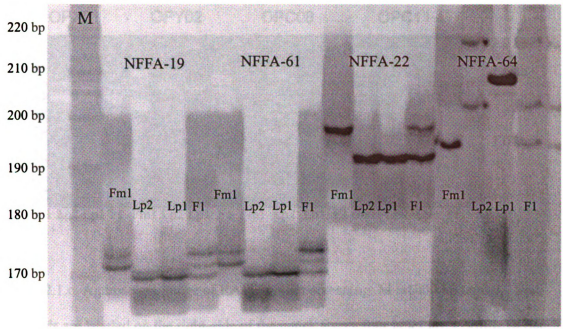

Figure 2.1.b. The image of SSR primer screening with silver staining. M is100 bp ladder. Size standards are labeled on the left-side of the panel. Every four lanes are a profile of one primer pair screened against Fm (*Festuca mairei*), Lp1 (*Lolium perenne,* Citation II), Lp2 (*Lolium perenne,* Calypso) and F_1 (Fm1 x Lp2). Primer names are at the top of the panel.

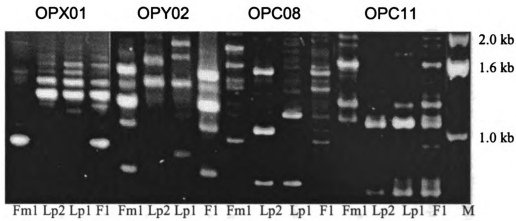

Figure 2.1.c. Agarose gel image of RAPD primer screening. M is1000 bp ladder. Size standards are labeled on the right-side of the panel. Every four lanes are a profile of one primer screened against Fm (*Festuca mairei*), Lp1 (*Lolium perenne,* Citation II), Lp2 (*Lolium perenne,* Calypso) and F_1 (Fm1 x Lp2). Primer names are at the top of the panel.

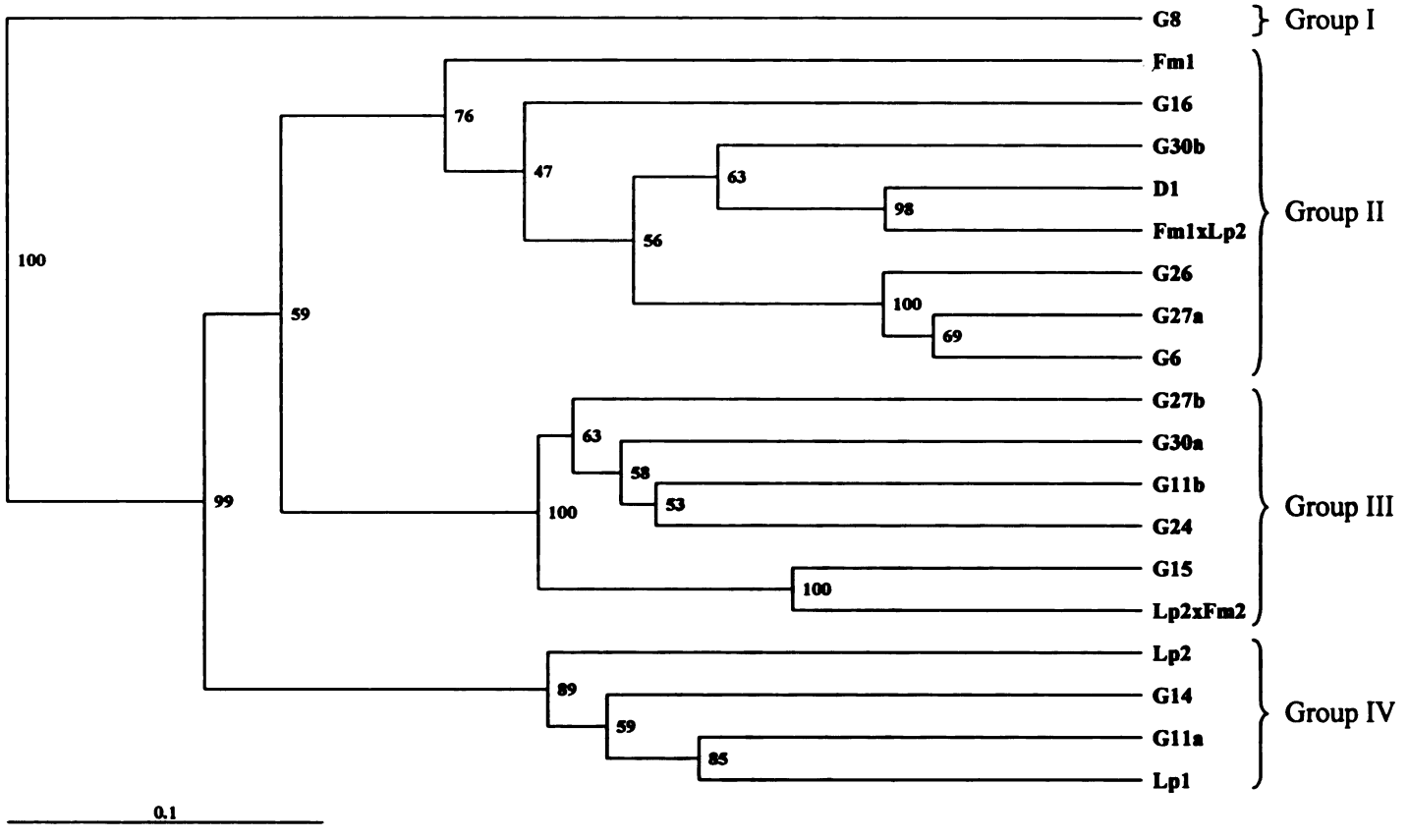

Figure 2.2. The dendrogram from UPGMA cluster analysis based on the merged SSR and RAPD data. The accessions include Fm1 (*Festuca mairei*), Lp1 (*Lolium perenne,* Citation II), Lp2 (*Lolium perenne,* Calypso), D1 (amphidiploid), G6 - G30b (backcross progeny). Numbers at branches are bootstrapping value (%). Group numbers are indicated at right-side of the figure.

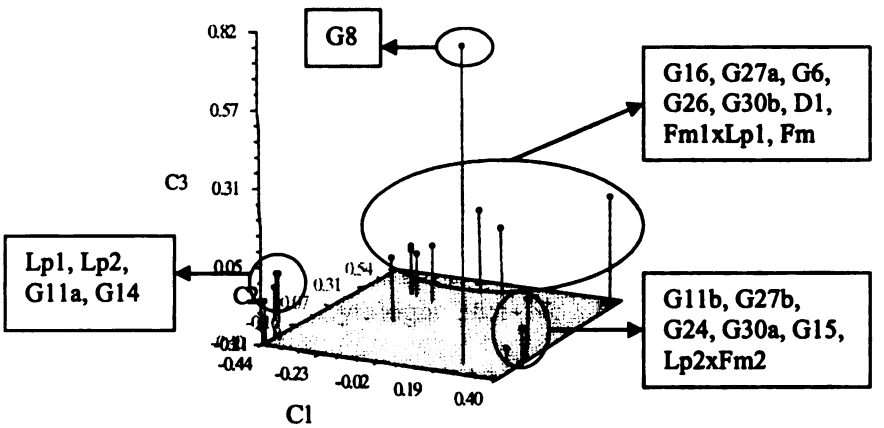

Figure 2.3. The 3-dimensional scatterplot of principle component analysis based on the merged SSR and RAPD data. The four groups were defined following the cluster analysis. C1, C2, and C3 are axes of the three principle components, which explained 76 % of the total variation. Accessions include Fm1 (*Festuca mairei*), Lp1 (*Lolium perenne,* Citation II), Lp2 (*Lolium perenne,* Calypso), D1 (amphidiploid), G6 - G30b (backcross progeny).

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CHAPTER III

Detection of An Efficient Restriction Enzyme Combination and Evaluation of the Experimental Conditions for cDNA-AFLP Analysis in *Festuca mairei*

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ABSTRACT

In cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis, it is critical to choose a suitable pair of restriction enzymes for tagging sites in cDNA for amplification. The experimental conditions might affect the efficiency of cDNA-AFLP technique in detecting transcript derived fragments (TDF). Possibility of production of chimeric fragments from cDNA-AFLP analysis remains to be researched. The objectives of this study were to (1) detect an efficient restriction enzyme combination for cDNA-AFLP analysis when *Festuca* species was used as template; (2) evaluate the effect of the experimental conditions on the efficiency of discovering TDF; and (3) evaluate the identity of transcript-derived fragments (TDFs) from cDNA-AFLP analysis. We found that NspI coupled with TaqI was a pair of highly efficient enzymes by generating a much higher number of TDFs than the commonly used EcoRI and TaqI. This was the first study to apply NspI for AFLP analysis, suggesting that this enzyme may have valuable

application potential for other species. The number of repeatable bands was not significantly affected by magnesium concentration and dilution of pre-amplification products, suggesting that the cDNA-AFLP analysis was relatively insensitive to amplification conditions and had high reproducibility across treatments. The identity of TDF was evaluated by sequencing a TDF and comparing it with the sequence of the template cDNA. The result showed that the chimeric fragments derived from ligation between digested fragments was generated and could not be eliminated by increasing adapter concentration. Although the existence of chimeric fragments should be carefully considered, the unexpected sequence in the chimeric TDF may not seriously influence the sequencing and BLAST searching analyses. Conclusively, cDNA-AFLP is a reliable and high throughput transcript profiling technique suitable to TDFs discovery in grasses such as *F. mairei*.

Key words: cDNA-amplified fragment length polymorphism (cDNA-AFLP), Transcript derived fragments (TDFs), Restriction enzyme combination, *Festuca mairei*.

INTRODUCTION

Transcript profiling is playing a substantial role in annotating and determining gene functions, having advanced from methods of one-gene-at-a-time to technologies that provide a holistic review of the genome (Donson, et al., 2002). A variety of high-throughput transcript profiling techniques have been established, which can be categorized into two types. First is a direct analysis including procedures involving

nucleotide sequencing and fragment sizing, such as cDNA-amplified restriction length polymorphism (cDNA-AFLP), serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), expressed sequence tag (EST) sequencing, and differential display PCR (DD-PCR). Second is an indirect analysis involving nucleic acid hybridization of mRNA or cDNA fragments, such as cDNA microarray, oligo-chips and suppression subtractive hybridization (SSH). These techniques differ in their expense, convenience, sensitivity, and repeatability (Kuhn, 2001).

The cDNA-AFLP analysis is an mRNA fingerprinting technique showing high reproducibility and sensitivity, good correlation with northern blot analysis and low set-up cost, even though it requires a comprehensive reference database (Donson et al., 2002). In contrast to the direct analysis techniques, cDNA-AFLP does not require prior sequence information and can reveal differential expression of any gene carrying suitable restriction sites (Bachem et al., 1996). cDNA-AFLP also overcomes the limitations of other PCR-based techniques by using selective fragment amplification under stringent PCR conditions (Jones and Harrower, 1998). In cDNA-AFLP analysis, non-selective PCR products can be eliminated by increasing the length of selective primers for amplification (Bachem et al., 1996). cDNA-AFLP has been successfully used to identify differentially expressed transcript derived fragments (TDFs) from almond (*Prunus amygdalus*) treated with abscisic acid (ABA) (Campalans et al., 2001); tissue-specific TDFs during potato (*Solanum tuberosum* L.) tuber development (Bachem et al., 2001); and TDFs associated with putative pathogenicity factors during infection of tubers by the potato cyst nematode (*Globodera rostochiensis*) (Qin et al., 2000).

The cDNA-AFLP procedure involves five major steps: i) double strand (ds) cDNA synthesis; ii) digestion of the ds cDNA with two restriction enzymes and ligation of adapters to the termini of the digested cDNA fragments; iii) pre-amplification of the ligated fragments with primers corresponding to the adapters; iv) selective amplification of the pre-amplified fragments with selective primers; and v) visualization of the final amplification products to generate the fingerprint (Bachem et al., 1998). A detailed cDNA-AFLP procedure has been reported and effects of PCR cycle number, template dilution level, and Mg^{2+} concentration during amplification on number of TDFs have been researched in potato (Bachem et al., 1998).

Efficiency of cDNA-AFLP, as a PCR-based technique, in discovering TDFs depends on the type of polymerase, base composition of template and primers, composition of buffer and the PCR program for the reaction (Bachem et al., 1998). Choosing a suitable pair of restriction enzymes is critical for the cDNA-AFLP technique. Restriction enzymes are used to tag sites in cDNA molecules to generate fragments with appropriate sizes, and also to introduce sticky termini for ligation to adapters (Bachem et al., 1998). To investigate the whole genome expression pattern or to discover TDFs throughout the target genome, restriction enzymes should optimally recognize and cut every single cDNA molecule derived from the target genome. If transcribed genes could not be recognized by the enzyme, an opportunity to discover these genes will be missed. Currently, EcoRI/MseI is the most widely used enzyme pair for genomic DNA AFLP, and AseI/TaqI was the first enzyme used for cDNA AFLP. It has also been reported that EcoRI, BamHI, and PstI in combination with TaqI or MseI have equal potential for identification of TDFs (Bachem et al., 1998).

Festuca is a large diverse genus comprising of about 450 species and is widely distributed across the cool regions of the world. It contains the most useful grass species (Clayton and Renvoize, 1986). *Festuca* has good drought resistance and has desired value for both turfgrass and forage usage (Aronson et al., 1987; Fry and Butler, 1989). Although the cDNA-AFLP technique has been successfully used for gene discovery and global gene expression in many species (Campalans et al., 2001; Bachem et al., 2001; Qin et al., 2000), it has not been reported on the *Festuca* species. It remains to be determined whether a specific restriction enzyme combination is needed to achieve high efficiency of cDNA-AFLP for *Festuca*. Magnesium concentration for the PCR reaction is a key parameter affecting the efficiency of the AFLP in detecting individual fragments (Bachem et al., 1998; Du-Toit et al., 1993). A magnesium concentration of 2.5 mM was recommended to generate bands with high clarity (Bachem et al., 1998). However, our preliminary study showed that bands generated from PCR at 1.5 mM Mg^{2+} were clearer (visually adjusted) than at 2.5 mM. It is necessary to determine whether these two magnesium concentrations will significantly affect the number of reliable or repeatable TDFs. Quantity of cDNA for cDNA-AFLP analysis is usually limited because of i) inefficiency of obtaining total RNA from some specific tissues (such as wood, old leaves, potato tubers, and anthers), ii) small mRNA proportion in total RNA (1 to 5%), and iii) low yield of ds cDNA synthesis (around 10%). The dilution level of pre-amplification product can reflect the amount of starting template cDNA. It is applicable to determine how dilution levels affect efficiency of TDFs discovery. In previous studies, the chimeric fragments generated from the cDNA-AFLP analysis had not been adequately addressed. The objectives of present study were to (1) select an effective restriction enzyme

combination to achieve high efficiency of cDNA-AFLP in the monocotyledon species such as *Festuca*; (2) detect the effect of cDNA-AFLP conditions on TDFs discovery, and (3) evaluate the chimeric fragment by sequencing a TDF and comparing it with the sequence of the template.

MATERIALS AND METHODS

Plant material and template preparation

Fresh leaf samples of the *F. mairei* plant were collected and immediately frozen in liquid nitrogen. For each sample, total RNA was extracted from approximately 100 mg of leaf tissue using plant RNA purification reagent (Invitrogen Life Technologies, Carlsbad, CA). The total RNA quantity was measured using a spectrophotometer at a wavelength of 260 nm. The purity of the RNA was evaluated by the ratio of absorbency at 260 nm to 280 nm. Quality of the RNA was checked by running 2 μg of the total RNA on 1.2% denature agarose gel with 2.5% formaldehyde in 40 mM 3-(N-morpholino) propan sulfonic acid (MOPS) with a running buffer for 2.5 hr. The mRNA was isolated from total RNA using PolyATract mRNA isolation systems III (Promega, Madison, WI). The mRNA solution in 250 μl was concentrated to a final volume of 10 μl using a speed vacuum.

Double-stranded (ds) cDNA was synthesized from mRNA using the Universal RiboClone cDNA Synthesis System (Promega). The mRNA sample of a kanamycin resistant gene (Promega) was used as a control for ds cDNA synthesis (sequence in

Figure 3.1 A). Synthesized ds cDNA was extracted with an equal volume of TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1). The quantity of ds cDNA was measured using Hoechst 33258 (bisbenzimide) dye, on a DyNA quant 200 fluorometer.

Digestion and ligation

Thirty nanograms of cDNA was digested with 5 U EcoRI or NspI (rare cutters) (as comparison) at 37°C for 2.5 hr, and then immediately digested with 5 U of TaqI (frequent cutter) at 65°C for 2.5 hr, followed by heat inactivation at 80°C for 20 min. The digestions were conducted in NEbuffer 2 (New England Biolabs, Beverly, MA) with a total volume of 30 µl.

Adapters were prepared by annealing two linkers (see Table 3.1 for sequences) on peltier thermal cycler (PTC-225) (MJ Research, Waltham, MA) using the following program profiles: 70°C, 10 min; 70°C (-1°C/cycle), 30 s (+2 s /cycle) for 45 cycles. All oligo-nucleotides used in this study were synthesized by MWG Biotech, Inc. (Charlotte, NC) (see Table 3.1 for sequences). The digestion mix (20 µl) was ligated with 0.5 mM adapter for rare cutter and 2.5 mM adapter for frequent cutter, using 1 U of T4 ligase supplemented with T4 DNA ligase buffer (Promega) in a total volume of 30 µl. The ligation mix was incubated at room temperature for 12 hr.

Pre-amplification and selective amplification

Reaction solution (20 µl) for pre-amplification contained 1 µl ligation mix, 0.5 µM of each primer, 0.3 mM dNTP mix, and 0.5 U Taq polymerase supplemented with 1x

Taq polymerase buffer (Promega). For optimization, two levels of Mg^{2+} concentration, 1.5 mM or 2.5 mM, were used. The PCR reaction was conducted on the PTC-225 using the profile: 72°C, 2 min; 94°C, 1 min; 15 cycles of 94°C, 30 s; 56°C, 30 s; and 72°C, 1 min; then followed by 10 min at 72°C for a final extension. Product was diluted to 2x, 5x, and 10x for optimization. The PCR mixture for selective amplification included 1 µl of 5x diluted pre-amplification product, 0.4 µM of each selective primer (Table 3.1), 0.3 mM dNTP mix, and 0.4 U Taq polymerase supplemented with 1x Taq polymerase buffer to total 15 µl reaction volume. The selective amplification was performed using the program: 10 cycles: 94°C, 30 s; 65°C (- 0.7°C /cycle), 30 s; 72°C, 1 min and 25 cycles: 94°C, 30 s; 56°C, 30 s; 72°C, 1 min; followed by a final extension step of 10 min at 72°C.

AFLP profiling

The selective amplification product (15 µl) was denatured at 96°C for 6 min after adding 9 µl loading buffer (98% formamide). Six microliters of the sample were fractionated on a 5% polyacrylamide sequencing gel with 45.4% urea at 90 W for 2.5 hr and the gel was stained using the Silver Sequence DNA staining reagents (Promega). The gel was allowed to dry overnight at room temperature for scoring on a light box.

Fragment isolation, cloning and sequencing

The band of interest was excised from the polyacrylamide gel with a sterile surgical blade. DNA was eluted by soaking the excised gel in 50 µl water 12 hr. The

DNA fragment was re-amplified using the same condition used for selective amplification and was run on 1% agarose gel for separation from possible DNA contamination. The re-amplified DNA fragment with a target size was excised and purified from the gel with QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA).

The purified DNA fragment was cloned into the pGEM-T easy vector (Promega) and transformed into *E. coli* by electroporation using electroporator II (Invitrogen Life Technologies, Carlsbad, CA). Plasmid DNA was extracted using the Wizard plus SV minipreps DNA purification system (Promega). Purified plasmid DNA with the desired insert was sequenced on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Sequence search and restriction map analysis

To choose an enzyme combination that would have at least one recognition site in each cDNA molecule, the information of *Festuca* cDNA sequences was obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The restriction maps of the cDNA sequences were analyzed using the restriction analysis program from the website (http://www.arabidopsis.org/cgi-bin/patmatch/RestrictionMapper.pl). Recognition sites of eight restriction enzymes including six rare cutters (ApoI, AseI, BamHI, EcoRI, NspI, and PstI) with a recognition site of 6 bases, and two frequent cutters (MseI and TaqI) with a 4-base recognition site were surveyed on the restriction maps of cDNA sequences.

Experimental design and data analysis

Under direction of the information from the website, the efficiency of the selected enzyme combination (NspI/TaqI) in detecting TDFs was compared with a commonly applied and acknowledged enzyme pair (EcoRI/TaqI) (Qin et al.; 2000, Bachem et al., 1998; Qin et al., 2001). The efficiency of the cDNA-AFLP in detecting TDFs using the enzyme combination was determined by the number of TDFs generated from all of the possible primer pairs designed for an enzyme combination. For EcoRI/TaqI, the possible selective primer pairs were 256 (16x16), while for NspI/TaqI, there were 128 (8x16) selective primer combinations. In the cDNA-AFLP procedure, 128 selective primer combinations for NspI/TaqI and 136 randomly chosen selective primer combinations for EcoRI/TaqI were screened using Festuca mairei cDNA as a template. The number of scorable (clearly visualized by the naked eye) bands in the AFLP profiles was recorded.

For detecting the effect of cDNA-AFLP conditions on TDFs discovery, a split plot design having two factors with four replications was used. Factor 1 was Mg^{2+} concentration at two levels: 1.5 mM and 2.5 mM. Factor 2 was dilution of pre-amplification product at three levels: 2x, 5x, and 10x. Three dilution levels represented approximately 500 pg, 200 pg, and 100 pg of starting cDNA respectively. After pre-amplification, each of four replications at two levels of factor 1 was divided into three aliquots, which were then diluted 2x, 5x, and 10x respectively. Selective amplification was then conducted at a Mg^{2+} concentration consistent with the level in pre-amplification. Four randomly chosen selective primer combinations (N7/T7, N4/T14, N3/T11, and N7/T4) were used to perform the selective amplification (Table 3.1). Number of repeatable bands in the AFLP profile was counted if two bands with same size

in two adjacent lanes of replications were clearly visualized. ANOVA analysis was conducted using PROC MIXED function in SAS system V8 (SAS Institute, 2002, Cary, NC).

To evaluate the identity of amplified TDFs, a cDNA sample from a kanamycin resistance gene (Promega) was used as a template and subjected to four randomly picked selective primer combinations (N7/T7, N4/T14, N3/T11, and N7/T4) in the AFLP process. The number of scorable bands in the AFLP profiles was counted. The template cDNA sequence has five restriction sites for TaqI, and has no restriction sites for NspI (Figure 3.1.A). The resulted fragments from the digestion of TaqI (and NspI) are 92 bp, 274 bp, 403 bp, and 93 bp (Figure 3.1.B).

RESULTS AND DISCUSSION

Selection of the optimal restriction enzymes

From NCBI website, 18 complete cDNA sequences of the *Festuca* species were available and obtained (Table 3.2). Restriction mapping analyses of these 18 sequences were conducted using 8 restriction enzymes. Results showed that of the six rare cutters (ApoI, AseI, BamHI, EcoRI, NspI, and PstI), NspI could recognize 94.4% of the cDNA sequences of the Festuca species (Table 3.2). Of the two frequent cutters (MseI and TaqI), TaqI could cut all of the cDNA sequences of the Festuca species (Table 3.2). Therefore, NspI and TaqI were initially chosen as the enzyme combination for the cDNA-AFLP analysis in the Festuca species.

The efficiency of NspI/TaqI in detecting TDFs was compared with a pair of commonly applied and acknowledged enzymes, EcoRI/TaqI. All of the 128 primer pairs for NspI/TaqI were screened, and in total 11,364 scorable bands (TDFs) ranging from 50bp to 1000bp were generated. Most of the bands (48.26%) had a size of 100-200bp (Table 3.3). In contrast, 136 out of 256 primer pairs for EcoRI/TaqI enzyme combination were randomly screened and in total generated 532 scorable bands. Only 17 (12.5%) of the 256 primer pairs generated more than 10 bands (Figure 3.2). This result demonstrated that the NspI/TaqI enzyme combination was more efficient than EcoRI/TaqI to discover the TDFs in the *Festuca* species.

The linkers and primers for the NspI enzyme were designed in this study based on the same principle applied in designing other enzymes (Table 3.1), since this enzyme has never been used before in AFLP analysis. Clear and abundant bands were generated from these primers, indicating the designing of the linkers and primers was successful. Application value of NspI was also confirmed when comparing with EcoRI (Table 3.3, Figure 3.2). NspI was not reported to be used for AFLP analysis before, and thus was not compared with other enzymes. This study prompts the potential value of NspI/TaqI used for cDNA-AFLP procedure across species. Or at least, NspI/TaqI could be put into consideration when selecting restriction enzyme for cDNA-AFLP analysis.

Because EcoRI recognizes 5' G^AATT C 3', whereas NspI recognizes 5'Pu CATG^Py 3' (Pu=A or G and Py = T or C), our study suggested that most of the cDNAs from *F. mairei* do not have AATT but do have CATG sequences. Two possible explanations are that (1) AATT is mostly present in uncoding regions of genomic DNA, while the template molecules are transcripts from coding regions in the cDNA-AFLP

procedure, and (2) the restriction site of NspI is not unique. To test the first hypothesis, both the genomic DNA and cDNA from *F. mairei* were subjected to AFLP analysis using the two enzyme combinations, NspI/TaqI verses EcoRI/TaqI. Results showed that when the NspI/TaqI enzyme combination was applied, more bands were generated with either genomic DNA or cDNA template. This result implied that when compared to EcoRI, NspI was an efficient enzyme irrespective of genomic DNA or cDNA molecule due to its non-unique-restriction-site property. In general, restriction enzyme selection is critical for high efficiency of cDNA-AFLP technique, even though it has been reported that other enzyme combinations had similar potential for identification of TDFs (Bachem et al., 1998).

Effects of magnesium concentration and pre-amplification dilution on number of TDFs in AFLP profiles

The number of repeatable bands in AFLP profiles generated from four randomly selected primer-combinations was evaluated to determine the effects of Mg^{2+} concentration and pre-amplification product dilution on the TDFs in AFLP profile. The ANOVA showed that differences in the number of repeatable bands at two levels of Mg^{2+} concentration were not statistically significant (P=0.22), although the mean value of 94.86 at 1.5 mM Mg^{2+} was greater than 84.89 at 2.5 mM Mg^{2+} (Table 3.4). Dilutions of the pre-amplification product did not significantly affect the number of repeatable bands in AFLP profiles (Table 3.4, Figure 3.3). Interaction between Mg^{2+} concentration and primer combination was not significant. The results indicated that neither of the two Mg^{2+} concentrations nor three dilutions significantly affected the number of repeatable

bands generated from cDNA-AFLP, which suggested that the cDNA-AFLP analysis was relatively insensitive to amplification conditions used and thus high reproducibility across treatments can be obtained. The statistic analysis in this study supports the experimental conditions optimized in potato (*Solanum tuberosum*) (Bachem et al., 1998).

In general, the number and pattern of AFLP bands were not significantly affected by Mg^{2+} concentrations and dilutions of the pre-amplification implying that the technique is relatively independent on the experimental conditions. A dilution level corresponding to 100 pg of starting cDNA material generated the same amount of information as the dilution level corresponding to 200 pg and 500 pg of starting cDNA material (Table 3.4). This result indicated that the real amount of cDNA required for AFLPs could be at least as low as 100 pg for around 25 selective amplifications, implying the high sensitivity of this technique in discovery of TDFs. This result also proved that cDNA- AFLP was an applicable technique when the starting material is limited. A higher DNA input concentration would lead to a background smear and the adverse effects on amplification of some individual TDFs likely due to competitive inhibition between fragments during the PCR (Bachem et al., 1998).

Evaluation of chimeric TDF identity

A cDNA sample from a kanamycin resistance gene had been subjected to standard AFLP procedure with four randomly picked selective primer pairs: N7/T7, N4/T14, N3/T11, and N7/T4 (see Table 3.1 for sequences). Numbers of resultingbands from each primer pair were 32, 35, 20 and 6. In addition to the four expected bands, 92 bp, 93 bp, 274 bp and 403 bp plus the primer sequence (Figure 3.1 B), many bands of

unexpected size were produced. To investigate the origin of these unexpected bands, a fragment of 710 bp was randomly selected for sequencing. Pair-wise BLAST analysis between the sequenced fragment and the original sequence of the template molecule was performed. Results showed that the 710 bp fragment contained a 403 bp and a 274 bp fragment, which were 99% and 98% identical to the original template sequences respectively. The 274 bp fragment was reversely joined with the 403 bp fragment (Figure 3.1 B and Figure 3.4). This result indicated that the 710 bp fragment was amplified from a reversely ligated chimeric fragment between two digested template fragments (a 403 bp and a 274 bp fragment) plus the two adapters at the end.

To investigate whether the chimeric bands derived from ligation between digested fragments could be prevented or at least minimized by increasing the adapter concentration in ligation reaction, ligations were conducted by adding five different amounts of adapter mixture (7 μM TaqI adapter) to each 20 μl of digestion solution: 10 μl, 15 μl, 20 μl, 25 μl, and 30 μl. The final molecular ratios of the template cDNA to the adapter in the ligations were 1:1842, 1:2763, 1:3684, 1:4605, and 1:5526 respectively. The five ligations at different adapter concentrations were replicated twice and subjected to four randomly picked selective primer combinations (N7/T7, N4/T14, N3/T11, and N7/T4) in the AFLP process. The number of bands was compared. Results showed that there was no significant difference among the number of bands generated from the five different adapter concentrations. The chimeric bands were not minimized by increasing the adapter concentration.

The existence of chimeric fragments should be carefully considered when a mixture of thousands of TDF is identified from the profile. A set of more specific

experiments could be planned to address the possibility of elimination or minimization of the accidental production of chimeric fragments from the established cDNA-AFLP protocol. Fortunately, the density and size of the band derived from ligation of the two fragments was highly reproducible in independent experiments (Figure 3.5). The high reproducibility of these fragments suggested that they did not severely affect the discovery of TDFs, but the number of TDFs derived from the same transcript was increased (Figure 3.5). When obtaining the sequence of TDF and using BLAST search to find the potential function of the TDF, the unexpected sequence in the chimeric TDF can be viewed as an uninformative tail that may not severely influence the sequencing and BLAST searching analysis.

In summery, NspI/TaqI is a high efficient enzyme combination in the AFLP analysis. It has less primer combinations, and might have valuable application potential in other species. cDNA-AFLP is relatively insensitive to the experimental conditions by showing consistent band number, density, and pattern in independent and variable conditioning experiments. Even though some chimeric fragments could be produced using the cDNA-AFLP procedure, similar to many other techniques, the discovery of TDFs will not be badly affected with the awareness of the possibility of chimeric fragment existence. In conclusion, cDNA-AFLP is a reliable and high throughput transcript profiling technique suitable to TDFs discovery in grasses such as *F. mairei.*

Table 3.1. Sequences of the primers and linkers used for AFLP analysis.

Primers and linkers	Initial	Sequences (5'-3')
EcoRI linker 1		CTCGTAGACTGCGTACC
EcoRI linker 2		AATTGGTACGCAGTCTAC
EcoRI pre-amplification primer		GACTGCGTACCAATTC
EcoRI selective amplification primer 1	E1	GACTGCGTACCAATTCAA
EcoRI selective amplification primer 2	E2	GACTGCGTACCAATTCAT
EcoRI selective amplification primer 3	E3	GACTGCGTACCAATTCAC
EcoRI selective amplification primer 4	E4	GACTGCGTACCAATTCAG
EcoRI selective amplification primer 5	E5	GACTGCGTACCAATTCTA
EcoRI selective amplification primer 6	E6	GACTGCGTACCAATTCTT
EcoRI selective amplification primer 7	E7	GACTGCGTACCAATTCTC
EcoRI selective amplification primer 8	E8	GACTGCGTACCAATTCTG
EcoRI selective amplification primer 9	E9	GACTGCGTACCAATTCCA
EcoRI selective amplification primer 10	E10	GACTGCGTACCAATTCCT
EcoRI selective amplification primer 11	E11	GACTGCGTACCAATTCCC
EcoRI selective amplification primer 12	E12	GACTGCGTACCAATTCCG
EcoRI selective amplification primer 13	E13	GACTGCGTACCAATTCGA
EcoRI selective amplification primer 14	E14	GACTGCGTACCAATTCGT
EcoRI selective amplification primer 15	E15	GACTGCGTACCAATTCGC
EcoRI selective amplification primer 16	E16	GACTGCGTACCAATTCGG
NspI linker 1		GTAGACTGCGTTCCCATG
NspI linker 2		GGAACGCAGTCTACGAG
NspI pre-amplification primer		GTAGACTGCGTTCCCATG
NspI selective amplification primer 1	N1	GTAGACTGCGTTCCCATGTA
NspI selective amplification primer 2	N2	GTAGACTGCGTTCCCATGTT
NspI selective amplification primer 3	N3	GTAGACTGCGTTCCCATGTC
NspI selective amplification primer 4	N4	GTAGACTGCGTTCCCATGTG
NspI selective amplification primer 5	N5	GTAGACTGCGTTCCCATGCA
NspI selective amplification primer 6	N6	GTAGACTGCGTTCCCATGCT
NspI selective amplification primer 7	N7	GTAGACTGCGTTCCCATGCC
NspI selective amplification primer 8	N8	GTAGACTGCGTTCCCATGCG
TaqI linker 1		AAGTCCTGAGTAGCAC
TaqI linker 2		CGTTCAGGACTCATC
TaqI pre-amplification primer		CACGATGAGTCCTGAACG
TaqI selective amplification primer 1	T1	CACGATGAGTCCTGAACGAAA
TaqI selective amplification primer 2	T2	CACGATGAGTCCTGAACGAAT
TaqI selective amplification primer 3	T3	CACGATGAGTCCTGAACGAAC
TaqI selective amplification primer 4	T4	CACGATGAGTCCTGAACGAAG
TaqI selective amplification primer 5	T5	CACGATGAGTCCTGAACGATA
TaqI selective amplification primer 6	T6	CACGATGAGTCCTGAACGATT
TaqI selective amplification primer 7	T7	CACGATGAGTCCTGAACGATC
TaqI selective amplification primer 8	T8	CACGATGAGTCCTGAACGATG

Table 3.1. Sequences of the primers and linkers used for AFLP analysis (cont'd).

Primers and linkers	**Initial**	**Sequences (5'-3')**
TaqI selective amplification primer 9	T9	CACGATGAGTCCTGAACGACA
TaqI selective amplification primer 10	T10	CACGATGAGTCCTGAACGACT
TaqI selective amplification primer 11	T11	CACGATGAGTCCTGAACGACC
TaqI selective amplification primer 12	T12	CACGATGAGTCCTGAACGACG
TaqI selective amplification primer 13	T13	CACGATGAGTCCTGAACGAGA
TaqI selective amplification primer14	T14	CACGATGAGTCCTGAACGAGT
TaqI selective amplification primer 15	T15	CACGATGAGTCCTGAACGAGC
TaqI selective amplification primer 16	T16	CACGATGAGTCCTGAACGAGG

Table 3.2. The recognition of 8 restriction enzymes on the complete cDNA sequences from *Festuca* genera searched in the web site.

Accession no. of complete cDNA sequences from *Festuca*	***Festuca* species**	**ApoI**	**AseI**	**BamHI**	**EcoRI**	**MseI**	**NspI**	**PstI**	**TaqI**
AF188295	*F. arundinacea*	+	-	-	+	+	+	+	+
AF188294	*F. arundinacea*	+	-	-	-	-	+	+	+
AF188293	*F. arundinacea*	-	-	-	-	-	+	-	+
AF188292	*F. arundinacea*	-	-	-	-	-	+	-	+
AF153826	*F. arundinacea*	+	+	+	+	+	+	-	+
AF153825	*F. arundinacea*	+	+	+	+	+	+	-	+
AF153824	*F. arundinacea*	+	-	+	+	-	+	-	+
AF153823	*F. arundinacea*	+	+	+	-	+	+	-	+
AJ295942	*F. pratensis*	-	-	+	-	-	+	-	+
AJ295941	*F. pratensis*	+	-	+	-	-	+	-	+
AJ295940	*F. pratensis*	-	-	+	-	+	-	-	+
AJ295946	*F. pratensis*	-	+	-	-	+	+	+	+
AJ295945	*F. pratensis*	-	-	+	-	+	+	+	+
AJ295944	*F. pratensis*	+	+	-	-	+	+	-	+
AJ295943	*F. pratensis*	-	-	-	-	+	+	-	+
AJ297369	*F. arundinacea*	-	-	-	-	-	+	+	+
AF104258	*F. rubra*	+	-	+	+	+	+	+	+
U96646	*F. rubra*	-	+	-	-	+	+	-	+
Total	18	9	6	9	5	11	17	6	18
(Percentage %)	(100)	(50)	(33.3)	(50)	(27.8)	(61.1)	(94.4)	(33.3)	(100)

Note: "+" indicates that there is at least one restriction site by the enzyme.

"-" indicates that there is no restriction site by the enzyme.

Table 3.3. The size distribution of the bands generated from AFLP analysis with EcoRI/TaqI and NspI/TaqI restriction enzyme system, respectively.

Size range of the band	EcoRI/TaqI (%)	NspI/TaqI (%)
700-1000bp	24 (4.51)	103 (0.91)
600-700bp	9 (1.69)	131 (1.15)
500-600bp	24 (4.51)	337 (2.97)
400-500bp	15 (2.82)	565 (4.97)
300-400bp	49 (9.21)	1,140 (10.03)
200-300bp	67 (12.59)	2,480 (21.82)
100-200bp	197 (37.03)	5,484 (48.26)
50-100bp	147 (27.63)	1,124 (9.89)
Total	532 (100.00)	11,364 (100.00)

Table 3.4. ANOVA of the number of repeatable bands generated from *F. mairei* cDNA-AFLP profile at two magnesium concentrations and 3 dilutions.

Effect	**Magnesium**	**Dilution**	**Estimated lsmean**	**Standard error**	***Pr* > F**
Magnesium	1.5 mM		94.8611	10.9463	0.2212 ns
Magnesium	2.5 mM		84.8889		
Dilution		2x	91.7917	10.5813	0.4900 ns
Dilution		5x	89.2083		
Dilution		10x	88.6250		
Magnesium*Dilution	1.5 mM	2x	96.7500	11.1834	0.6915 ns
Magnesium*Dilution	1.5 mM	5x	95.4167		
Magnesium*Dilution	1.5 mM	10x	92.4167		
Magnesium*Dilution	2.5 mM	2x	86.8333		
Magnesium*Dilution	2.5 mM	5x	83.0000		
Magnesium*Dilution	2.5 mM	10x	84.8333		

ns: not significant.

Table 3.5. ANOVA of the number of bands generated from kanamycin cDNA-AFLP profile at five different adapter concentrations.

Adapter concentrations*	**Estimated means**	**Standard error**	***Pr* > F**
1	22.6250	7.1675	0.4632ns*
2	23.6250		
3	22.5000		
4	23.0000		
5	19.5000		

*: 1 to 5 indicates 5 different volumes of adapter mixture added to each 20ul of digestion solution: 10ul, 15ul, 20ul, 25ul and 30ul.

ns, not significant.

A

1 GAATACAAGC TTGGGCGTGT CTCAAAATCT CTGATGTTAC ATTGCACAAG
51 ATAAAAATAT ATCATCATGA ACAATAAAAC TGTCTGCTTA CATAAACAGT

101 AATACAAGGG GTGTTATGAG CCATATTCAA CGGGAAACGT CTTGCTCG AG
151 GCCGCGATTA AATTCCAACA TGGATGCTGA TTTATATGGG TATAAATGGG

201 CTCGCGATAA TGTCGGGCAA TCAGGTGCGA CAATCTATCG ATTGTATGGG
251 AAGCCCGATG CGCCAGAGTT GTTTCTGAAA CATGGCAAAG GTAGCGTTGC
301 CAATGATGTT ACAGATGAGA TGGTCAGACT AAACTGGCTG ACGGAATTTA
351 TGCCTCTTCC GACCATCAAG CATTTTATCC GTACTCCTGA TGATGCATGG
401 TTACTCACCA CTGCGATCCC CGGGAAAACA GCATTCCAGG TATTAGAAGA
451 ATATCCTGAG TCAGGTGAAA ATATTGTTGA TGCGCTGGCA GTGTTCCTGC

501 GCCGGTTGCA TTCG ATTCCT GTTTGTAATT GTCCTTTTAA CAGCGATCGC
551 GTATTTCGTC TCGCTCAGGC GCAATCACGA ATGAATAACG GTTTGGTTGA
601 TGCGAGTGAT TTTGATGACG AGCGTAATGG CTGGCCTGTT GAACAAGTCT
651 GGAAAGAAAT GCATAAGCTT TTGCCATTCT CACCGGATTC AGTCGTCACT
701 CATGGTGATT TCTCACTTGA TAACCTTATT TTTGACGAGG GGAAATTAAT
751 AGGTTGTATT GATGTTGGAC GAGTCGGAAT CGCAGACCGA TACCAGGATC
801 TTGCCATCCT ATGGAACTGC CTCGGTGAGT TTTCTCCTTC ATTACAGAAA
851 CGGCTTTTTC AAAAATATGG TATTGATAAT CCTGATATGA ATAAATTGCA

901 GTTTCATTTG ATGCTCG ATG AGTTTTTCTA ATCAGAATTG GTTAATTGGT
951 TGTAACACTG GCAGAGCATT ACGCTGACTT GACGGGACGG CGGCTTTGTT

1001 GAATAAATCG AACTTTTGCT GAGTTGAAGG ATCAGATCAC GCATCTTCCC
1051 GACAACGCAG ACCGTTCCGT GGCAAAGCAA AAGTTCAAAA TCACCAACTG
1101 GTCCACCTAC AACAAAGCTC TCATCAACCG TGGCGACTCT AGAGGATCCC
1151 CGGGCGAGCT CCCAAAAAAA AAAAAAAAAA AAAAAAAAAA AAACCGAATT

Figure 3.1. The restriction diagram of the cDNA of the kanamycin resistant gene with TaqI enzyme. A. Single strand cDNA sequence. The arrows point to the restriction sites of TaqI. B. Fragments with double sticky ends restricted by TaqI.

B

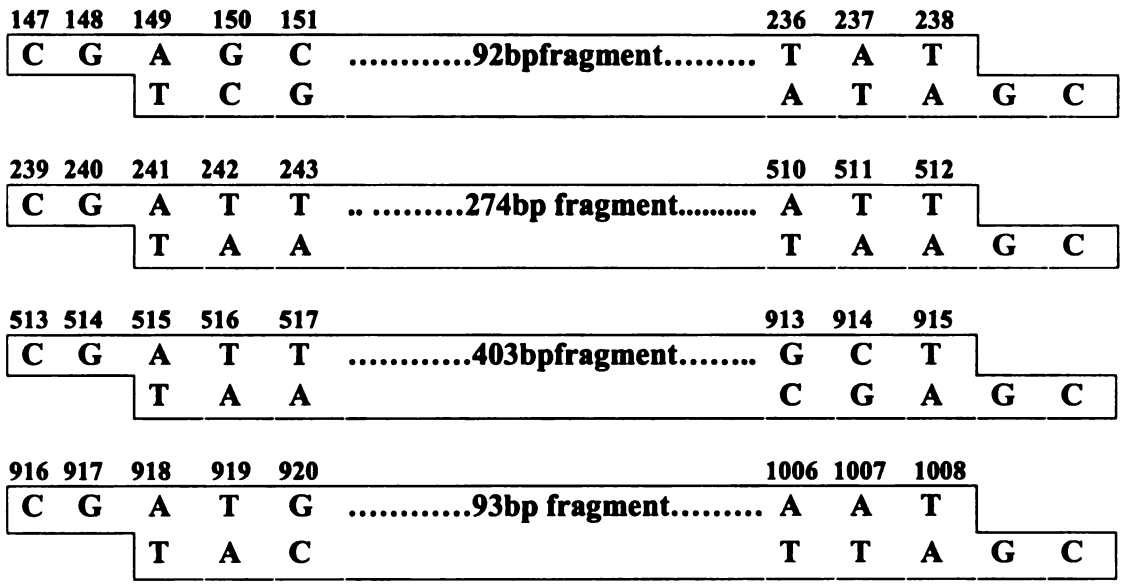

Figure 3.1. The restriction diagram of the cDNA of the kanamycin resistant gene with TaqI enzyme. A. Single strand cDNA sequence. The arrows point to the restriction sites of TaqI. B. Fragments with double sticky ends restricted by TaqI (cont'd).

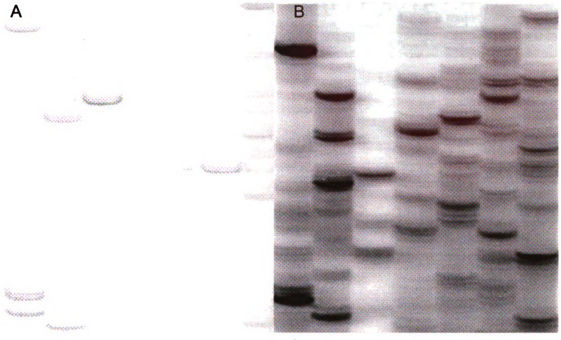

Figure 3.2. The cDNA-AFLP banding pattern generated from the EcoRI/TaqI enzyme combination (A) and the NspI/TaqI enzyme combination (B).

1.5mM Magnesium			2.5mM magnesium		
Dilution 1	Dilution 2	Dilution 3	Dilution 1	Dilution 2	Dilution 3

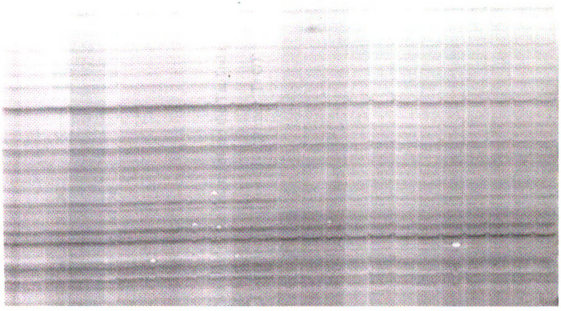

Figure 3.3. Effect of magnesium concentration and dilution levels on the cDNA-AFLP profiling. Every four lanes are four replications.

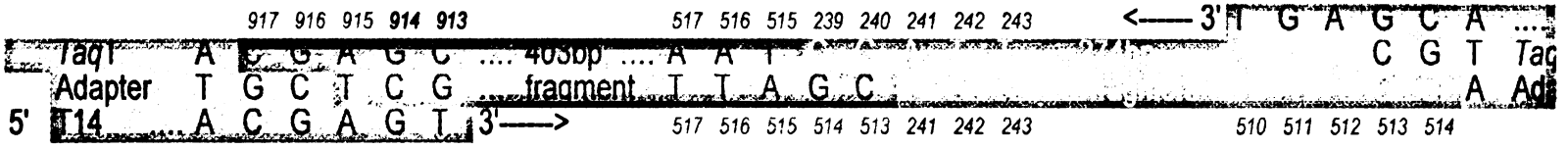

Figure 3.4. The diagram of the composition of the 710 bp band. Two ends of the fragment are the TaqI adapters. Two middle fragments are 403bp (917-515) (left) and 274bp (239-512) (right). T14 is the TaqI selective primer: T+GT.

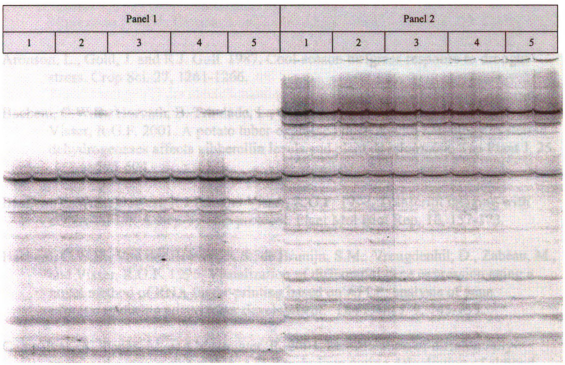

Figure 3.5. Effect of adapter concentration on the cDNA-AFLP profiling. Panels 1 and 2 show primer pairs of N4/T14 and N3/T11 respectively. 1, 2, 3, 4, and 5 are five levels of adapter concentration with two replications.

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CHAPTER IV

Gene Expression Profiles and Candidate Genes Involved in Drought Tolerance in *Festuca mairei*

(Submitted to Plant Mol. Bio.)

ABSTRACT

To understand the molecular genetic basis underlying drought tolerance in grasses, the cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique was applied for identification of genes responding to drought stress in *Festuca mairei* showing xerophytic adaptation. One hundred and twenty eight primer combinations for cDNA-AFLP were conducted on nine plant samples with one sample per day during drought stress treatment. A total of 11,346 transcript derived fragments (TDFs) were detected with size distribution between 50 and 1000bp, and, 464 (4.1%) TDFs were identified as differentially expressed fragments (DEFs) across the nine days. The expression patterns of these DEFs included up-regulated (29.7%), down-regulated (54.3%), transiently-expressed (12.3%), and up-then-down-regulated (3.7%). To confirm the differential expression pattern, 406 DEFs were subjected to macroarray hybridization analysis. Consequently 171 (42.1%) DEFs showed a consistent expression pattern with

the cDNA-AFLP analysis. Sequences of 163 confirmed DEFs were compared to the GenBank plant protein database by using BLASTX to target the potential function of these gene fragments. Results showed 62 sequences had no significant hits in the database. Predicted functions of the 101 sequences were subdivided into 17 functional categories and 11 DEFs were found to be function-unknown, hypothetical or unclassified protein. The down-regulated genes involved in metabolism and cellular biogenesis were nearly twice the number of the up-regulated. On the other hand, more than two times the amount of up-regulated DEFs were involved in transcription, defense, cell cycle and DNA processing compared to down-regulated DEFs. The results suggested that during drought stress generally more metabolic function and biogenesis of cellular components in the plant were under degenerative processes, and the plant system may rescue energy for new gene transcription and stress defense. Predicted function of some DEFs had previously been reported as stress induced genes in other species indicating our analysis system was functioning properly to find stress-inducible genes. Some novel genes were identified in *F. mairei* during the drought response procedure. The combination of data from studies on the genetic model plant and on diverse plant species will help us to better understand the underlying mechanism of drought tolerance in plants.

Key words: Drought stress, cDNA-amplified fragment length polymorphism (cDNA-AFLP), Macroarray, Transcript derived fragments (TDFs), Differential expression.

INTRODUCTION

Abiotic stresses such as drought, high salinity, and extreme temperatures can severely impair plant growth and performance. Thus, the plant response to these abiotic stresses has been the focus of a number of researchers for decades at physiological and genetic levels (Levitt, 1980; Quarrie et al., 1994) and more recently targeting the molecular and genomic aspects (Seki et al., 2001; Ozturk, et al., 2002). Among these stresses, drought or water deficit is the most severe and complex limiting factor on plant growth and crop production (Seki et al., 2002). Drought stress triggers a wide range of plant responses manifested by changes in growth rates, physiological, and metabolic processes, to altered gene expression. A drought stress response is initiated when a plant recognizes the stress, which then activates signal transduction pathways to transmit the information within individual cells and throughout the plants. Ultimately, changes in gene expression will occur and are integrated into plant adaptive response to modify growth and development.

Several hundred genes are differentially expressed in response to dehydration in the resurrection plant *Craterostigma plantagineum*, as evidenced by transcript profiling (Bockel et al., 1998). In *Arabidopsis*, which has been extensively studied as a model plant that tolerates moderate water deficit, genes involved in many different pathways are expressed in response to drought stress (Seki et al., 2002). All these identified genes can be assigned to diverse metabolic pathways. In one example, genes encoding enzymes involved in sugar metabolism and biosynthesis of other compounds acting as compatible solutes have been found up-regulated in response to drought in many species (Bohnert,

1995). In another example, the ion and water channel proteins are likely to be important in regulating water flux, which has been supported by isolation of channel protein genes from pea (*Pisum sativum*) in response to water deficit (Guerrero et al., 1990). In addition, enzymes required for cell wall lignification processes seem to be increased in drought stressed *Arabidopsis thaliana* tissue (Peleman et al., 1989). Genes encoding proteins similar to proteases and enzymes that detoxify active oxygen species have also been induced by drought in some species such as *Arabidopsis* and pea (Williams et al., 1994; Mittler et al., 1994). Although the precise function of these genes has not been demonstrated yet, Bartels and Salamini (2001) have summarized all the drought inducible genes and grouped them into five main categories, as genes encoding (a) proteins with protective properties, (b) membrane proteins involved in transport processes, (c) enzymes related to carbohydrate metabolism, (d) regulatory molecules, such as transcription factors, kinases, or other putative signaling molecules, and (e) open reading frames that show no homologies to known sequences.

It has become obvious that a network of signal transduction pathways allows the plant to adjust its metabolism to the demands imposed by water deficit (Shinozaki and Yamaguchi-Shinozaki, 2000; Kirch et al., 2001). The molecular complexity of the process during drought stress response was illustrated nicely by recent microarray experiments on *Arabidopsis* (Seki et al., 2002). The complex signal transduction cascade can be divided into three basic steps (Ingram and Bartels, 1996): (a) perception of stimulus, (b) signal amplification and integration, and (c) response reaction in the form of de novo gene expression. Signaling molecules involved in the signal transmission process and the activation of gene expression in response to stress have been identified

through several experimental approaches. Most information is derived from promoter analyses and from differential screening procedures. One molecule that is central to dehydration-regulated gene expression is the plant hormone abscisic acid (ABA). Endogenous ABA levels have been reported to increase as a result of water deficit in many physiological studies, and therefore ABA is thought to be involved in the signal transduction (Chandler 1994; Giraudat 1994). Besides the ABA-mediated gene expression, the investigation of drought-induced genes in *A. thaliana* has revealed ABA-independent signal transduction pathways (Yamaguchi, 1994). Both ABA-dependent and -independent stress signaling first modifies constitutively expressed transcription factors, leading to the expression of early response transcriptional activators, which then activate downstream stress tolerance effective genes (Zhu, 2001). Even though a large number of drought induced genes have been identified in a wide range of species and an impressive progress has been made in this area, the molecular basis still remains far from being understood (Ingram and Bartels, 1996).

Festuca mairei St. Yves is a tetraploid (2n=4x=28, $M_1M_1M_2M_2$) species, commonly known as atlas fescue. It shares the M_1M_2 genomes with *F. arundinacea* var. *atlantigena* ($G_1G_1G_2G_2M_1M_1M_2M_2$), which is only found near the Atlas Mountain ranges in northwest Africa. The M genome in *Festuca* is associated with a xerophytic adaptation allowing the plant to survive long summers under drought stress (Marlatt et al., 1997). We consider that *F. mairei* must have evolved highly developed systems for growing under severe drought conditions. As a polyploidy monocot species, *F. mairei* can serve as a reference system for drought tolerance study on some grass species, such as ornamental grasses, turfgrass and forage, which are grown for their desired traits of

vegetative growth and performance, rather than for grain production. The functional genomics of these species lags far behind other plant systems. Molecular genetic mechanisms conditioning the expression of drought tolerance in these species also remains to be illustrated, though a significant effort has been invested into the studies of physiological mechanisms (Levitt, 1980; Youngner, 1985; Qian, 1997) and developing and evaluating drought resistance in grass species (Aronson et al., 1987; Fry and Butler, 1989). It is important to identify more drought inducible genes and analyze the functions of those genes. Increasing knowledge of the function of genes induced by drought in *F. mairei* would be essential to understand the molecular mechanism of drought tolerance in grasses and to facilitate gene manipulation for grass breeding programs.

The advent of whole genomic-related technology for differential gene expression provides the necessary and efficient tools to identify the key genes on a large scale that respond to drought stress and relate their regulation to adaptive events occurring during stress. A variety of techniques allow insight to be gained into differential gene expression during stress. cDNA amplified fragment length polymorphism (cDNA-AFLP) is an extremely efficient method for isolating differentially expressed fragments (DEFs) or transcript derived fragments (TDFs) in a genome wide scale (Bachem et al. 1996). cDNA-AFLP shows high reproducibility and sensitivity, good correlation with northern blot analysis and low set-up cost, even though it requires a comprehensive reference database (Donson et al., 2002). Recently, the creation of a large-scale EST database from various species, and the complete sequencing of *Arabidopsis* (Arabidopsis genome initiative [AGI], 2000) and rice genome (Yu et al., 2002) were made public. This genomic information source can provide a vast reference database for evaluating the

coordinated function and expression of genes identified by using the cDNA-AFLP approach.

The objectives of this study were to monitor the gene expression profiles of drought response in *F. mairei* by cDNA-AFLP procedure, to identify genes involved in drought stress response, and thus to gain insight into the molecular genetic basis of drought tolerance in grass species.

MATERIALS AND METHODS

Plant materials and drought treatment

Ten plants of *F. mairei* clone were transplanted into polyethylene pots (20.32 cm diameter at the top, 15.24 cm diameter at the bottom, and 35.56 cm height) filled with 90% sand and 10% silt and clay. The plants had been established for three months with regular irrigation and fertilization in a uniform greenhouse environment condition. After establishment, five *F. mairei* plants were deprived of water until they were severely stressed and the other five plants, as a treatment control, were watered daily throughout the drought stress period. During the drought stress treatment, leaf samples from both the stressed and the control *F. mairei* plants were collected at noon every day to eliminate the variation due to any diurnal changes of gene expression and the samples were immediately frozen in liquid nitrogen and stored in – 80 °C for consequent RNA isolation.

Relative leaf water content measurement

Fully extended leaves of the *F. mairei* plant were detached every other day during the drought stress treatment for relative leaf water content measurement. The fresh weight (FW) (weigh the leaf immediately after detachment), turgor weight (TW) (weigh the leaf after being soaked in the miniQ water for 24 hr at room temperature), and dry weight (DW) (weigh the leaf after being dried in an oven at 80°C for 24 hr) of the leaf were used to calculate the relative leaf water content (RWC) following the formula: RWC (%) = (FW-DW)/(TW-DW) x 100 (White et al., 1992).

RNA isolation and cDNA synthesis

Total RNA was isolated with plant RNA purification reagent (Invitrogen Life Technologies, Carlsbad, CA) and then quantified using a spectrophotometer at a wavelength of 260 nm. Quality of the RNA was checked by running 2 μg of the total RNA on 1.2% denature agarose gel with 2.5% formaldehyde in 40 mM 3-(N-morpholino) propan sulfonic acid (MOPS) running buffer for 2.5 hrs. Poly $(A)^+$ RNA was isolated from the total RNA by using PolyATract mRNA isolation systems III (Promega, Madison, WI). Double-stranded (ds) cDNA was synthesized from Poly$(A)^+$ RNA using the Universal RiboClone cDNA Synthesis System (Promega) and purified with an equal volume of TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1). The ds cDNA was quantified using Hoechst 33258 (bisbenzimide) dye on DyNA quant 200 fluorometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA).

cDNA-AFLP analysis

The cDNA-AFLP procedure followed the description of Bachem et al., (1998) with some modifications. Thirty nanograms of cDNA were digested with 5 U NspI at 37°C for 2.5 hrs., and then immediately digested with 5 U of TaqI at 65 °C for 2.5 hrs. followed by heat inactivation at 80°C for 20 min. The two steps of digestion were conducted in NEbuffer 2 (New England Biolabs, Beverly, MA) in a total volume of 30 µl. The digestion mix (20µl) was ligated to 0.5 mM NspI adapter and 2.5 mM TaqI adapter using 1 U of T4 ligase supplemented with T4 DNA ligase buffer (Promega). All oligo-nucleotides (adapters and primers) in this study were synthesized by MWG Biotech, Inc. (Charlotte, NC) (see Table 4.1 for sequences). PCR reaction solution (20 µl) for pre-amplification contained 1 µl digestion mix, 0.5 µM of each primer, 0.3 mM dNTP mix, 1.5 mM Mg^{2+}, and 0.5 U Taq polymerase (Promega). The PCR reaction was conducted on the PTC-225 at: 72°C, 2 min; 94°C, 1 min; 15 cycles of 94°C, 30 s; 56°C, 30 s; and 72°C, 1 min; then followed by 10 min at 72°C for a final extension. For selective amplification, the PCR solution included 1 µl of 5x diluted pre-amplification product, 0.4 µM of each selective primer, 1.5 mM Mg^{2+}, 0.3 mM dNTP mix, and 0.4 U Taq polymerase in 15 µl total reaction volume. The PCR reaction was performed following the program: 10 cycles: 94°C, 30 s; 65°C (- 0.7°C /cycle), 30 s; 72°C, 1 min and 25 cycles: 94°C, 30 s; 56°C, 30 s; 72°C, 1 min; followed by a final extension step of 10 min at 72°C.

The selective PCR product (15 µl) was denatured at 96°C for 6 min after adding 9 µl of 98% formamide loading buffer. The denatured PCR product (6 µl) was loaded into a 5% denatured polyacrylamide sequencing gel with 45.4% urea for fractionation by

electrophoresis at 90 W for 2.5 hrs. The fractionated fragments on the gel were then detected by using the Silver Sequence DNA Sequencing System (Promega). The gel on the back plate was allowed to dry overnight at room temperature for scoring on a light box. For recovery of TDFs from the polyacrylamide gel, silver staining has advantages over radioactive fingerprints by being directly visualized and excised from the gel.

Identification of differentially expressed fragments and fragment recovery from polyacrylamide gel

For each primer combination, the final PCR products from a series of days of drought stress were loaded in order into lanes next to each other in the sequencing gel for comparison of band density for bands of the same size. If the density of the bands increases from lane to lane gradually across the time points, the bands were identified as up-regulated differentially expressed fragments. If they decrease gradually, the bands were identified as down-regulated DEFs (Bachem et al., 1996). If the bands show up only at a specific time point, these bands were identified as transient expressed DEFs. A few bands were also identified as up-then-down regulated DEFs, which means the density of the bands increases at first several lanes and then decreases at the last several lanes.

The fiur types of DEFs were excised from the polyacrylamide gel with a sterile surgical blade. DNA was eluted by soaking the excised gel in 50 µl water for 12 hrs and then was used as the template to re-amplify the DNA fragment using the same PCR condition as used for selective amplification. The re-amplified product was run on an 1% agarose gel in 1 x TBE buffer for confirmation of the target fragment and separation from

possible DNA contamination. DNA fragments with the target size were purified from the agarose gel with a QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA) and eluted in 50 µl sterile water.

Macroarray hybridization and data analysis

Double stranded cDNA samples from control and stressed plants at different time points were labeled with DIG-dUTP by using a PCR DIG probe synthesis kit (Roche Applied Science, Penzberg, Germany). The labeled product was purified with a high pure PCR product purification kit (Roche).

Ten microliters of both the recovered DEF DNA samples and control samples were denatured with 10 µl denature solution (0.4 N sodium hydroxide, 0.01 M EDTA, pH=8.0) at 37°C for 15 minutes and then neutralized with 10 µl 2 M ammonium acetate (pH=7.0). Controls included a negative control, which contained sterile water but not DNA, and a housekeeping control, which contained only DNA fragment with the same expression level (constitutively expressed) throughout the control and the different days of the stressed plant. All of the above denatured solutions of the DEF and controls were spotted on a nylon membrane (115 x 76 mm) (Nalge Nunc International, Naperville, IL) with two replications using the Beckman BioMek® 2000 laboratory automation workstation (Dilks et al., 2003). The controls were spotted in different sections of the membrane to compensate for variable background levels. The spot arrangement on the nylon array is shown in Figure 4.1. Five identical nylon arrays were prepared serially and then subjected to separate hybridization with labeled ds cDNA probes. The hybridization and washing were performed by using DIG high prime DNA labeling and

detection starter kit II (Roche). The luminescent signal on the membrane was exposed to Lumi-Film Chemiluminescent detection film (Roche).

The array image on the film was scanned and saved as an individual ".TIFF" file and was then analyzed with BIORAD® Quantity One Software 4.2.3 (Bio-Rad Laboratories, Hercules, CA). For each array image, all of the spots were delimitated with the same size circles, which could include all the pixels in the spot. The volume (=pixel intensity x area of the circled spot) of each spot was reported from the software. The average volume of negative control spots on the array image was subtracted from the volume of each of the other spots to eliminate background effect. The average volume of housekeeping control spots on the array image was used to normalize the spots of unknown DEFs between array images. The ratio of the average volume of housekeeping spots between images was applied as a scaling factor for the volume of unknown DEF spots, which were compared to its counterpart between membranes to confirm the differential expression pattern. For up-regulated and down-regulated DEFs, the membrane arrays hybridized with a treatment control cDNA probe were compared to the 5-day stressed cDNA probe. For up-then-down regulated DEFs, the membrane arrays hybridized with the 3-day stressed cDNA probe were compared to the control cDNA probe for up-regulation and to the 5-day stress for down-regulation. For the transiently expressed DEFs, the membrane hybridized with the cDNA probe of a certain number of day's stress, at which the DEFs were exclusively expressed, were compared to the probe of the day before and after respectively. The volume ratio of the DNA dots from the two replications greater than 1 were accepted as confirmed DEF.

DNA fragment sequencing and sequence analysis

The DEFs recovered from the polyacrylamide gel whose differential expression pattern were confirmed with macroarray analysis, were cloned into pGEM-T easy vector (Promega) and transformed into JM109 component cells (Promega) by heat shock. Plasmid DNA was extracted from successful transformants using the Wizard plus SV minipreps DNA purification system (Promega), and plasmid inserts were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA) at The Genomics Technology Support Facility (Michigan State University).

The sequences of the DEFs were searched against the AGI (Arabidopsis Genome Initiative) protein database using BLASTX (http://www.arabidopsis.org/Blast/). Additional analysis using BLASTX against GenBank plant protein database and TBLASTX against GenBank plant dbEST were performed for DEFs with zero matches or low similarity (E value greater than 1E-6) in AGI protein database. A tool of the "Blast 2 sequences", which can be found at: http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi, was used for sequence comparisons.

RESULTS

Plant performance during the drought stress

The treatment control plants of *F. mairei* remained green and survived throughout the stress treatment period. Plants under stress were green for the first three days after

being deprived of water, and then began discoloring and firing at the 4th day after water deprivation. The RWC of the stressed plant decreased dramatically from 83% to 26% between 3rd and 5th day of drought stress (Figure 4.2). At 8th day of drought stress, the leaves of the stressed plants were completely fired with a RWC around 17%. These eight days covered dynamic changes of the plants responding to drought stress, and the 4th day under stress was a critical time point during the response (Figure 4.2). The eight days plus the day before application of stress (a total of 9 days) were considered to be a whole drought stress period. With each day as a time point, there were nine time points within this stress period.

cDNA-AFLP analysis

To investigate the possibility of the DEFs induced by plant development or changes of greenhouse conditions during the drought stress period, cDNA-AFLP analysis was performed on the treatment control plants of *F. mairei* over the nine days using five randomly picked primer combinations. No DEF was detected across the nine time-points in control plants (Figure 4.3), suggesting both greenhouse conditions and plant development did not affect gene expression in *F. mairei* plants during the days of application of the drought stress.

cDNA-AFLP analysis was conducted using all of the 128 primer combinations over nine days of the stressed *F. mairei* plants and revealed 11,346 transcript derived fragments (TDFs) with an average of 89 fragments obtained per primer pair. The size distribution of the fragments was between 50 and 1000bp (Figure 4.4). Of these TDFs, 464 fragments (4.1%) were identified as being differentially expressed across the nine

time-points during the drought stress, indicating the gene expression had been altered by the drought conditions. The expression pattern of these DEFs included up-regulated (138, 29.7%), down-regulated (252, 54.3%), transient-expressed (57, 12.3%), and up-then-down-regulated (17, 3.7%) (Figure 4.5). Of these 464 DEFs, 434 (94%) fragments were recovered from acrylamide gel and isolated as genes potentially related with plant response to drought stress.

Macroarray hybridization analysis

In addition to 13 positive and 13 negative controls, 406 samples of 434 recovered DEFs were printed on the membrane with two replications (Figure 4.1). Twenty eight DEFs with sizes of less than 100 bp were not included in the macroarray analysis. The dot intensity volume of two or three identical membrane arrays hybridized with cDNA probes from different time-point respectively were compared for confirmation of the differential expression pattern of the 406 DEFs (see Materials and Methods). Figure 4.6 shows an image of a portion of the hybridized macroarray. The comparison results revealed that 54 of 128 (42.2%) up-regulated, 97 of 210 (46.2%) down-regulated, 14 of 51 (27.5%) transiently expressed, and 6 of 17 (35.3%) up-then-down regulated DEFs showed the consistent differential expression pattern. In total, the expression pattern of 171 (42.1%) DEFs were confirmed. These 171 DEFs were cloned as drought inducible and repressible gene fragments.

Sequence analysis and functional category

All 171 DEFs were sequenced and an expected size sequence was obtained for 166. Among the 166 sequences, three pairs of fragments were aligned with above 98% similarity and one of each pair was excluded from further analysis. BLASTX analysis was conducted against the GenBank protein database of the 163 sequences (92 down-regulated, 50 up-regulated, 15 transiently expressed, and 6 up-then-down-regulated). The results revealed that 101 DEFs (62.0%) showed significant homology to a protein sequence in the database (E value less than 1E-6) and the other 62 DEFs (38.0%) showed zero match (no hits found) or no significant homology (E value higher than 1E-6). When the entire GenBank EST database was screened for the presence of sequences similar to these 62 DEFs (TBLASTX analysis), 23 DEFs showed statistically significant degrees of similarity to the public available ESTs. The remaining 39 DEFs were defined as novel sequences, which have not previously been described from other organisms. The 39 novel DEFs included 13 down-regulated, 6 transiently expressed, and 20 up-regulated sequences. Therefore, 40% of the 50 up-regulated, 15 transiently expressed DEFs respectively and 14.1% of the 92 down-regulated DEF were found to be novel in *F. mairei* under drought stress.

The function predicted for the 101 DEFs could be subdivided into 17 functional categories and 4.8% of these DEFs were function unknown, hypothetical or unclassified protein based on function classification criteria in the website of MIPS (Munich Information Center for Protein Sequences) (http://mips.gsf.de) (Table 4.2). Most of the proteins fell into more than one functional category with statistical significance, and all of those categories were counted. In comparison of the functional category between the up-

regulated and the down-regulated DEF (Figure 4.7), the down-regulated genes involved in metabolism and cellular biogenesis were found to be nearly twice that of the up-regulated. More than two times the amount of the up-regulated DEFs were involved in transcription, defense, cell cycle and DNA processing compared to down-regulated DEFs. During drought stress, the results reflected, generally more metabolic function and biogenesis of cellular components in the plants were under degenerative processes. The plant system appeared to save energy for new gene transcription and stress defense. More genes involved in cell cycle and DNA synthesis were up-regulated suggesting an increasing activity of growth in some specific guard cells for stress defense. The transiently expressed DEFs were primarily involved in subcellular localization, defense, or acted as heavy metal carriers for transport reflecting the temporary needs for sets of genes in defense, transport and subcellular localization during plant response to drought. The up-then-down regulated DEFs were mostly involved in transport, subcellular localization, and energy indicating that some genes for electron/hydrogen transport, subcellular localization, and photosynthesis were stimulated by drought stress signal firstly, and then inactivated by continued or severe stress.

Predicted function of up-regulated DEFs

Twenty two DEFs up-regulated or induced by drought stress in *F. mairei* had significant similarity with protein sequences in the GenBank database (Table 4.3). Most of them had the highest similar hits in the monocot species such as rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*). SSBII-H2, SSBI-B6, and SSBI-C09 encoded enzymes, respectively, involved in biosynthesis of purine nucleotide, raffinose,

which has also been induced by water stress in *Cicer arietinum* (Romo et al., 2001), and trehalose, the most effective osmoprotectant sugar in terms of minimum concentration required (Crowe et al., 1992). The SSBI-D11 encoding of an enzyme for C-compound and carbohydrate utilization has been identified as a transcript differentially expressed in response to high salinity in the mangrove, *Bruguiera gymnorrhiza* (Banzai et al., 2002). The farnesylated protein encoded by SSBI-B9 has been found to be a nuclear protein involved in stress response and leaf senescence in *Hordeum vulgare* (Barth et al., 2004). SSBI-D4, SSBI-D9, SSBII-A3, and SSBI-A4 encoded a fiber protein Fb19, a dehydration-responsive family protein, a type-1 pathogenesis-related protein, and a DNAJ heat shock N-terminal domain-containing protein, respectively, which have been widely studied in association with stress response. SSBI-E2 encoded a 34 kD fibrillin-like protein, the major constituent of elastin-associated extracellular microfibrils, and has been recently identified in a network of rice genes associated with stress response (Cooper et al., 2003), even though this protein was classified as categories of transcription and subcellular localization on the website of MIPS. SSBI-A5 encoded a brown planthopper susceptibility protein and shared similarity with the sequence of a rice gene induced in response to herbivore grazing. Several DEFs encoded proteins involved in heavy metal ion transport, electron/hydrogen transport, and membrane channel, reflecting the plant actively adjusted the ion and water status for homeostasis. The DEFs encoding proteins for transcription and translation regulation were induced such as zinc finger protein, MYB transcription factor, and peptide chain release factor suggesting the some stress-responsive genes are activated by these factors for positive stress defense.

Predicted function of down-regulated DEFs

In total, 70 down-regulated DEFs showed significant similarity to previously identified proteins (Table 4.4). A much larger quantity of down-regulated DEFs than up-regulated were isolated in *F. mairei* during drought stress indicating that the plant was mainly under degenerative processes imposed by the stress. Down-regulated genes were involved in a number of basic metabolic or biosynthetic functions and systemic development or growth. For example, light-inducible protein HV58 (SSBII-D7) was for photosynthesis; chlorophyll A-B binding family protein (SSBI-B10) was for respiration; victorin binding protein (a glycine dehydrogenase P protein) (SSBI-B12) was for amino acid metabolism; GTP-binding protein typA (SSBII-C2) was for oligopeptide synthesis; UDP-glycosyltransferase 88B1 (SSBII-G7) was for c-compound and carbohydrate metabolism; homeobox protein knotted-7 (SSBI-H3) was for tissue development; DNA helicase (DNA-binding protein) (SSBI-G10) was for cell division and so on. In addition, some proteins for transport facilitation were down-regulated, such as ADP-ribosylation factor for vesicular transport (SSBII-H8), iron-phytosiderophore transporter protein for aligopeptide transport (SSBII-B8), ferric reductase for electron transport (SSBII-B2), cation diffusion facilitator for ion transport (SSBII-C1), and triose phosphate translocator for c-compound transport (SSBI-E7). Moreover, several proteins involved in transcription and signal transduction were also down-regulated indicating some pathways for signaling and basic biosynthesis or metabolism were turned down in the plant during drought stress. Those proteins included cleavage and polyadenylation specificity factor (SSBII-F10), homeobox gene knotted 7 (SSBI-H3), TATA-binding protein associated factor (SSBII-F3), SEUSS transcriptional co-regulator (repressor) (SSBI-B7),

EREBP1transcription factor (SSBI-C10), zinc finger protein (SSBII-E5), and phosphatidylinositol-4-phosphate 5-kinase (SSBII-G2).

Predicted function of up-then-down regulated and transiently expressed DEFs

Five up-then-down regulated and four transiently expressed DEFs were identified sharing significant similarity with proteins in the public database (Table 4.5). The rieske Fe-S precursor protein (SSBI-F10), a chlorophyll a/b-binding protein (SSBII-D5), digalactosyldiacylglycerol synthase (SSBI-D3), and a disease resistance protein (SSBI-D5) were up-regulated at the earlier stress period and then turned down with the stress continuing, indicating that these proteins may have a positive response to the mild stress but were not retained during the severe stress. The glutamine-dependent asparagine synthase, plasma membrane H+ ATPase, small heat shock protein Hsp23.5, and type 2 metallothioneine were temporally expressed at only around day 4 stress suggesting the transient regulation of these protein might be critical for drought stress response.

DISCUSSION

Functional approach to identify stress response genes

In this study, an effective enzyme combination, NspI/TaqI was used to conduct the cDNA-AFLP procedure for a sufficient data extraction (Wang et al., 2005). The DEF was first identified with cDNA-AFLP analysis, and then the macroarray analysis was

performed to verify the differential expression pattern. Around 42% DEFs were consistently expressed, but the expression of 58% DEFs were not consistent between the two techniques. The inconsistency could be due to: (1) the subjective evaluation on the DEF in the cDNA-AFLP gel; (2) the different macroarray hybridization intensities and or background between the membranes compared; (3) possible cross hybridization of closely related sequences in macroarray; and (4) low expression genes in the probe for macroarray hybridization (Miller et al., 2002). Therefore, we excluded more than half (58%) of the DEF from subsequent sequence analysis to have a more correct data set. Some of the drought inducible DEFs identified by cDNA-AFLP coupled with macroarray had previously been reported as stress-inducible genes in other species (see Results). These discoveries indicated our analysis system functioned properly to find stress-inducible genes.

Previously, a large number of identified genes, transcripts and proteins were stress inducible or up-regulated. The down-regulated genes were basically underestimated. Only one or two time-point stresses were compared with the control, especially by microarray analysis. Some genes, such as transiently expressed genes, and up-then-down regulated genes, could not be identified. However, the plant response to stress is a complicated procedure. Down-regulation or other types of regulation may also play important roles in stress response or even tolerance. Investigation of the systemic and dynamic changes of the gene expression will provide more complete information for understanding the molecular mechanism of stress response. In this study, nine days in the drought stress cycle were taken for analysis, which covered the whole dynamic change of the plant responding to the stress. Four different differential expression

patterns were detected by the cDNA-AFLP analysis, even though the transient and up-then-down expression patterns were not abundant. Further study on genes with all of these differential expression patterns, including spatial and temporal regulation patterns, will lead to a programmed control of the desiccation response, and thus, will increase our knowledge on the stress response mechanism at the gene regulation level. The cDNA-AFLP technique not only provided a means to generate genomic sequence information and functional analysis but also served as a powerful tool for the identification of genes with different kinds of differential expression patterns for stress response.

Drought inducible genes

Stress-inducible genes have been transferred into several crops to improve the stress tolerance of plants (Bajaj et al., 1999). Functional analyses of the stress-inducible genes are important for increasing our knowledge on molecular mechanisms of stress response and for stress tolerance improvement of crops. To date, more than 200 drought inducible genes have been reported (Seki et al., 2002, Ozturk et al., 2002). Identification of more novel drought-inducible genes will provide more complete information about genes involved in stress tolerance and *cis*-acting promoter elements that function in drought specific gene expression (Seki et al., 2001). In our study, 50 drought inducible gene fragments were identified from the drought adaptive monocot plant, *F. mairei*. Only 22 (44%) had hits with significant similarity in the protein database and were assigned functions. The remaining 28 (56%) either had hits with no significant similarity in the protein database, or had significant hits in the EST database with unknown function, or had no hits in either database and were defined as novel drought-inducible

gene fragments. Among the gene products of the 22 informative DEFs, several have been reported as up-regulated by stress in *Arabidopsis* such as zinc finger and MYB family transcription factors, raffinose synthases and trehalose-6-phosphate synthase, heat-shock protein, auxin-regulated protein, and so on (Seki et al., 2002).

The products of the stress-inducible genes can be classified into two groups: (1) those that directly protect against environmental stresses; and (2) those that regulate gene expressions and signal transductions in stress response (Seki et al., 2002). The proteins in the first group have the ability to function in stress tolerance. The raffinose synthases and trehalose-6-phosphate synthase were osmoprotectant biosynthesis-related proteins for adjusting the osmotic pressure under stress conditions. The heat-shock proteins have been reported to be involved in protecting macromolecules such as enzymes and lipids (Shinozaki and Yamaguchi-Shinozaki, 1999). The fibrillin and fiber proteins might contribute to cell wall structure modification. The type-1 pathogenesis-related protein is considered to be a protein with antifungal activity (Antoniw et al., 1980) that may have multiple stress-related roles even though the function is still unknown. Tonoplast intrinsic protein (aquaporin) functions as a water channel to transport water through plasma membrane and tonoplast to adjust the osmotic pressure under stress conditions (Daniels et al., 1994). The transporters for anion and zinc may function in adjustment of ion homeostasis. The second group contains regulatory proteins involved in regulation of signal transduction and gene expression in stress responses. The zinc finger and MYB family transcription factors may function in the regulation of stress-inducible gene expression. The peptide chain release factor induced by drought stress reflected that the post-transcriptional regulatory mechanism also affected the gene expression. Ankyrin

protein kinase is thought to be involved in signal transduction and in further regulating the functional genes under stress conditions. The auxin-regulated gene was identified as drought-inducible suggesting a link between auxin and drought stress signaling pathways.

Some DEFs annotated to the same functional genes were probably derived from the same gene or from redundant homologous genes. Currently, functions of most of these genes are not fully understood. Moreover, 56% of the drought inducible gene fragments in this study were still functionally unknown and remain to be elucidated. Functional analysis of such sets of DEFs might be informative to follow up in later experiments based on more natural drying plants in the field.

Drought repressible genes

Analysis of drought-repressible genes is as important as analysis of drought-inducible genes in understanding the molecular mechanism of plant response to stress. In this study, many photosynthesis-related genes were found such as chlorophyll a/b binding protein, ribulose-1,5-bisphosphate carboxylase, high molecular mass early light-inducible protein HV58, etc., all reflecting that photosynthesis was inhibited by the water deficit. This can be due to a reduction in light interception as leaf senesce, or to a reduction of intercellular CO_2 concentration as closure of stomata (Bartels D. and Salamini F., 2001). The benefit of the depressed photosynthesis appears to be the switch toward another carbohydrate utilization pathway, which leads to the production of valuable stress tolerance molecules (Pattanagul and Madore, 1999). Bockel & Bartels (unpublished data) proposed that down-regulation of photosynthesis-related genes possibly contributed to reduced photooxidative stress.

Lipoxygenase, glutamate synthase, malate dehydrogenase up-regulated under drought in barley (Ozturk et al., 2002) but were down-regulated in this study. Some clones have been up-regulated by drought in Arabidopsis encoding protein products the same as protein encoded by down-regulated clones, eg. Cytochrome P450 protein and malate dehydrogenase have shown in both up-regulated and down regulated groups (Seki et al., 2002). This distinct behavior has also been found in barley and rice (Kawasaki et al., 2001). Their role and importance in tolerance or sensitivity is impossible to judge based on the experiments alone under controlled environment conditions. But these DEFs, at least, provide clues about the genes differentially expressed with a reference database for comparison later on with data from nature field drought conditions.

Application of drought stress and DEFs

Plant response to drought stress will vary as the time, duration, and intensity of the stress applied on plants differs. Growth chambers and greenhouses allow very precise control of the timing, duration and intensity of stress control, but they are least like the plant's natural environment or farmers' fields, where the crops are ultimately evaluated in breeding programs (Bruce et al., 2002). If water loss occurs slowly enough to allow metabolism to adapt the stress by activating a specific program of gene expression, plants will develop the ability to survive desiccation. If dehydration occurs too rapidly, plants will not acquire enough tolerance to desiccation (Bartels and Salamini, 2001). However, in recent functional genomic studies, the drought-inducible transcripts have been identified in hours or one day, which might lead to omission of some important tolerance transcripts or genes. In this study responses to the whole stress cycle were evaluated, but

more information will need to be collected before breeders can use this information. All the DEFs including the novel ones should be analyzed with breeding lines under more natural drought conditions to further confirm the correlation of expression pattern of the transcripts with drought tolerance. The particular functions of these DEFs need to be studied by using knock-out mutants and transgenics, such as over-expression, antisense suppression, and double-stranded RNA interference (RNAi). It has been found that some genes induced by drought stress have no effect on drought tolerance in transgenic plants (Karakas et al., 1997). Therefore, a challenge for future research is to distinguish between gene products with a potential in osmoprotection and those that are only involved in secondary reaction. The combination of quantitative transcript profiles with an appropriate QTL analysis could possibly lead to the identification of candidate genes for agronomically valuable traits.

In general, most advances in understanding the drought tolerance mechanism will be obtained from studies on the mild drought tolerant model species, *Arabidopsis*. However, these studies will not be sufficient to explain the adaptation of the *Festuca* species to severe drought stress. The research on *F. mairei* has uncovered a number of GenBank novel genes. The combination of data from studies on the genetic model plant and on diverse plant species should help us to better understand the underlying mechanism of drought tolerance in plants. Existence of variety of drought responsive genes suggests a complex process of plant response to the stress. The genes are involved in drought stress tolerance and stress responses. Although more work is necessary to define gene functions and dissect the complex regulation of gene expression, the genes isolated and characterized to date give us many intriguing insights into the protective

mechanisms that determine desiccation tolerance. Reverse genetic approach as well as classical genetics will become more important to understand not only functions of stress-inducible genes but also the complex signaling process in environmental stress response.

Table 4.1. Sequences of the linkers and primers used for cDNA-AFLP analysis synthesized by MWG Biotech Inc.

Primers and linkers	**Sequences (5'-3')**
*Nsp*I linker 1	GTAGACTGCGTTCCCATG
*Nsp*I linker 2	GGAACGCAGTCTACGAG
*Nsp*I pre-amplification primer	GTAGACTGCGTTCCCATG
*Nsp*I selective amplification primer 1	GTAGACTGCGTTCCCATGTA
*Nsp*I selective amplification primer 2	GTAGACTGCGTTCCCATGTT
*Nsp*I selective amplification primer 3	GTAGACTGCGTTCCCATGTC
*Nsp*I selective amplification primer 4	GTAGACTGCGTTCCCATGTG
*Nsp*I selective amplification primer 5	GTAGACTGCGTTCCCATGCA
*Nsp*I selective amplification primer 6	GTAGACTGCGTTCCCATGCT
*Nsp*I selective amplification primer 7	GTAGACTGCGTTCCCATGCC
*Nsp*I selective amplification primer 8	GTAGACTGCGTTCCCATGCG
*Taq*I linker 1	AAGTCCTGAGTAGCAC
*Taq*I linker 2	CGTTCAGGACTCATC
*Taq*I pre-amplification primer	CACGATGAGTCCTGAACG
*Taq*I selective amplification primer 1	CACGATGAGTCCTGAACGAAA
*Taq*I selective amplification primer 2	CACGATGAGTCCTGAACGAAT
*Taq*I selective amplification primer 3	CACGATGAGTCCTGAACGAAC
*Taq*I selective amplification primer 4	CACGATGAGTCCTGAACGAAG
*Taq*I selective amplification primer 5	CACGATGAGTCCTGAACGATA
*Taq*I selective amplification primer 6	CACGATGAGTCCTGAACGATT
*Taq*I selective amplification primer 7	CACGATGAGTCCTGAACGATC
*Taq*I selective amplification primer 8	CACGATGAGTCCTGAACGATG
*Taq*I selective amplification primer 9	CACGATGAGTCCTGAACGACA
*Taq*I selective amplification primer 10	CACGATGAGTCCTGAACGACT
*Taq*I selective amplification primer 11	CACGATGAGTCCTGAACGACC
*Taq*I selective amplification primer 12	CACGATGAGTCCTGAACGACG
*Taq*I selective amplification primer 13	CACGATGAGTCCTGAACGAGA
*Taq*I selective amplification primer 14	CACGATGAGTCCTGAACGAGT
*Taq*I selective amplification primer 15	CACGATGAGTCCTGAACGAGC
*Taq*I selective amplification primer 16	CACGATGAGTCCTGAACGAGG

Table 4.2. Distribution of functional category of the differentially expressed fragments (DEFs) during drought stress cycle in *F. mairei*. Classification was performed for 101 DEFs with strong statistical similarity to GenBank plant protein sequence (E values lower than 1.00E-06) by BLASTX search. The functional category was assigned based on function classification criteria in the website of Munich information center for protein sequences (MIPS) (http://mips.gsf.de).

Function category	**Total (%)**	**Up-regulated (%)**	**Down-regulated (%)**	**Transiently expressed (%)**	**Up-then-down regulated (%)**
Metabolism	16.45	9.76	19.63	9.09	6.25
Energy	7.36	4.88	7.98	0.00	12.5
Biogenesis of cellular components	4.76	2.44	5.52	0.00	6.25
Subcellular localization	22.94	17.07	24.54	27.27	18.75
Transport	10.39	9.76	9.20	9.09	25
Transcription	5.19	12.20	4.29	0.00	0
Signal transduction	2.60	0.00	3.07	0.00	6.25
Interaction with the cellular environment	3.90	4.88	3.68	9.09	0
Protein synthesis	1.73	2.44	1.84	0.00	0
Protein with binding function	5.63	4.88	4.91	18.18	6.25
Defense	6.93	17.07	3.68	18.18	6.25
Development	2.16	2.44	2.45	0.00	0
Cell fate	1.30	2.44	1.23	0.00	0
Cell cycle and DNA processing	0.87	2.44	0.61	0.00	0
Protein fate	2.16	0.00	2.45	0.00	6.25
Cell type differentiation	0.43	0.00	0.00	9.09	0
Protein activity regulation	0.43	0.00	0.61	0.00	0
Function unknown, hypothetical, and unclassified protein	4.76	7.32	4.29	0.00	6.25

Table 4.3. Function annotation and size of the differentially expressed fragments (DEFs) up-regulated by drought stress in *F. mairei*.

DEF	Top hit in GenBank	E-value[1]	Description	Organisium	Size (bp)
SSBII-H2	gi|46560602|	2.00E-44	putative inosine-uridine preferring nucleoside hydrolase	[Zea mays]	245
SSBI-B6	gi|50540754|	7.00E-44	putative raffinose synthase or seed imbibition protein	[Oryza sativa]	248
SSBI-B9	gi|28866019|	3.00E-40	farnesylated protein 3	[Hordeum vulgare]	263
SSBI-C9	gi|50252610|	1.00E-33	putative trehalose-6-phosphate synthase/phosphatase	[Oryza sativa]	251
SSBI-A4	gi|52353404|	1.00E-29	DNAJ heat shock N-terminal domain-containing protein	[Oryza sativa]	194
SSBI-E2	gi|50898740|	1.00E-25	putative chloroplast drought-induced stress protein, 34 kD/ fibrillin-like protein	[Oryza sativa]	306
SSBI-A5	gi|33771376|	2.00E-25	putative brown planthopper susceptibility protein Hd002A	[Oryza sativa]	228
SSBI-C7	gi|49328143|	2.00E-24	putative peptide chain release factor subunit 1 (eRF1)	[Oryza sativa]	172
SSBI-D11	gi|34894800|	6.00E-19	putative dihydrolipoamide dehydrogenase precursor	[Oryza sativa]	156
SSBI-D4	gi|38016525|	9.00E-19	fiber protein Fb19/universal stress protein USP1-like protein	[Gossypium barbadense]	273
SSBI-C2	gi|56784864|	8.00E-18	auxin-regulated protein-like	[Oryza sativa]	310
SSBI-F9	gi|53749298|	8.00E-16	putative polyprotein/ GAG-POL precursor	[Oryza sativa]	166
SSBII-A3	gi|3702665|	1.00E-13	type-1 pathogenesis-related protein	[Triticum aestivum]	100
SSBI-B2	gi|50919725|	2.00E-13	Putative anion transporter	[Oryza sativa]	114
SSBI-D9	gi|51469000|	3.00E-13	Ankyrin protein kinase-like/dehydration-responsive protein-like	[Poa pratensis]	103
SSBII-G4	gi|30420736|	4.00E-13	zinc transporter	[Oryza sativa]	108
SSBI-E11	gi|168473|	9.00E-13	ferredoxin	[Zea mays]	119
SSBII-D9	gi|25090853|	2.00E-09	NADH-ubiquinone oxidoreductase18 kDa subunit, mitochondrial precursor	[Arabidopsis thaliana]	77
SSBII-H9	gi|4996646|	1.00E-08	Dof zinc finger protein	[Oryza sativa]	185
SSBI-C11	gi|4996646|	1.00E-08	Dof zinc finger protein	[Oryza sativa]	199
SSBII-A4	gi|32879770|	5.00E-07	tonoplast intrinsic protein	[Oryza sativa]	70
SSBI-F2	gi|51572282|	5.00E-07	MYB transcription factor	[Triticum aestivum]	80

[1]E-value < 1.00E-06 means the significant similarity with the protein sequences in the Genbank database

Table 4.4. Functional annotation and size of the differentially expressed fragments (DEFs) down-regulated by drought stress in *F. mairei.*

DEF	Top hit in GenBank	E-value	Description	Organisium	Size (bp)
SSBII-A5	gi|710308|	1.00E-108	victorin binding protein/ glycine dehydrogenase P protein	[Avena sativa]	582
SSBI-C5	gi|50940811|	2.00E-75	putative non-phototropic hypocotyl 3 (NPH3)/phototropic response protein	[Oryza sativa]	449
SSBI-G5	gi|14861035|	2.00E-71	protoporphyrin IX Mg-chelatase subunit XANTHA-F	[Hordeum vulgare]	399
SSBII-C11	gi|710308|	5.00E-71	victorin binding protein/ glycine dehydrogenase P protein	[Avena sativa]	398
SSBI-H3	gi|33333542|	1.00E-70	knotted 7 /homeobox gene	[Hordeum vulgare]	460
SSBII-E9	gi|1181331|	9.00E-69	calnexin	[Zea mays]	356
SSBII-E4	gi|861199|	1.00E-68	protoporphyrin IX Mg-chelatase subunit precursor	[Hordeum vulgare]	398
SSBII-F10	gi|50921909|	3.00E-65	OSJNBa0032B23.5 /cleavage and polyadenylation specificity factor (CPSF)	[Oryza sativa]	398
SSBII-G10	gi|13661772|	2.00E-64	putative cytochrome P450	[Lolium rigidum]	357
SSBI-E8	gi|68599|	2.00E-59	glutamate-ammonia ligase precursor, chloroplast - barley	[Hordeum vulgare]	390
SSBII-H8	gi|861205|	3.00E-55	ADP-ribosylation factor	[Chlamydomonas reinhardtii]	309
SSBII-E2	gi|6409335|	7.00E-54	ribulose-1,5-bisphosphate carboxylase small subunit	[Avena clauda]	431
SSBI-F5	gi|50912639|	4.00E-51	putative serine-threonine rich antigen	[Oryza sativa]	473
SSBII-G8	gi|18147771|	3.00E-49	cycloartenol synthase	[Costus speciosus]	345
SSBII-D1	gi|54290318|	1.00E-48	unknown protein/IWS1 C-terminus family protein [Arabidopsis thaliana]	[Oryza sativa]	318
SSBII-B8	gi|40888826|	1.00E-46	iron-phytosiderophore transporter protein yellow stripe 1	[Oryza sativa]	338
SSBII-F2	gi|21842139|	1.00E-43	cytochrome P450 monooxygenase CYP72A28	[Zea mays]	390
SSBI-E10	gi|32251039|	3.00E-38	glyoxysomal malate dehydrogenase	[Triticum aestivum]	238
SSBII-F3	gi|49388196|	4.00E-37	putative TATA-binding protein associated factor (IID (TFIID) component TAF4 family)	[Oryza sativa]	400
SSBII-F8	gi|51091645|	2.00E-35	rab3-GAP regulatory domain-like	[Oryza sativa]	235
SSBII-H7	gi|50872458|	1.00E-34	putative c-type cytochrome synthesis protein	[Oryza sativa]	223

Table 4.4. Functional annotation and size of the differentially expressed fragments (DEFs) down-regulated by drought stress in *F. mairei* (cont'd).

DEF	Top hit in GenBank	E-value	Description	Organisium	Size (bp)
SSBII-A2	gi|4325354|	1.00E-34	contains similarity to retrovirus-related polyproteins and to CCHC zinc finger protein /gag-pol polyprotein	[Arabidopsis thaliana]	223
SSBI-H1	gi|14334888|	1.00E-34	putative glycine hydroxymethyltransferase	[Arabidopsis thaliana]	224
SSBII-E7	gi|2072727|	1.00E-34	ferredoxin-dependent glutamate synthase	[Oryza sativa]	232
SSBI-B7	gi|18033922|	2.00E-34	SEUSS transcriptional co-regulator (Repressor?)	[Arabidopsis thaliana]	286
SSBI-C10	gi|50726040|	3.00E-34	putative transcription factor EREBP1/BTH-induced ERF transcriptional factor 1	[Oryza sativa]	280
SSBII-F5	gi|45357045|	5.00E-34	coatomer alpha subunit	[Hordeum vulgare]	209
SSBII-A11	gi|2072727|	7.00E-31	ferredoxin-dependent glutamate synthase	[Oryza sativa]	215
SSBII-B6	gi|2072727|	7.00E-31	ferredoxin-dependent glutamate synthase	[Oryza sativa]	215
SSBII-F4	gi|20153218|	9.00E-31	putative sucrose:sucrose 1-fructosyltransferase	[Lolium perenne]	192
SSBI-E4	gi|50948231|	1.00E-30	putative cytochrome p450 (CYP78A9)	[Oryza sativa]	230
SSBI-E7	gi|13195734|	2.00E-30	triose phosphate translocator	[Triticum aestivum]	201
SSBII-D4	gi|34922538|	1.00E-28	Lipoxygenase 2.3, chloroplast precursor	[Hordeum vulgare]	194
SSBII-H10	gi|21839|	2.00E-28	phosphoribulokinase; ribulose-5-phosphate kinase	[Triticum aestivum]	254
SSBII-G7	gi|54290956|	1.00E-27	putative UDP-glycosyltransferase 88B1	[Oryza sativa]	245
SSBI-C4	gi|51451358|	3.00E-27	putative o-methyltransferase ZRP4	[Oryza sativa]	206
SSBI-G9	gi|50912885|	8.00E-26	putative Ribosomal RNA processing protein/ S1 self-incompatibility locus-linked pollen G211 protein	[Oryza sativa]	285
SSBII-F6	gi|13785467|	9.00E-26	phosphoenolpyruvate carboxykinase (ATP-dependent)	[Flaveria trinervia]	172
SSBII-B2	gi|47169677|	2.00E-25	ferric reductase	[Oryza sativa]	178
SSBII-H3	gi|39750999|	1.00E-24	unnamed protein product/ Alpha-glucan water dikinase, chloroplast precursor (Starch-related R1 protein)	[Lolium perenne]	232
SSBI-B12	gi|710308|	1.00E-23	victorin binding protein	[Avena sativa]	166
SSBI-E6	gi|50905199|	1.00E-23	cycloartenol synthase	[Oryza sativa]	185

Table 4.4. Functional annotation and size of the differentially expressed fragments (DEFs) down-regulated by drought stress in *F. mairei* (cont'd).

DEF	Top hit in GenBank	E-value	Description	Organisium	Size (bp)
SSBI-C6	gi|32481061|	1.00E-23	Rubisco activase alpha form precursor/ ribulose-bisphosphate carboxylase activase	[Deschampsia antarctica]	233
SSBII-H6	gi|38347077|	9.00E-23	OSJNBa0006A01.18/ unknown protein	[Oryza sativa]	161
SSBII-E5	gi|50906397|	1.00E-22	zinc finger protein-like /GATA-1 zinc finger protein	[Oryza sativa]	231
SSBII-C1	gi|56784641|	4.00E-21	putative cation diffusion facilitator 9	[Oryza sativa]	167
SSBII-B4	gi|51964240|	3.00E-19	PREDICTED P0666E12.10 gene product	[Oryza sativa]	278
SSBII-G3	gi|48716905|	2.00E-18	apospory-associated protein C-like	[Oryza sativa]	129
SSBI-E12	gi|710308|	2.00E-17	victorin binding protein	[Avena sativa]	122
SSBII-G9	gi|50251471|	3.00E-17	unknown protein	[Oryza sativa]	323
SSBI-H8	gi|50907447|	6.00E-17	unknown protein	[Oryza sativa]	132
SSBII-C2	gi|50906979|	1.00E-15	putative GTP-binding protein typA (tyrosine phosphorylated protein A)/ elongation factor family protein	[Oryza sativa]	134
SSBII-D7	gi|119284|	1.00E-15	High molecular mass early light-inducible protein HV58, chloroplast precursor (ELIP)	[Hordeum vulgare]	170
SSBII-F7	gi|121343|	2.00E-15	Glutamine synthetase shoot isozyme, chloroplast precursor (Glutamate--ammonia ligase)	[Oryza sativa]	109
SSBI-D6	gi|53749331|	1.00E-13	putative 2-oxoglutarate/malate translocator	[Oryza sativa]	128
SSBII-C3	gi|119748|	7.00E-13	Fructose-1,6-bisphosphatase, cytosolic (D-fructose-1,6-bisphosphate 1-phosphohydrolase) (FBPase)	[Spinacia oleracea]	121
SSBI-G6	gi|50905143|	7.00E-12	putative 50S ribosomal protein L3	[Oryza sativa]	204
SSBII-E6	gi|11761654|	2.00E-11	peroxiredoxin / thioredoxin peroxidase CATP	[Oryza sativa]	178
SSBI-F6	gi|32400293|	7.00E-11	hydroxyanthranilate hydroxycinnamoyltransferase 2	[Avena sativa]	91
SSBII-C10	gi|12060390|	2.00E-10	response regulator 7	[Zea mays]	148
SSBI-H6	gi|38345616|	4.00E-10	OSJNBb0003B01.8 (BAC clone)/unknown protein	[Oryza sativa]	91
SSBII-A8	gi|1769849|	3.00E-09	photosystem II type I chlorophyll a/b binding protein	[Apium graveolens]	166
SSBII-D8	gi|15223823|	2.00E-08	armadillo/beta-catenin repeat family protein /unknown protein	[Arabidopsis thaliana]	204

Table 4.4. Functional annotation and size of the differentially expressed fragments (DEFs) down-regulated by drought stress in *F. mairei* (cont'd).

DEF	Top hit in GenBank	E-value	Description	Organisium	Size (bp)
SSBI-A7	gi|82619|	6.00E-08	ribulose-bisphosphate carboxylase	[Triticum aestivum]	212
SSBII-G2	gi|50915986|	2.00E-07	putative phosphatidylinositol-4-phosphate 5-kinase	[Oryza sativa]	90
SSBI-B8	gi|31323256|	2.00E-07	photosystem II type I chlorophyll a/b binding protein	[Brassica oleracea]	116
SSBI-G10	gi|50911901|	2.00E-07	putative DNA helicase/ DNA-binding protein, putative	[Oryza sativa] [Arabidopsis thaliana]	82
SSBI-B10	gi|2196772|	4.00E-07	chlorophyll a/b-binding protein	[Mesembryanthemum crystallinum]	116
SSBII-C8	gi|50928287|	6.00E-07	OSJNBa0013K16.8/ putative glutamate receptor	[Oryza sativa]	113
SSBII-G1	gi|133872|	2.00E-06	30S ribosomal protein S1, chloroplast precursor (CS1)	[Spinacia oleracea]	114

Table 4.5. Functional annotation and size of the differentially expressed fragments (DEFs) transiently expressed and up-then-down-regulated during drought stress in *F. mairei.*

DEF	Top hit in GenBank	E-value	Description	Organisium	Expression pattern[1]	Size (bp)
SSBII-D5	gi|53680379|	3.00E-51	glutamine-dependent asparagine synthetase	[Triticum aestivum]	T	290
SSBI-A10	gi|20302435	1.00E-33	plasma membrane H+ ATPase	[Oryza sativa]	T	222
SSBI-H7	gi|4138869|	9.00E-16	small heat shock protein Hsp23.5	[Triticum aestivum]	T	128
SSBI-H5	gi|23954355|	8.00E-15	metallothioneine type2	[Hordeum vulgare]	T	287
SSBI-F10	gi|32394644|	3.00E-33	putative Rieske Fe-S precursor protein	[Triticum aestivum]	UD	259
SSBI-B5	gi|82682|	4.00E-31	chlorophyll a/b-binding protein precursor	[Zea mays]	UD	197
SSBI-D3	gi|50252668|	1.00E-21	putative digalactosyldiacylglycerol synthase	[Oryza sativa]	UD	309
SSBI-D5	gi|50943213|	5.00E-12	putative disease resistance protein	[Oryza sativa]	UD	231
SSBII-E3	gi|50912463|	8.00E-07	unknown protein	[Oryza sativa]	UD	84

[1]T means transiently expressed DEF; UD means up-then-down-regulated DEF

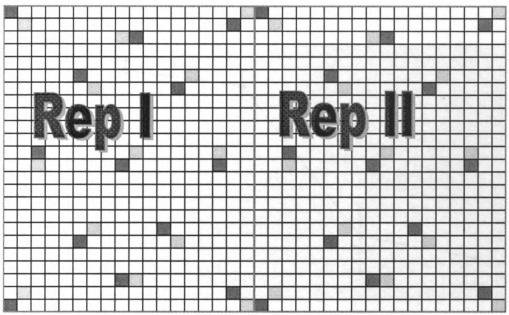


Figure 4.1. The spot arrangement on the nylon array for macroarray hybridization. The controls were spotted in different sections of the membrane to compensate for variable background levels. All samples were presented with two replications on each array.

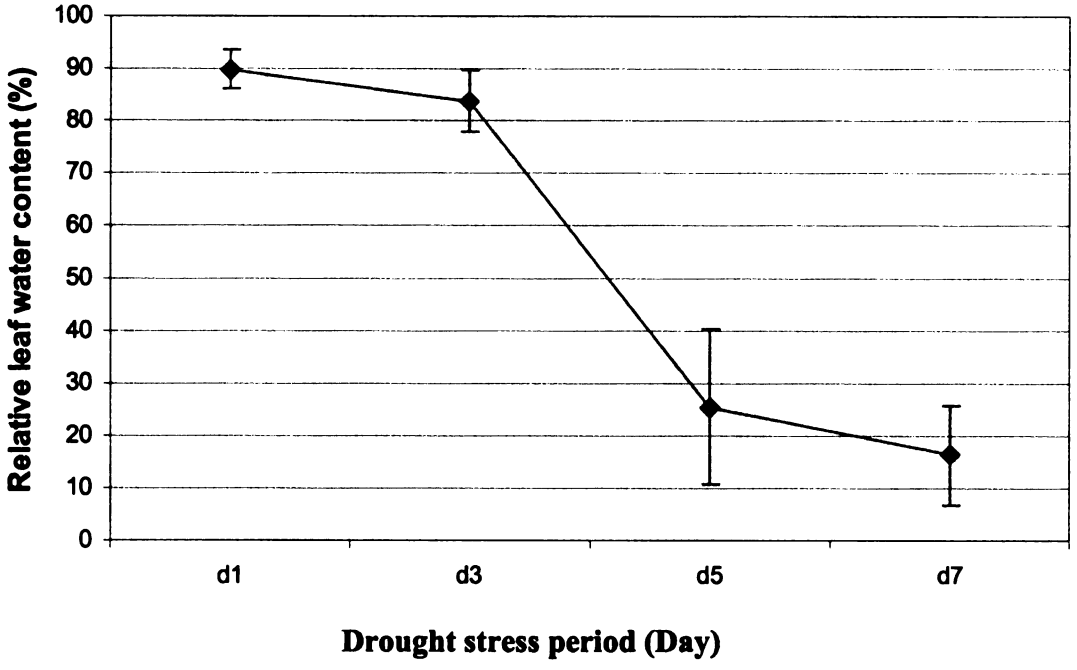


Figure 4.2. Relative leaf water content of *F. mairei* during the drought stress cycle.

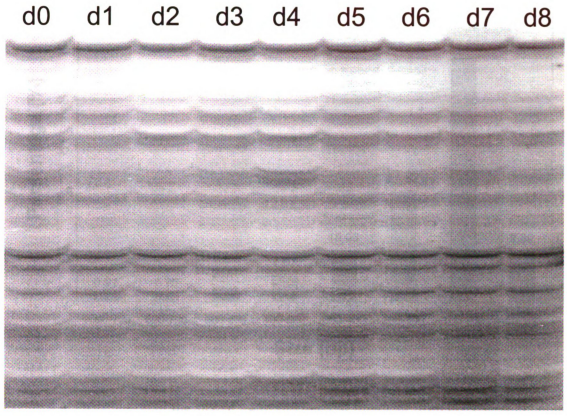


Figure 4.3. An example of cDNA-AFLP profile for treatment control *F. mairei.* All transcript derived fragments were constitutively expressed at the same level.

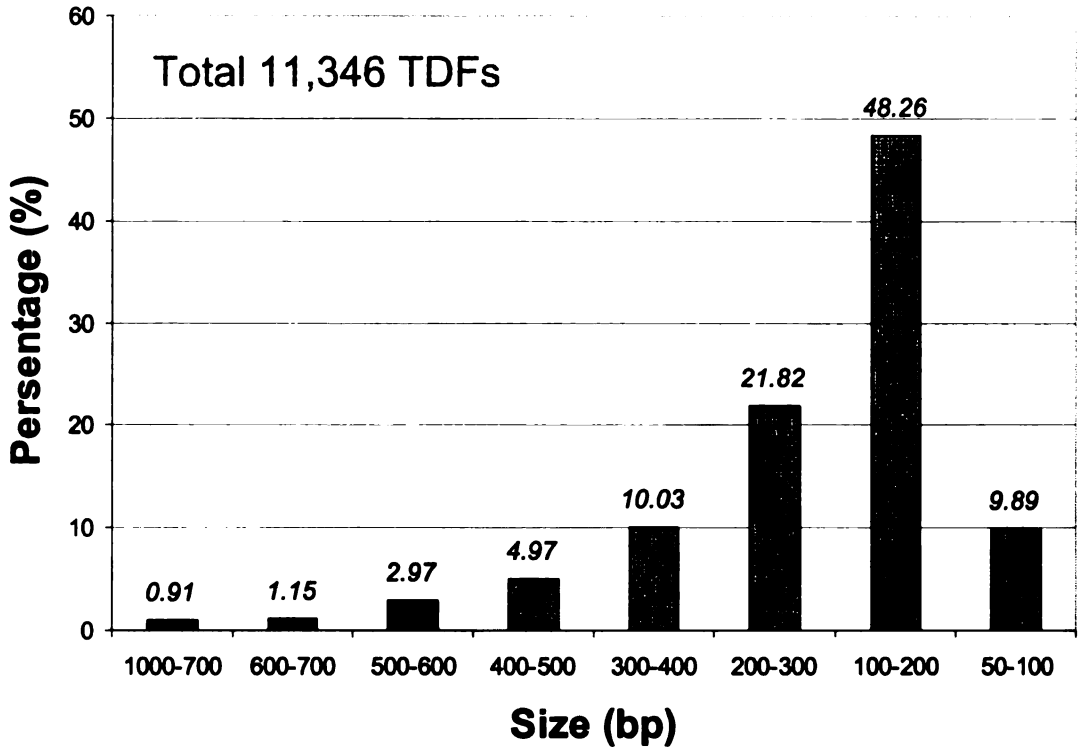


Figure 4.4. Distribution of the size of transcript derived fragment from cDNA-AFLP performed on *F. mairei*.

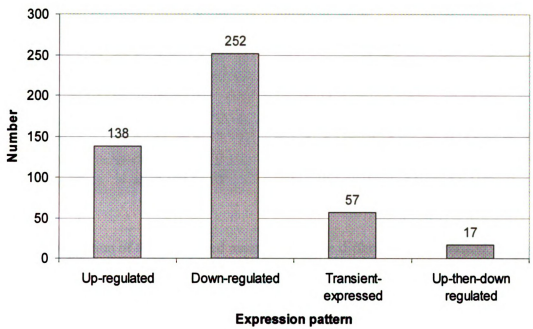


Figure 4.5. Distribution of the pattern of differentially expressed fragments revealed by cDNA-AFLP during drought stress cycle in *F. mairei*.

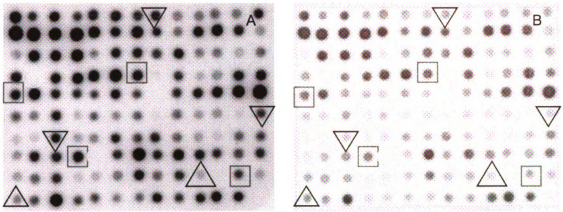
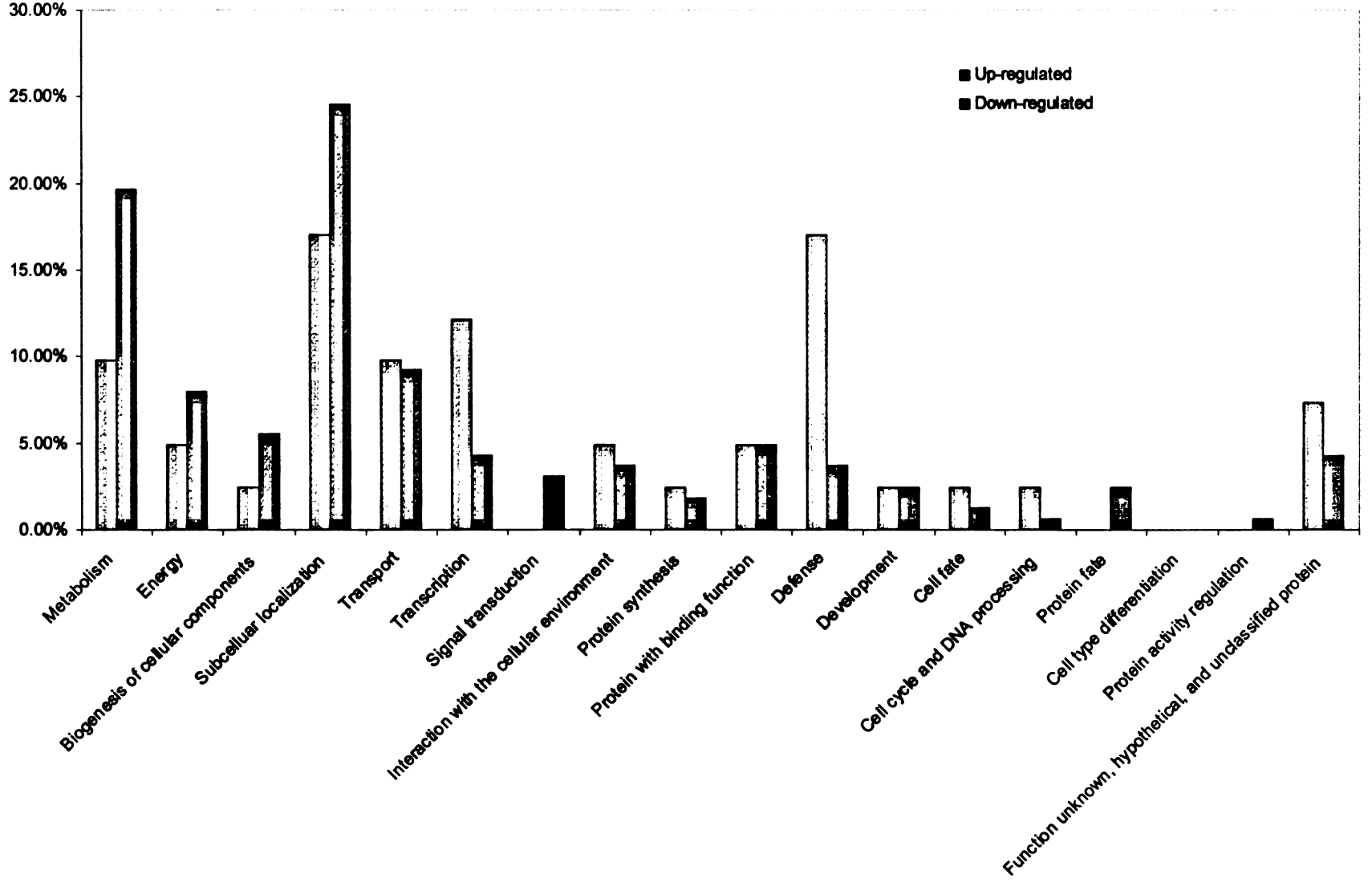


Figure 4.6. A portion of the hybridized macroarray. The differentially expressed fragments (DEFs) from cDNA-AFLP were arrayed on nylon membranes A and B identically. Membranes were seperatively hybridized to treatment control (A) and 5 days stress treated (B) cDNA probes, respectively. Spots in squares indicated the housekeeping controls used for normalization between arrays (In present case, signals in membrane A were stronger than B. Therefore, the housekeeping controls were used to balance the two arrays). Spots in the circle indicated the negative controls used to eliminate the background effect. Spots in the up-triangle indicated example of up-regulated DEFs. Spots in the down-triangle showed example of down-regulated DEFs.

Figure 4.7. Comparison of functional categories between up-regulated and down-regulated differentially expressed fragments (DEFs) during drought stress cycle in *F. mairei*. Each DEF was searched against the GenBank plant protein database by BLASTX. The functional category was assigned based on function classification criteria on the website of Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de).


30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Up-regulated
Down-regulated
Metabolism
Energy
Biogenesis of cellular components
Subcellular localization
Transport
Transcription
Signal transduction
Interaction with the cellular environment
Protein synthesis
Protein with binding function
Defense
Development
Cell fate
Cell cycle and DNA processing
Protein fate
Cell type differentiation
Protein activity regulation
Function unknown, hypothetical, and unclassified protein

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SUMMARIES

- Compared with three tall fescue (*F. arundinacea* Schreb.) cultivars, the leaf elongation and leaf water content of Atlas fescue (*Festuca mairei*) was less sensitive to the drought stress treatment.
- Atlas fescue had an exceptional ability to accumulate water in leaf tissue under severe drought stress.
- A mechanism may exist in Atlas fescue to maintain cell turgor necessary for cell expansion as soil water content declined and leaf water potential became more negative.
- Atlas fescue possessed drought tolerance and afforded potential to improve drought tolerance in turfgrass breeding program.
- The genome of Atlas fescue had been successfully transferred into drought susceptible perennial ryegrass through intergeneric hybridization.
- The parental genome composition of the hybrid progeny ranged widely when detected by SSR and RAPD markers.
- The non-coinheritance of the linked markers suggested chromosome crossover between the two parents.
- Cluster and principle component analyses of the progeny consistently revealed three major groups. Group I included progeny that introgressed more of the Atlas fescue than perennial ryegrass genome. Group II comprised of progeny showing similar amounts of genome introgression from both parents. Group III contained progeny that introgressed more of the perennial ryegrass genome.

- For the progeny with more Atlas fescue genome, more backcrosses should be conducted to recover the good turf quality of ryegrass.
- Drought tolerant cultivar can be released from these progeny after drought tolerance and turf quality evaluations.
- In cDNA-AFLP analysis, NspI coupled with TaqI was a pair of highly efficient enzymes for detecting transcript derived fragments in Atlas fescue species. This enzyme combination may have valuable application potential for other species.
- The cDNA-AFLP analysis was relatively insensitive to amplification conditions and had high reproducibility across treatments.
- The chimeric fragments derived from ligation between digested fragments were generated and could not be eliminated by increasing adapter concentration.
- The cDNA-AFLP analysis is a reliable and high throughput transcript profiling technique suitable to transcript derived fragments (TDFs) discovery in grasses such as Atlas fescue species.
- Among a total of 11,346 TDFs revealed by cDNA-AFLP in Atlas fescue species, 464 (4.1%) TDFs were identified as differentially expressed fragments (DEFs with size distribution between 50 and 1000bp. The expression patterns of these DEFs included up-regulated (29.7%), down-regulated (54.3%), transiently-expressed (12.3%), and up-then-down-regulated (3.7%).
- The differential expression pattern of 171 (42.1% of 406) DEFs from cDNA-AFLP analysis was confirmed by macroarray hybridization analysis.
- cDNA-AFLP technique coupled with macroarray hybridization analysis was an efficient procedure in detecting differentially expressed genes.

- Sequences of 163 confirmed DEFs were compared to the GenBank plant protein database by using BLASTX to target the potential function of these gene fragments. Predicted functions of the 101 sequences were subdivided into 17 functional categories, suggesting Atlas fescue responded to drought stress at a comprehensive molecular regulation level.
- Some DEFs discovered in Atlas fescue are novel genes, suggesting Atlas fescue might apply current unknown mechanism to defense drought stress.
- During drought stress treatment in Atlas fescue, more metabolic function and biogenesis of cellular components in the plant were under degenerative processes, and the plant system may rescue energy for new gene transcription and stress defense.