PHYSIOLOGICAL RESPONSES OF CONTINENTAL (SUMMER-ACTIVE) AND MEDITERRANEAN (SUMMER-DORMANT) TALL FESCUE TO COLD STRESS AND IDENTIFICATION OF UNDERLYING QTL FOR FALL GROWTH AND WINTER SURVIVAL

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DEDICATION

TO:
My wife Emily
My family
and
My parents Michael and Victoria Dierking

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ABSTRACT

We describe three experiments; the first two experiments were conducted in a greenhouse and with the use of cold chambers. The first experiment determined the effect of endophyte (*Neotyphodium ceonophialum*) presence under cold (4.5^{o}C) and freezing (-3, -6, -9, and -12^{o}C) conditions on two genotypes of Continental tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.], one Mediterranean tall fescue, and two *F. arundinacea* var. *atlantigena* St. Yves. The plants were assessed for growth, measured by leaf extension rate (LER), proline, fructan, and mono- and disaccharides concentrations at 25.0 and 4.5^{o}C. Additionally, the survivability of these plants was assessed at -3, -6, -9, and -12^{o}C. It was found that endophyte presence did not affect the LER or concentrations of proline and the mono- and disaccharides at 25.0 or 4.5^{o}C. In the second experiment four Continental (PI 172423, PI 283297, PI 314684, and Kentucky-31) and three Mediterranean accessions (PI 200339, PI 610956, Flecha) were used to determine the growth quantified by LER, abscisic acid (ABA), proline, fructan, and mono- and disaccharides concentrations. Mediterranean accessions were different from Continental accessions for all parameters measured at 4.5^{o}C. The final experiment included the development of a linkage map from a cross between a Mediterranean (103-2) x Continental (R43-64) cross, and identifying QTL relating to fall growth (FG), measured by LER, and winter survival (WS). This is the first reported linkage map of Mediterranean tall fescue as well as the use of DArTFest markers using array technology. Major QTL were found for FG and WS at each location across years. Based on all data in this study it appears these two types of tall fescue are unique species.

LITERATURE REVIEW

Introduction

Tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] is a cool-season grass native to Europe, Western Asia, and North Africa. Tall fescue types are broadly separated into two categories: 1) Continental types (summer-active) are found primarily in Western Asia and Central and Northern Europe, and 2) Mediterranean types (summer-dormant) are found primarily in Northern Africa and extreme Southern Europe. Continental tall fescue is a hexaploid comprised of three genomes P, G_1, and G_2. The 'P' genome originates from meadow fescue (*Lolium pratensis* [Huds.] Darbysh.= *Festcua pratensis* Huds.), and two genomes 'G_1' and 'G_2' are derived from the tetraploid fescue *Festuca arundinacea* var. *glaucescens* ($G_1G_1G_2G_2$). Mediterranean germplasm, however, is unique and genetically incompatible with Continental types (Hopkins et al., 2007). There are additional species of tall fescue with genomes ranging from tetraploid to decaploid. These different species can be utilized to determine which genomes are responsible for harboring specific genes underlying traits of interest.

It was not until the 1800s that tall fescue was grown in the United States as forage. It took until the early-twentieth century for tall fescue to become an important agricultural grass with the release of 'Kentucky 31' in the Eastern US and 'Alta' in the Pacific Northwest. After the release of these varieties, its popularity and geographical range grew immensely. It was widely accepted by producers due to its ability to produce and persist under a wide range of soil, climatic, and management conditions (Hoveland, 2007). Today tall fescue is found in nearly every state east of longitude -95^o covering

roughly 40 million acres in the US with 5 to 6 million in production in Missouri alone (Roberts, 2000).

One of the largest limitations for acceptance of tall fescue is its mutualistic relationship with the endophyte *Neotyphodium coenophialum* [Morgan-Jones and Gams] Glenn, Bacon, and Hanlin. Typically, tall fescue is infected with a wild-type endophyte (*Neotyphodium ceonophialum*) that produces toxic ergot-like alkaloids. Animals that graze tall fescue infected with a wild-type endophyte exhibit a number of physiological problems that limit livestock productivity. A recent development is the use of novel endophyte (E++) cultivars with low to no ergot alkaloid toxicity compared to the native endophyte (E+) that tall fescue contains. This advancement retains the competitive ability of E+ cultivars, but is superior for animal production.

Like all cool-season grasses, tall fescue grows most rapidly in cool, moist weather which typically occurs during the spring and autumn months in the Continental US. Tall fescue enters a dormant phase over winter which typically begins in December and lasts until mid-March in Missouri. This type of growth habit only occurs in Continental tall fescue types; Mediterranean tall fescue is more active during the winter months which is due to the environmental conditions of its native habitat. This trait could be useful in two ways. First, release of summer-dormant types derived from Mediterranean germplasm might allow producers in drier climates, such as California, Kansas, Oklahoma and Texas to use tall fescue as part of a grazing system. Second, the ability of Mediterranean tall fescue types to grow longer into the autumn could significantly reduce winter feeding costs by extending the grazing season. This might be possible by identifying genotypes that continue dry matter production into fall and survive through the winter months.

Abiotic Factors of Cold Tolerance

Many researchers have an interest in cold tolerance since most arable lands exist in temperate regions of the globe where cold and freezing conditions occur every year. However, there are only two ways of enduring cold temperatures in the biological world: (a) avoidance or (b) tolerance/resistance. Since plants are poikilotherms and assume the temperature of their environment, their only strategy is to tolerate extremes in temperature and overcome it by some mechanism(s) that permits survival (Levitt, 1980). Many factors affect the plant's ability to survive which include water availability, nutrients, light conditions, and combined light and temperature interactions. In order to exploit different winter hardiness traits, it is imperative to understand the mechanisms that facilitate plant survival and continued growth. These factors will be discussed in more detail below.

Moisture: Several experiments involving seeds, seedlings, or mature plants show that plants under water stress harden and are able to withstand colder temperatures than plants that are not "water stressed". Metcalf et al. (1970) compared three barley (*Hordeum vulgare* L.) and three wheat (*Triticum aestivum* L.) cultivars with adjusted crown moistures between 55 and 70%. As the wheat and barley crowns became drier, the ability of the plants to withstand colder temperatures increased until crown moisture was above 65%. However, water stress only impacts cold hardiness early in the growth cycle with the ability to withstand colder temperatures diminished approximately 16 days after cold initiation (Tyler et al., 1980).

Light and temperature effects on light reactions: Temperature shifts induce changes in the light reactions of photosynthesis; these changes are required for a plant to

survive a range of temperatures. In fact, light and the reactions necessary to shuttle the electrons through the photosystems are largely unaffected by cold temperatures. However the biochemical (enzymatic) reactions are subject to change due to fluctuating temperatures. This is known as the Q_{10} where a 10 degree temperature change will result in a 2 to 3 fold change in the biochemical reaction rate. Therefore, the biochemical reactions that were occurring at the warmer temperatures (25^{o}C) are now functions at 1/4 to 1/6 the rate at the lower temperatures (4^{o}C). This difference between the photosystem I and II reactions and the biochemical reactions causes congestion leading to an excess of electrons, which ultimately lead to either alterations in the photosystems or a degradation of the entire photosystem.

All plants use pigments such as chlorophyll, phytochromes, and carotenoids which are influenced by light intensity and quality. These pigments are vital for light sensing and day length determination. Of the pigments produced, phytochromes are the most responsive to changing light conditions. Different types of phytochromes (PHY) exist in the plant kingdom; monocots contain three (PHYA, B, and C) and dicots typically contain five (PHY A, B, C, D, and E) phytochrome genes (Taiz and Zieger, 2006). Phytochrome A is the most fully understood of the phytochromes due to its responses to red and far red light. By using PHYA mutants/transgenics, an understanding of how plants perceive light and adapt to the changing seasons can be gained.

Aspen (*Populus tremuloides*) and evergreen trees are commonly studied for responses to cold/freezing temperatures and low light levels due to their native habitats. Using oat *(Avena sativa) PHYA* gene in transgenic aspen, Olsen et al. (1997) showed the transgenic trees failed to respond to short days resulting in the loss of their ability to

harden before the onset of cold temperatures. Ultimately, the transgenic trees were unable to tolerate the freezing conditions compared to trees that could acclimate naturally. Similarly, evergreens need to acclimate to shorter days and low temperatures in the fall and winter months. Evergreen trees down-regulate the photosynthetic apparatus, more specifically the D1 protein in the reaction center, and dissipate the excess energy by non-photochemical quenching (NPQ) through the use of xanthophylls (Savitch et al., 2002; Sveshnikov et al., 2006). This process results in the constant production of sugars and complete conversion to storage carbohydrates which allows separation of photosynthesis from growth (Hjelm and Ogren, 2003). Unlike evergreens, which rely more heavily on light levels to change their photosynthetic apparatus, temperature regulates cold tolerance mechanisms to a greater extent in grass and herbaceous plants (Gray et al., 1994; Huner et al., 1993)

Grasses rely on the crown tissue where carbohydrates are stored for persistence, which is in contrast to evergreens that rely on their needles/leaves throughout the winter to survive, (Huner et al., 1993; Savitch et al., 2000; Ensminger et al., 2006). Therefore, grasses utilize photochemical quenching rather than NPQ, which is primary used by evergreens to dissipate excess energy as heat. Grasses tend to increase their tolerance to photoinhibition by increasing the rate at which electron acceptor quinone$_A$ (Q_A) is oxidized, by having a higher photochemical quenching (q_P) capacity which results in elevated sugar production (Huner et al., 1998). This process of photostasis can be difficult to obtain and is constantly changing under field conditions due to the effects of fluctuating temperatures on the different photo- and biochemical reaction rates. When temperatures decrease, the chemical reactions optimized at a higher temperature abruptly

become unbalanced due to the ability of the photochemical reactions to exceed biochemical metabolism rates. This imbalance requires adjustment of the photoinhibition effects, which is achieved by the plastoquinone (PQ) pool residing in the thylakoid membrane (Allen and Nilsson, 1997; Escoubas et al., 1995; Maxwell et al., 1995; Pfannschmidt, 2003). This PQ pool triggers genes in both the cell nucleus and chloroplast to alter either the source where the initial photochemical reactions occur, or the sink where the carbon metabolism takes place. This can be accomplished by one or many of the following mechanisms: 1) adjusting the PSII quantum (σ_{PSII}) functional absorption; 2) adjusting the σ_{PSII} by a physical decrease in size; 3) decreasing irradiance and/or 4) increasing the electron sink capacity by the up-regulation of CO_2 assimilation (Ensminger et al., 2006; Huner et al., 1998; Huner et al., 2003).

Photochemical quenching occurs when there is an increased rate of electrons transported away from PSII. This is the primary result of the increased activation of carbon metabolism enzymes and open stomata in tissues acclimated to cold temperatures (Maxwell and Johnson, 2000). Measurements of photochemical quenching (PQ) include: PSII quantum yield/operating efficiency (Φ_{PSII}), proportion of PSII open RC/efficiency factor (*qP*), PSII maximum quantum yield/efficiency (F_v/F_m) (Maxwell and Johnson, 2000; Baker and Rosenqvist, 2004). These processes are the main factors regulating the ability of grasses to acclimate to cold temperatures rather than the NPQ seen in trees; however, NPQ is a component in herbaceous plants.

Several mechanisms can limit photoinhibition at low temperatures other than the primary use of PQ. One mentioned above involves the use of NPQ that has several ways in dissipating excess energy. One involves the use of xanthophylls to dissipate excess

light energy. More specifically, the compound violaxanthin is thought to associate with the light harvesting complex (LHC) of PSII. When the LHC is flooded with excess light, the pH of the lumen decreases and violaxanthin converts to antheraxanthin and zeaxanthin which dissipates the energy as heat rather than passing it to chlorophyll *a* (Niyogi et al., 2005). Frank et al. (1994) hypothesized that the use of xanthophylls acts like a 'molecular gear shift' for the system transferring energy away from the PSII reaction centers. This lowers the potential of damage occurring to the PSII. This reaction occurs rapidly and is quickly reversible when temperatures increase especially in winter annual plants like cereals (Ensminger et al., 2006). Contrary to the 'molecular gear shift' hypothesis, Horton (1999) proposed that both the *trans*-thylakoid pH change occurring in the lumen and the xanthophylls together are regulating the LHC of PSII. Regardless of how plants use xanthophylls, they are of minor importance for any of the NPQ methods utilized by herbaceous plants. Xanthophylls are thought to be utilized primarily as absorptive molecules that protect membranes from lipid peroxidation by reactive oxygen species (ROS) rather than protecting PSII (Ivanov et al., 2003).

Another aspect of NPQ is the antenna size and how its association with PSI and PSII can adjust to changes in light and temperature. If alterations in temperature or light intensity are short, the antenna can migrate in a matter of minutes to the other photosystem. This migration is determined by the excitation pressure, which is dependent on the size of the PQ pool. Thus, the LHC associated with PSII migrate to PSI and effectively balance the energy load for efficient electron movement (Lunde et al., 2000). However, if the temperature/light shift lasts for an extended duration, the physical antenna size can be modified. This is accomplished by altering the transcription and

translation of *Lhcb* nuclear genes encoding for the LHC of PSII; this effectively alters the chlorophyll *a/b* ratio (Ensminger et al., 2006).

Lastly, the RC of PSII can have NPQ properties, termed reaction center quenching, that complement the xanthophyll cycle and antenna alterations via the protein PsbS. This mechanism was suggested by several authors in the late 1990s to occur; however, recently this form of NPQ was determined to occur with the D1 protein in the PSII RC along with the plastoquinone Q_A and Q_B (Ivanov et al., 2003). This type of NPQ, unlike the other methods, takes several weeks to develop. It was observed that *Arabidopsis*, under steady state conditions, acclimates to cold temperatures by shifting Q_A to a higher temperature while Q_B shifts to a lower temperature. This decreases the redox potential/free energy gap between Q_A and Q_B when compared to a non-acclimated plant when using thermoluminescence (Sane et al., 2003). This reduction in free energy, due to the increased reduction of the PQ pool, does not allow for the electron energy at PSII RC to reduce the Q_B site forcing a quinone to stay at the D1 protein. This process effectively protects the PSII RC from photoinhibition and degradation (Wilson et al., 2006). In order for this series of events to occur, it is thought Q_A must be reduced (Bukhov et al., 2001), which is consistent with results of Sane et al. (2003). By increasing the amount of reduced $Q_{A,}$ Sane et al. (2003) proposed that it creates a back reaction with $P680^+$, leading to the dissipation of excess light energy thought PSII RC.

Combined Light and Temperature: In addition to the distinct temperature and light responses of plants, there is growing evidence that some plant responses are due to a combination of both light and temperature or the reduction state of PSII and how that changes the excitation pressure observed in the PSII reaction center and PQ pool. Gray et

al. (1997) first reported on this type of response in winter rye (*Secale cereal* L.) and suggested that cold acclimation and tolerance is much more complex in the responses to both light and temperature than previously thought. By using a combination of temperatures and irradiance levels they deduced how growth alterations occur, transcript levels alter, and freezing tolerance is affected. Plants grown under 5^{o}C and 250 μmol $m^{-2}s^{-1}$ (5/250) had the greatest freezing tolerance; while plants grown under the 5/50 regime had a 52% decrease in freezing tolerance due to the lower light intensity. These results indicate that light levels are critical to obtaining greater levels of freezing tolerance. A similar trend was observed at 20^{o}C, but freezing tolerances were reduced to a greater extent than those grown at 5^{o}C regardless of light intensity.

Growth rates were also altered with rye plants receiving high irradiation having the greatest elongation rates regardless of temperature. Plants growing at 20/800 and 5/250 exhibited similar growth morphology and excitation pressure. However, these plants differed in morphology and excitation pressure compared to plants growing under a 20/250 and 5/50 regime (Gray et al., 1997). Ndong et al. (2001) were interested in determining the genes that control excitation pressure, temperature, light, or temperature and light responses. They furthered Gray et al. (1997) work and found that 28 out of 42 of the expressed genes identified were up- or down- regulated by PSII excitation pressure. However, four were specific to temperature, one to light, and four were influenced by both temperature and light. From these results it appears that excitation pressure along with a combination of light and temperature have the greatest influence on the plant's acclimation of the light reactions to cold temperatures rather than just temperature or light alone.

Biotic Factors of Cold Tolerance

A plant's response to cold stress temperatures depends upon the climate where it originated. Plants common to the tropics and subtropics, such as rice (*Oryza sativa* L.) and sugarcane (*Saccharum officinarum* L.), exhibit stress responses and cell death at temperatures around 10-15^{o}C. Alternatively, plants native to temperate regions can grow at temperatures near or below 0^{o}C (Levitt, 1980; Fowler et al., 2005). Leasure (1952) reported that tall fescue grows at weekly mean temperature of 4.4^{o}C and dormancy occurs at 1.1^{o}C. These differences among plants at low temperatures have a profound impact on the plant's ability to grow and sustain carbon metabolism. It also has a profound effect on the ability for sustained enzyme function, hormone production, and membrane alterations.

Growth Rates: Leaf elongation rate (LER) is one of many fundamental measures used in determining plant response to environmental conditions. During cold stress, leaves decrease their elongation rate, but the rate changes can be quite different between genotypes. Thomas and Stoddart (1995), comparing Mediterranean and Continental populations of tall fescue, reported that at 20^{o}C the Continental genotype S170 and Mediterranean genotypes Bn467 and Bn772 had similar growth rates, however, when grown at 5^{o}C, the Mediterranean lines had higher growth rates compared to the Continental types. It was also noted that chlorophyll degradation rates were higher for Mediterranean line Bn379 than for the other types. Similar trends were also described by Nelson et al. (1978) showing that the Mediterranean population Bn272 had a greater elongation rate than all Continental ecotypes at 8 to 10^{o}C while improved cultivars with

Continental backgrounds had elongations rates equal to the Mediterranean population at the same temperatures.

Carbon Metabolism-The Formation of Carbohydrates: Sugar concentrations in leaves and roots increase with the induction of cold temperatures. However, it is crucial to differentiate between plants or leaves abruptly subjected to a colder temperature compared to those developed at lower temperatures. Leaves subjected to the stress of acute temperature transition have limited sucrose production due the imbalance between the photo- and biochemical reactions leading to low inorganic phosphate (Pi) content. This reduced Pi concentration further impedes the production of ATP from the light reactions used to regenerate RuBP as shown using *pho* mutants in *Arabidopsis* (Hurry et al. 2000). Conversely, plants that have acclimated to cold temperature(s) are not only much more resistant to freezing temperatures, but are capable of adjustments in subsequent leaves and for continued growth. Prud'Homme et al. (1993) used labeled $^{14}CO_2$ to show that in tall fescue, sucrose is the primary sugar generated instead of glucose or fructose, when plants were subjected to cold stress. Additionally, concentrations of labeled fructans began to increase approximately 24hrs following exposure to cold stress, with low degree of polymerization (DP), short chain, fructans responding the greatest. This increase in labeled fructans supports the idea that sucrose is the main sugar used for fructan synthesis via fructosyltransferases (Pollock et al., 1989) and is not at the expense of other non-structural carbohydrates (Chatterton et al., 1989).

This increased biosynthesis of sucrose, as shown with transgenic sucrose phosphate synthase (sps) in *Arabidopsis*, allows for greater freezing tolerance as well as lowers the inhibition of photosynthesis typically experienced with cold temperatures

(Strand et al., 2003). This is further exemplified by Wanner and Junttila (1999), who showed that in plants subjected to cold temperatures without light, all cold-inducible mRNAs up-regulated, but plants accumulated low levels of sucrose compared to plants receiving light. Plants receiving no light did not achieve as great of cold tolerance as plants exposed to various light levels under the same cold conditions. Therefore, light is critical for the full development of cold tolerance.

In addition to the apparent cryoprotective status in the plant, sugars may also be involved in the regulation of abscisic acid (ABA) during cold/freezing conditions. High glucose and sucrose levels control ABA synthesis and its transcription factors (Arenas-Huertero et al., 2000; Leon and Sheen, 2003) both of which are elevated during a cold stress. The ABI4 and ABI5 (abscisic acid insensitive) genes are responsive to sugars, with ABI5 being hypersensitive to mannitol resulting in ABA and/or stress induction (Gusta et al., 2005). Therefore, sugars play an integral role in cold acclimation functioning as cryoprotectants, supplying carbohydrates for winter survival, and as signaling molecules.

Amino Acid Accumulation: Proline accumulation has also been known to accompany stress responses such as exposure to cold or salt. It was first related to cold resistance in plants by Chu et al. (1974). Nanjo et al. (1999) showed *Arabidopsis* plants that accumulated proline were more tolerant to freezing than control plants. Molecular work has played a role in discovering genes responsible for proline's initiation and subsequent degradation in response to stress. When plants are stressed, proline is synthesized from glutamate by *P5CS1*, *P5CS2* and *PDH* (Abraham et al., 2003) of which *P5CS2* is part of the *CBF* regulon (discussed later) (Fowler and Thomashow, 2002). It

was believed that proline synthesis occured through an ABA-independent manner, however, Knight et al. (2004) showed that CBF gene products accumulate in response to increased ABA. Indeed, Zeevaart and Creelman (1988) showed that proline accumulated after an endogenous increase in ABA. Proline functions as a protective compatible osmolite to scavenge free radicals and alters the redox potential though $NADP^+$ replenishment (Hare et al., 1999). This function appears to be light dependent, as plants grown in the dark do not accumulate proline when subjected to exogenous ABA treatment (Abrahams et al., 2003).

Lipid/Membrane Alterations: Even though the light sensing apparatuses are important to the transition to colder temperatures through PQ pool signaling, other mechanisms are independent of such responses (Ensminger et al., 2006). One such change is the thylakoid membrane and its fluidity. It has been suggested that for cells to survive and function, lipid membranes must alter their state when subjected to extreme temperatures. Upon cooling, the lipid membrane passes through a phase transition from a liquid crystalline to a solid crystalline (or gel) state. This transition occurs rapidly and is reversible upon returning to warmer temperatures. However, not every plant has the same transition point and the transition point is not necessarily correlated to chilling sensitivity. The transition temperature cannot fully predict the maximum and minimum growth temperatures. One purpose of this transition is to maintain the fluidity of the membrane for the attached enzymes to function at cold/freezing temperatures. Prior to phase transition, desaturase enzymes increase the palmitic, linoleic, and α-linolenic fatty acids and depress the transition phase to a lower temperature. This desaturation is apparently only completed in newly developing tissue. This increase in cell membrane desaturation

allows permeability to remain high and leads to the freezing of water outside the cell protecting it from rupture when the extracellular water freezes. However, not every plant responds by increasing the unsaturated lipid levels in the membranes such as trees that already possess a low transition phase (Levitt, 1980).

In addition to membrane fluidity and enzyme activity, the desaturation of membranes, such as the thylakoid, plays a role in the photosynthetic ability of the plant during cold stress. This desaturation to unsaturated fatty acids not only influences the survival of the plant, but also the plant's ability to capture light. Several *Arabidopsis* fatty acid desaturase (*fad)* mutants have been shown to significantly reduce chlorophyll fluorescence measurements (Vijayan et al., 1998). Additionally, the growth rate and the appearance of necrosis are different compared to wild types. Thus, normal photosynthetic functioning is dependent on a specific lipid composition of the chloroplast and other membranes at cold temperatures (0-10^{o}C). Specific fatty acids, trienoic (16:3 and 18:3), help plants tolerate cold temperatures and are necessary for continued quantum yield (Φ_{PSII}) in *Arabidopsis* (Vijayan et al., 1998). This fatty acid adjustment was also observed in tobacco (*Nicotiana tabacum* L.), a cold sensitive species, when transformed with FAD7 from *Arabidopsis* and subjected to cold treatment. The levels of trienoic acids increased making the transformed tobacco chilling tolerant (Kodama et al., 1994, 1995; Vijayan et al., 1998). Other examples of the influence of desaturase genes on cold tolerance have been documented in maize (Berberich et al., 1998). Recently, increases in *trans*16:1 have been implicated as a fatty acid that imparts chilling tolerance. This increase appears to be controlled by both light and temperature (Gray et al., 2005).

Lipid membranes not only undergo chemical changes, but they are also thought to contain an enzyme, that is at least in part responsible for sensing low temperatures and releasing signaling molecules. Evidence of this attribute was first shown by Murata and Los (1997) where changes in microdomain fluidity of the lipid membranes go through a phase transition before entering the gel state. This triggers a conformational change in a histidine kinase causing an autophosphorylation event to occur. This phosphorylation event then induces the expression of desaturase genes ultimately increasing the unsaturation of the membrane. This system has yet to be identified in any plant system, but occurs in the outer membrane of the cyanobacterium, *Synechocystis*. The histidine kinase Hik33 was responsive to temperature fluctuations by changing the viscosity of the membrane (Murata and Los, 2006).

Protein Responses: With the onset of cold temperatures, plants must overcome the potential damage caused by water that begins to freeze in the intercellular spaces. The water begins to freeze since the extracellular spaces contain a low solute concentration. Thus, as this area begins freezing it draws water out of the cell until a water potential equilibrium is established. Some of the impacts of freezing water include dehydration and cell destruction due to hypertonicity. However, plants can delay or stop these processes from occurring by the use of dehydrins. Dehydrins are members of the cold responsive/late embryo abundant/dehydrin (COR/LEA/*dhn*) genes proteins, which are discussed below in the genetic control section. These proteins of various sizes are thought to stabilize membranes, protect enzymes from freeze-thaw cycles, and delay the formation of ice from cellular dehydration during cold stress (Fowler et al., 2005). During cold acclimation many of these proteins are produced prior to freezing.

Hormone Responses: The hormones ABA, indole-3-acetic acid i.e. auxin (IAA), gibberellic acid, and cytokinins have been investigated regarding their relationship to cold tolerance and acclimation in several species. Often, levels of these hormones dramatically increase or decrease in response to changes in temperature. Abscisic acid is the most studied and best understood of these hormones when plants are subjected to environmental stresses. In addition to stress, ABA is involved in dormancy, germination, stomatal closure and other physiological adjustments (Gusta et al., 2005). The role of ABA is somewhat controversial with regard to cold stress due to the fact that some studies have found that ABA elicits a response, where in other studies no clear conclusion can be made. This has led to researchers proposing both an ABA-dependent and -independent pathways. Genes under ABA regulation have an ABA-responsive element (ABRE) that allows transcription when ABA is released and recognized by the upstream promoter (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Many of the cold-regulated (COR) genes and the C-repeat binding factor (CBF) genes do not contain this binding element and this has led to the proposal of two distinct pathways. Part of the controversy may be due to the fact that in many experiments exogenous ABA is applied at predetermined concentrations which generates a response not typically found in nature. This is because in studies where ABA levels were determined they were found to be a momentary response to cold temperatures. This short ABA response was therefore thought to have a non-significant role (Shinozaki and Yamaguchi-Shinozaki, 2000). Abscisic acid inducible pathways not only use ABREs, but can also use myelocytomatosis (MYC) and myeloblastosis (MYB) transcription factors which are known to occur in many of the COR genes promoters (Mazzucotelli et al., 2008). Knight

et al. (2004) revealed that transcribed CBF1, 2 and 3 genes increase in response to ABA treatment. Therefore, it seems likely that ABA has a role in cold tolerance although its impact remains a question.

Similar to other biotic factors, ABA not only responds to temperature changes but also to light intensity and has an influence on PSII. Li et al. (2003) exposed silver birch (*Betula pendula*) to decreasing photoperiods and demonstrated that ABA levels increased four-fold prior to maximum cold hardiness and dormancy. Hybrid aspen transformed with *PHYA*, which perceives light and regulates plant dormancy, has also been shown to withstand freezing temperatures similar to the wild type. The ABA levels dramatically increased upon exposure to cold temperatures in both wild type and transformed plants. It was concluded that Phytochrome A is regulated by photoperiods but differently than low temperature regulation (Eriksson, 2000). Chlorophyll *a* fluorescence has also been reported to respond to ABA treatment in *Stylosanthes guianensis*, a tropical legume used in pastures. With the addition of ABA, *S. guianensis* was able to maintain a higher chlorophyll fluorescence ratio compared to control plants, which could be indicative of protecting PSII from photoinhibition. Also, a higher quantum efficiency of PSII (Φ_{psII}) in the ABA treatment was maintained while plants were subjected to chilling temperatures which is indicative of higher electron flow rates for CO_2 assimilation. Plants in the ABA treatment were also able to accumulate 11.4% more dry matter when compared to the control (Zhour et al., 2006).

Unlike ABA, both gibberellic acid and IAA are shown to decrease in both birch and hybrid aspen in relation to shorter days and/or cold treatment (Eriksson 2000; Li et al., 2003; Olsen et al., 1997). The wild type and transformed plants that did not express

PHYA had a large reduction in IAA from the long to the short day photoperiod while those expressing *PHYA* exhibited no or little decrease in IAA.

Endophyte response: Endophyte infection of tall fescue by the fungus *Neotyphodium coenophialum* is common throughout naturalized populations. Through a mutualistic relationship, it confers specific traits to tall fescue that would otherwise make the plant less ecologically fit. In return, tall fescue supplies carbohydrates as well as provides a protective environment for it to persist. Endophyte infected plants show increased resistance to biotic stresses such as insect feeding (Latch, 1993; Popay and Rowan, 1994), fungal pathogens (Gwinn and Gavin, 1992) and nematodes (Kimmons et al., 1990; Elmi et al., 2000). Additionally, endophyte infection improves the ability of tall fescue plants to tolerate abiotic stresses such as drought and heat (Arechavaleta et al., 1989; Bacon, 1993; West, 1994). However, only two publications discuss what role the endophyte may play in response to cold and freezing temperatures. Ju et al. (2006) determined that endophyte growth in the plant ceases at 10^{o}C while plants continue to function near the freezing point. Additionally, it was concluded that winter survivability and spring yield are not affected by the absence of an endophyte (Casler and van Santen, 2008). Johnson et al. (2003) found several genes either up or down regulated based on endophyte infection. As mentioned above, desaturase enzymes alter the saturated to unsaturated fatty acid ratio, which ultimately affects the fluidity of lipid membranes in response to alterations in temperature. One of the genes up-regulated due to endophyte infection was matched to *FAD7* and *FAD8* from wheat and *Arabidopsis*. These genes are known as desaturase genes responsible for maintaining fluidity in chloroplast membranes (Johnson et al. 2003). Additionally, a gene related to Chlorophyll A–B binding protein of

the *LHCII* gene (*CAB/lhbc gene family*) in barley was identified as being down regulated in the presence of the endophyte (Johnson et al. 2003). Consequently, harboring an endophyte might prove advantageous for a response to cold conditions. These genes, as discussed elsewhere can be regulated to differing degrees based on the plant's current temperature.

Genetic Control and Responses to Cold Temperatures

With the advent of molecular genetics, the ability to correlate a specific cold induced phenotype with a specific gene is possible and extremely powerful. Every cultivated species grown has numerous genes that become active when the plant encounters a temperature change from mild growing conditions to that of cold or freezing temperatures. The plants most often studied include: wheat, rye, barley, rapeseed rapeseed (*Brassica napus*), perennial ryegrass (lolium perenne), and *Arabidopsis* due to their ability to thrive in and/or withstand cold and freezing temperatures. Some of these genes related to cold/freezing tolerance are also active during cell dehydration events which occurs during drought stress. Therefore, an important consideration is that a gene or genetic pathway may be involved in multiple stress responses.

Currently the only pathway defined for cold tolerance is the c-repeat binding factors/dehydration-responsive element binding-factor (CBF/DREB) regulon in *Arabidopsis*. In *Arabidopsis*, the CBF/DREB has three genes known as *CBF1-3* or *DREB1a-c*. These genes are specifically induced by cold temperatures while other *DREB* genes are specific to cell dehydration. The CBF/DREB transcription factor induced under cold conditions acts in trans on genes containing the upstream CRT/DRE (c-repeat/dehydration-responsive element) element sequence A/GCCGAC. The *CBF/DREB*

genes become active within a short period of time and are detectable within 15 minutes of the plants exposure to cold temperatures (Gilmour et al., 1998). Further down the pathway, approximately 109 genes have been determined to be a part of the CBF regulon (Fowler et al., 2005), which is determined by the genes ability to respond to both low temperatures and CBF overexpression (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). Genes that make up this regulon are placed in one of four groups: 'unknown function', cryoprotective, regulatory, and biosynthetic proteins (Fowler et al., 2005).

Currently, the largest gene group of known function is the cryoprotective group. This group is made up of the low temperature-induced (LTI), cold-regulated (COR) or cold-induced (KIN), responsive to dehydration (RD), and early responsive to dehydration (ERD) genes (Thomashow, 1999). Of the many genes grouped in this category, *COR15a* is one of the best characterized. It is known to target membranes specifically in the chloroplast to aid against freezing injury by acting as a cryoprotective peptide. There are many other COR/LEA (late embryo abundant) proteins produced but it is difficult to determine their exact nature and purpose since many are 'natively unfolded' or 'intrinsically disordered'. Additionally, genes that belong to the COR413 family are speculated to target plasma and/or the thylakoid membrane. Other functions of the COR/LEA/Dehydrin proteins include metabolism, transport, protein processing and fate, transcription, signal transduction, cellular biogenesis, and DNA replication and repair (Fowler et al., 2005).

This cryoprotective regulatory group usually consists of transcription factors and components of signal transduction pathways. It is suspected that there is a subregulon

portion of the CBF pathway due to the fact that approximately 25% of the CBF regulon does not contain the core CRT sequence, A/GCCGAC, for recognition. Therefore, it is recognized that the CBF proteins trigger transcription factors in AP2 (apetella), which is a member of the MYB (myeloblastosis) family, which activates other genes responsible for increased cold/freezing tolerance. This reasoning is founded on the fact that some of the genes activated during a cold treatment do not contain the CRT elements in the promoter region (Fowler and Thomashow, 2002).

Lastly, the biosynthetic proteins, which are used in the creation or alteration of compounds such as proline, carbohydrates, and lipid composition, are just as crucial to the adaptation to cold. Proline, as mentioned above, is critical to this process. This compound is monitored by the protein Δ^1-pyrroline-5-carboxylate synthase (*P5CS1*and *2*). The latter of these, known to be part of the CBF regulon, is up-regulated during cold stress and increases the amount of free proline within the cell. Saccharides, like proline, are also useful for the stabilization of membranes, as well as increasing the osmotic potential within a cell to maintain turgidity as the plant experiences freezing temperatures. Galactinol synthase, catalyzes the first committed step in the production of raffinose, increases in response to low temperatures and CBF3 overexpression. Thus after a chilling event, production of galactinol synthase is highly expressed so that raffinose can be quickly synthesized for cellular protection (Zuthera et al., 2004).

Also, during chilling, cell membranes can become rigid and have the potential to burst, which results in electrolyte leakage and ultimately cell death. One mechanism the plant employs to protect against this event is increasing the fluidity of the membrane by desaturating many of the saturated and monounsaturated fatty acids. Desaturation is

accomplished by the Δ^9- acyl-lipid desaturase (*ADS2*) which is also known to be a part of the CBF regulon (Fukuchi-Mizutani et al., 1998; Maruyama et al., 2004). This trait may be enhanced by the presence of an endophyte which was shown to upregulate *FAD7* and *8* in tall fescue (Johnson et al., 2003).

There are some genes known to influence *CBF*'s function. One of these is the *ICE1* (inducer of *CBF* expression) and it is known to encode a MYC-like bHLH (basic helix-loop-helix) protein crucial for *CBF3* regulation (Figure 1.1) (Chinnusamy et al., 2003). However, it is possible that other *ICE* or *ICE*-like genes control *CBF1* and *2* since they both contain bHLB binding sites (Fowler et al., 2005). Upstream regions of the *CBF* genes contain the sequence CACATG and are recognized by bHLH proteins (Massari and Murre, 2000). The current thought is that *ICE* is present but inactive at warm temperatures. However, when temperatures fall *ICE* becomes active and can interact with itself or another protein to induce *CBF* transcription (Gilmour et al., 1998).

Additionally, genes such as *hos1* (high expression of osmotically responsive genes) and *hos2/fry1* are known to influence *ICE* expression. However, it is not known what signals are perceived or engaged by the plant to signal the pathway into action. The current idea is that inositol-1, 4, 5-triphosphate (IP3) controls the release of Ca^{2+} within the cell, and Ca^{2+} signals the cascade to become active. Plants containing *fry1/hos2* had increased and sustained levels of IP3 which were induced by cold and ABA treatment (Xiong et al., 2001). The *hos2* mutant however, is completely insensitive to ABA treatment, but has enhanced *CBF* expression (Xiong et al., 2004).

There are other positive and negative regulators of the *CBF* genes. Like *ICE*, *LOS4* positively affects *CBF* expression, but the whole mechanism is currently not

known (Fowler et al., 2005). It is known that *LOS4/CRYOPHYTE* is a DEAD-box RNA helicase believed to be located at the nuclear envelope. There it is thought to be critical for *CBF* responses to cold either through direct export of *CBF* mRNAs or as an indicator of temperature - 'a thermosensor' (Gong et al., 2002; 2005). Negative regulators of the *CBF* genes include *HOS1* and *HOS2/FRY*, *LOS1,* and *CBF2*. They are thought of as negative regulators because nonfunctional mutants have elevated expression of CBF and COR genes while positive regulators show decreased expression. *HOS1* encodes for a RING finger ubiquitin E3 ligase and is thought to target *ICE* for degradation. *LOS1* encodes for a translation elongation factor and is thought to have an effect on the *COR* genes protein formation (Fowler et al., 2005). Lastly, it is interesting that the *CBF2* gene regulates other CBF genes. By identifying a *cbf2 Arabidopsis* mutant, it was shown that *CBF1* and *CBF3* had higher levels of expression meaning CBF2 regulates their expression. Additionally, *CBF2* was maximally expressed about 90 min after *CBF1* and *CBF3* became active showing that the *CBF* genes are expressed differentially in time (Novillo et al., 2004).

Like the function of *HOS1,* many of the genes used in the CBF pathway are regulated by RNA and proteins for degradation (ubiquitination), phosphorylation, or sumoylation events in addition to being targeted by microRNAs (miRNAs) and small interfering RNAs (siRNAs), or by having alternative splicing rather than acting in any of the other functions described previously. Alternative splicing can occur by exon skipping, intron retention, or by 5' or 3' alternative splicing (Mazzucotelli et al., 2008). These actions usually occur in mRNAs utilized for signal transduction, receptors, and

transcription factors (Dinesh-Kumar and Baker, 2000). However, only a handful of alternatively spliced gene products have been determined in relation to a stress response.

The miRNAs and siRNAs cause gene silencing when targeted for degradation by ubiquitination. *ICE1* is thought to be the target of ubiquitination by *HOS1*. This ubiquitination event involves the covalent addition of the molecule ubiquitin, which occurs in a three step process using an activating enzyme (E1), conjugating enzyme (E2), and a ligase (E3). However, *ICE1* can counter ubiquitination by the process of sumoylation, which post-translationally modifies proteins with the SUMO (Small Ubiquitin-like Modifier) peptide by a similar E1, E2, and E3 process. This process allows *ICE1* to activate *CBF* genes which in turn can activate the *CBF* dependent genes. Therefore, in conjunction with its phosphorylation status, *ICE1* activity can be fine-tuned to the environmental conditions (Mazzucotelli et al., 2008). In addition to the COR pathway, there is evidence of other independent pathways that respond to ABA and evidence of other transcription factors that do not contain the usual CRT/DRE, MYB, or MYC recognition sequences (Chinnusamy et al., 2006).

Although the cold response is best understood in *Arabidopsis*, many aspects of the CBF pathway are highly conserved across species. There is progress being made in other crop species such as rapeseed, tomato (*Solanum lycopersicum*), rice, wheat, barley, and perennial ryegrass with portions of the CBF pathway being better understood. For instance, as many as 10 *CBF/DREB* genes related to the CBF genes in *Arabidopsis*, have been discovered in rice thus far. However, there may be many more genes not yet identified. Barley is known to contain 20 CBF homologues (Skinner et al., 2005). Perennial ryegrass was also found to contain a homologue of *CBF3* which is induced

only under cold conditions (Zhao and Bughrara, 2008) and it also has 10 other *CBF* cDNAs that have similar relationships and properties to the barley *CBF* genes (Tamura and Yamada, 2007). Lastly, Tang et al. (2005) identified a putative DREB transcription factor in tall fescue (*FaDREB1)* which contains the characteristic gene sequences common to other CBF/DREB sequences and is phylogenetically similar to *Lp*CBF3 (Tamura and Yamada, 2007). The *FaDREB1* gene was up-regulated when exposed to cold temperatures, however, ABA did not induce transcription and therefore it appears to be ABA-independent (Tang et al., 2005). It is likely that tall fescue will have many of the same homologous genes that have been conserved throughout the divergence of the plant species.

Other genes that are up- or down-regulated due to cold temperatures are thought to be separate from the CBF/DREB pathway. They include genes for photosynthetic function such as the *Lhcb* nuclear genes that encode for the LHC of PSII (Gray et al., 1997), and many of the genes responsible for the construction and alterations of the RC (Ensminger et al., 2006). Additionally, 42 genes that were found to be regulated by excitation pressure will have some value in the plant's ability to cope with temperature stress. A majority of these genes are activated when a particular excitation pressure threshold is reached. Of these 21 genes, 5 are reported to be involved with sugar production, 7 with electron transport, and the others with lipid biosynthesis (Ndong et al., 2001). All of these are crucial for regulation during the cold temperature response and acclimation. Therefore, many of these genes along with the CBF homologues would be good candidates for association analysis. Like other plant traits, cold/freezing tolerance is

complex, and will take a concerted effort to make advancements in our crop species to increase their tolerance and productivity during cold stress.

Although the *CBF* genes have been shown to be responsible for cold/freezing tolerance in controlled lab experiments their actual function in field studies are lacking and a relatively new revelation. Prior to *CBF* identification one method used for identifying these potential genes is by performing a QTL analysis where genomic regions for a phenotypic are located. These QTL studies have been primarily focused on wheat and barley, due to their use as winter crops, as well as perennial ryegrass, due to its perennial nature, to identifying genomic regions vital for winter survival. Typically these QTL studies are conducted in structured crosses where one parent is highly susceptible while the other is completely winter hardy. The first low-temperature tolerance QTL was reported by Hayes et al. (1993) in the 'Dicktoo' × 'Morex' population. This QTL for winter survival was found consistently on the region of the long arm of chromosome 5H (Pan et al., 1994; Francia et al., 2004). This QTL was reported to explain between 21.5 to 36.6% of the variation in barley (Francia et al. (2004). It was discovered that this major QTL region for winter survival/freezing tolerance was consistently found near the mapped locations of *CBF* genes and other cold tolerance related genes (Skinner et al., 2006; Tondelli et al., 2006). Fine mapping revealed a cluster of *CBF* genes on 5H of barley which is associated with freezing tolerance (Francia et al., 2007). In an annual × perennial ryegrass interspecific hybrid population winter survival was mapped to genomic regions on the same homologous LGs of the parental maps across years (Xiong et al., 2007). These regions correspond to the same 5H chromosomal regions in barley. Therefore, it appears *CBF* genes are necessary for superior winter survival.

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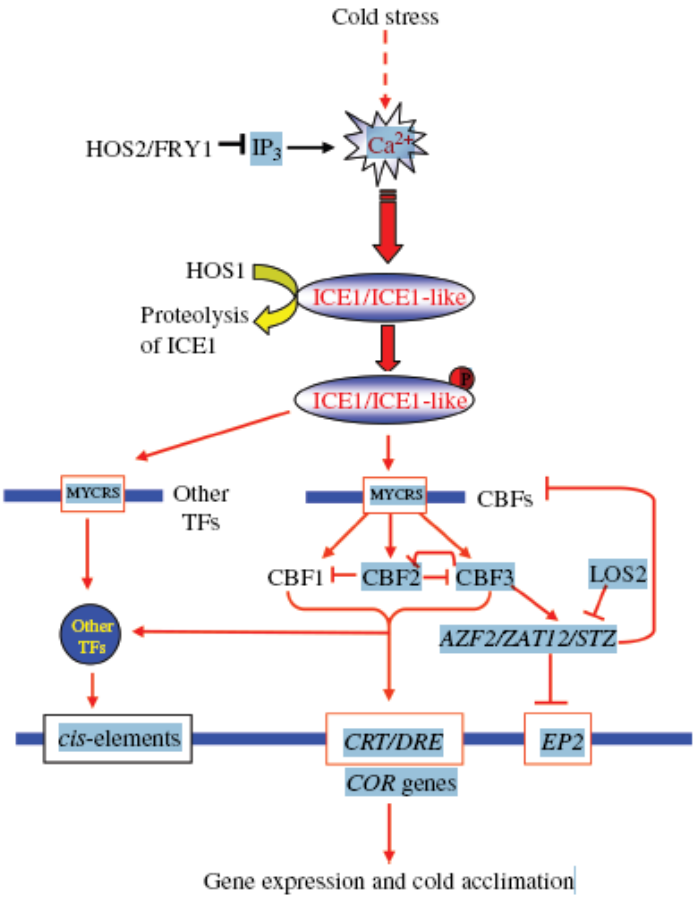

Figure 1.1. Schematic of the proposed induction factors that trigger the CBF pathway and related genes regulated during cold stress. (taken from Chinnusamy et al., 2006)

EFFECTS OF ENDOPHYTE STATUS ON LEAF EXTENSION, PROLINE, MONO- AND DISACCHARIDES, FRUCTAN, AND FREEZING SURVIVABILITY

ABSTRACT

Tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] is widely used for hay and pasture in the Lower Midwest and Southeast regions of the U.S. New types of Mediterranean or "summer-dormant" tall fescue have superior growth under cool growing conditions, but do not survive winters well in the Midwest. To date, little research has examined the role of endophyte infection (*Neotyphodium coenophialum* [Morgan-Jones and Gams] Glenn, Bacon, and Hanlin) on the ability of tall fescue to grow under cold conditions or to survive freezing temperatures. The objectives of this study was to determine the endophyte's role in tall fescue growth, storage compounds, and osmoprotectants under cold, but non-freezing conditions, as well as to determine the role of endophyte infection on the ability of plants to survive freezing temperatures. For this experiment five genotypes of tall fescue were used. Half of the plants from each genotype were endophyte infected (E+), with each genotype containing a unique endophyte strain, while the remaining genotypes were endophyte free (E-). We report here for the first time the effects of endophyte status on proline, non-structural carbohydrates, and fructan accumulation when plants were grown under cold, but nonfreezing temperatures and the survivability at various freezing temperatures. Endophyte removal had no effect on any of the traits except for fructan accumulation at 4.5^{o}C; however, plant genotype had an effect on LER (leaf extension rate), proline, simple sugars, and fructan concentration. For the freezing test, the endophyte had no effect on tiller survival, but fescue genotype was significant. These data indicate that endophyte removal has little influence on the measured traits for this abiotic stress, and plant genotype is more critical for survival under cold conditions.

INTRODUCTION

Tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] is widely used by forage-livestock producers in the humid-temperate U.S. due to its ability to produce and persist under a wide range of soil, climatic, and management conditions (Hoveland, 2007). Many of these agronomic attributes are enhanced by an endemic endophyte (*Neotyphodium coenophialum*) known to confer to the host plant resistance to abiotic and biotic stresses (Bacon and Siegel, 1988). These benefits have also been observed in perennial ryegrass (Eerens et al., 1992, 1997, 1998a, 1998b). An in-depth review on tall fescue's mutualistic relationship with its endophyte can be found in the following resources (Arachavaleta et al., 1989; Cheplick et al., 1989; Pedersen et al., 1990; White et al., 1992; Joost, 1995; Hill et al., 1996).

Little is known about how endophyte infection impacts growth and/or survival of tall fescue during autumn and winter. Considering the multitude of benefits the endophyte provides in other stress situations, it is plausible that its presence may be important when plants are subjected to cold/freezing stresses. However, some evidence indicates that this may not be the case. Casler and van Santen (2008) did not find an effect of endophyte infection on winter survival or spring yield in Wisconsin. Further, Ju et al. (2006) showed that endophyte growth ceases around 10^{o}C in tall fescue which is well above the minimum temperature for plant growth. However, it is unclear if the presence of an endophyte alters the process of winter hardening during autumn.

Recently, tall fescue cultivars selected from Mediterranean germplasm have been released (Miller, 2000). These Mediterranean types produce more forage during cooler temperatures, especially in autumn, than Continental varieties like 'Kentucky 31'. This

increased growth in autumn would be of great value to forage-livestock producers in the Midwest, as forage production at this time of year could be used to extend the grazing season and/or reduce winter feeding costs substantially (May et al., 1999; Prevatt et al., 2001). However, these Mediterranean types typically do not survive north of 36° N latitude in the humid-temperate region of the US. It is unknown if endophyte infection would enhance the ability of Mediterranean genotypes to grow and/or survive at more northern latitudes.

Our objective was to assess the effect of endophyte infection on the ability of one Mediterranean, two Continental, and two genotypes of *F. arundinacea* var. *atlantigena* St. Yves to produce metabolites and survive freezing temperatures. Two experiments were conducted; Experiment I was a growth analysis and metabolite production experiment conducted at cool but non-freezing conditions. Experiment II assessed the survivability of plants when subjected to a range of freezing temperatures.

METHODS AND MATERIALS

Plant material

For both experiments, five (genotypes 103-2, GA80-85, GA80-88, 9, and 11) of tall fescue having varying degrees of ploidy were used. The first genotype, 103-2 (2n=6x=42), was Mediterranean in origin, and was derived from Grasslands 'Flecha' (Miller, 2000). A note about the Mediterranean hexaploids' genome: it is uncertain at this time if the M_1 and M_2 genomes from (*Festuca mairei* St. Yves) constitute the remaining genomic structure; however, there has been some putative information that this might be the case (Saha et al., 2010). Genotypes GA80-85 and GA80-88, (2n=6x=42, $PPG_1G_1G_2G_2$) were selections from 'Kentucky-31' tall fescue and are considered

Continental types. GA80-85 was described previously by Bouton et al. (1992) and GA80-88 was described by D'Uva et al. (1983). GA-8085 is only moderately rhizomatous while GA80-88 is highly rhizomatous. Both of these genotypes are located at the Americus Plant Materials Center (SCS), Americus , GA. Genotypes 9 and 11 are *F. arundinacea* var. *atlantigena* St. Yves tall fescue octoploids (2n=8x=56, $G_1G_1G_2G_2M_1M_1M_2M_2$). The octoploids originate from Morocco, but were collected at different elevations. Genotype 11 was collected at an elevation of 1770m while genotype 9 was collected at 1140m. For each genotype, an endophtye infected (E+) and an endophtye-free (E-) clone were tested. Endophyte presence and absence was confirmed by PCR. All plants were clonally replicated 12 times, except for 103-2, which had 14 endophyte infected plants and 6 endophyte free plants.

Planting, Growing Conditions, and Growth Chambers

Plants were clonally split and grown in D40H (6.4 cm dia. x 25 cm tall) Deepots™ (Stuewe and Sons Inc., Tangent, OR) filled with Pro-Mix (Premier Tech Horticulture, Rivière-du-Loup, Québec, Canada) potting soil. The pots were arranged inside modified glass-topped freezers (described below) located in a greenhouse. A 12-h day length was used at 25.0 and 4.5^{o}C to ensure day length would not affect the results. In addition to natural sunlight, plants received supplemental light from high-pressure sodium lamps. During the 12-h photoperiod, photosynthetic photon flux density (PPFD) ranged from 500 to 1600 μmol $m^{-2}s^{-1}$ which depended largely on sunlight. The greenhouse temperature was 25.0 +/- 5.0^{o}C, while that of the chambers was maintained at 4.5 +/- 0.5^{o}C during the plant's acclimation and growth periods. Plants received 200 +/- 25 μmol $m^{-2}s^{-1}$ of artificial light while in the chambers. This light intensity was chosen to

mimic the lower light levels of winter, and because increased light intensity can produce effects that may not be associated with acclimated plants at low temperatures (Ndong et al., 2001). Lastly, plants were given the equivalent of 9.0 kg ha^{-1} of nitrogen, 3.0 kg ha^{-1} of phosphorus, and 6.0 kg ha^{-1} of potassium fertilizer weekly to counteract the leaching from the potting soil.

The chambers were made from vencold freezers, model GST65-C22 (Guelph, Ontario, Canada). Low emissivity glass was used to prevent the chamber's internal temperature from becoming too warm during the point at which the lights were turned on. Sofasco (Winchester, VA) fans measuring 92x92x25 mm (model: D9225V12H) and 60x60x25 mm (model: D6025V12H), were used to circulate air within each chamber so that air would not stratify into warm and cold layers. Evercool (San-Chung City, Taiwan) fans measuring 30x30x7 mm (model: EC3007M05CA) were used to push fresh air into each chamber through the duration of the experiment. The chamber temperature was monitored using thermocouples in each chamber placed near the crown tissue of the plants. Each freezer had a heating tape applied to the inside of the freezer adjacent to the cooling coils. This heat tape was thermostatically controlled by a personal computer running custom LabVIEW (National Instruments, Austin, TX) software.

LER, Proline, and Fructan Determination

To measure leaf extension rate (LER), one tiller was selected from each plant where the newest leaf was between one-fourth and one-half the length of the second newest leaf. The third newest leaf sheath had a permanent mark placed below the leaf collar (Figure 2.1). This mark was used as a reference mark each day when measuring the LER. Additionally, the leaf being evaluated was either the third, fourth, or fifth emerging

leaf for that particular tiller. A clear plastic ruler was used to measure the leaf's length each day. The measurements were completed on a 24-h period for 5 days. These measurements were averaged to calculate the LER for each individual plant at both 25.0 and 4.5^{o}C. Since LER is a measure of leaf growth and is positively correlated to potential yield and regrowth (Horst et al., 1978), it was necessary to select leaves that would have the same growth characteristics so that leaf number would have little impact on the measurement. From preliminary testing in our lab and work by Robson (1974) and Durand et al. (1999), it has been shown that in tillers with three or more leaves that the third and successive leaves have nearly the same growth rate.

To determine free proline, both leaves and stems were harvested prior to placement in the cold growth chamber and again after plants had grown for 30 d at 4.5 ^{o}C. The tissue for each plant was cut at approximately 2.5 to 3.0 cm above the soil level and immediately frozen using liquid nitrogen. Samples were then lyophilized and kept at -20^{o}C. Because of the small sample size, samples were ground using Braun (Cincinnati, OH) model KSM2 coffee grinders for approximately 30 sec. and then returned to the freezer. Free proline was determined by using the method described by Bates (1973) with the following modifications. Samples harvested at 25.0^{o}C used 100 mg of tissue while samples harvested at 4.5^{o}C needed only 50 mg of tissue for proline extraction to remain within the standard curve due to their higher concentrations. Additionally, only 1 mL of filtrate was used for samples collected at 4.5^{o}C due to the high proline concentration while 2 mL were used for samples harvested at 25.0^{o}C for the reaction process. For each set of samples analyzed, a new standard curve was used at concentrations of 1, 3, 5, 7, 9, 10, and 20 μg ml^{-1}. This curve was used to calculate free proline in the tissue. Then using

the equation provided by Bates (1973), the values determined by the standard curve were converted to μmol mg^{-1} dry matter (DM).

Lastly, fructan concentration of samples was determined using the Megazyme© (Wicklow, Ireland) fructan assay procedure according to the manufacturer's instructions except for sample quantity. Since the samples contained between 0.5 and 20% fructan, a 200 mg sample was used and diluted in a 50 mL volumetric flask. The remaining protocol was followed according to the manufacturer's instructions.

Mono- and Disaccharide Determination

The same tissue used for proline, and fructan determination was used to determine glucose, fructose, and sucrose. To determine the mono- and disaccharide concentrations, a 12.5 mg sample was extracted with 1.2 mL of 40% ethanol. This solution was placed on a shaker at 200 rpm for 30 min at 50.0°C. The samples were vortexed prior to their placement in the incubator and half-way through the 30 min. extraction. After the incubation period, 100 μl were filtered through a Millipore (Billerica, MA) 96 well filter plate with 45 μm filter for 2 min. at 2000g. This filtrate was then used for injection on a Agilent 1100 series high performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) HPLC with an ESA Coulochem III detector (Agilent Technologies, Chesterfield, MO). The filtrate was separated using a Dionex Carbo Pac PA 10 analytical column (250 mm x 4 mm, 10 μm) connected to a Carbo Pac 10 guard column (50 mm x 4 mm). The mobile phase included 90 mM NaOH solution with a flow rate of 1.5 ml min^{-1}, maintained at 30°C. Detection settings were: time 0, 0.1 v, time 0.41, -2.0 v, time 0.42 0.6 v, and time 0.44, -0.1 v.

Experiment I (Growth Analysis at Cool but Non-Freezing Temperatures)

In Experiment I, the five tall fescue accessions, described previously, were grown in the greenhouse at 25.0^{o}C (±5^{o}C) to determine their optimum LER, proline, and carbohydrate concentrations. Once the initial growth analysis was completed at 25.0^{o}C, these same plants were placed into growth chambers and held at 4.5^{o}C (±0.5^{o}C) for 30 days and the same characters quantified.

Experiment II (Freezing Survivability)

Plants were placed into the chambers described above and allowed to acclimate an additional 5 d at 4.5^{o}C for a total of 35 d. After the acclimation period, the number of live tillers plant^{-1} was recorded and plant tops were cut to a 2.5 to 3.0 cm residual. The roots were left uncut since tall fescue accumulates root mass throughout the winter and uses those roots for storage of remobilized nutrients from the senesced leaves in the fall and winter (Remley, 2010). After counting and clipping, the chambers were set to 0^{o}C and this temperature was maintained for 24h. Then the chambers were programmed to decrease in temperature at approximately 1.5^{o}C h^{-1} until reaching the final temperature of -12^{o}C. For each genotype, three replicates of E+ and E-plants were removed at -3, -6, -9 and -12^{o}C. Upon removal from the freezing chamber, plants were placed into a similar growth chamber set a 5^{o}C for 24 h. After this 24 h period elapsed, plants were then grown in the greenhouse at 25^{o}C for 2 wk. After the 2 wk. regrowth period, the remaining live tillers were counted and the percentage of the remaining live tillers calculated.

Statistical Analysis

For both experiments, plants were arranged in a randomized complete block design (RCBD) with a factorial arrangement of treatments (Steel and Torrie, 1980). In

Experiment I, a factorial arrangement of five genotypes x two endophyte levels was replicated 12 times (n=120 plants). Each genotype was replicated 12 times by clonal propagation except for 103-2 where 6 clones were E- and 14 clones were E+. In Experiment II, a split-plot arrangement of a RCBD was used with five genotypes x two endophyte levels (factorially arranged) and blocks as main plots and temperatures (-3, -6, -9, and -12^{o}C) as sub-plots (n=120 plants). Main effects and all interactions were analyzed by analysis of variance using PROC GLM of SAS version 9.1 (SAS Inst. Inc., Cary, NC). Differences were considered significant if P values were less than 0.05 unless otherwise noted. Correlations to LER were performed using PROC CORR in SAS statistical software (SAS Inst. Inc.).

RESULTS and DISCUSSION

Experiment I

Leaf Extension Rate (LER)

Leaf extension rate at either 25.0 or 4.5^{o}C was not influenced by endophyte status of the plants. This is in agreement with Belesky et al. (1989) and Elbersen and West (1996), who suggested that differences in LER were mainly due to plant genotypes. Additionally, Casler and van Santen (2008) found endophyte status had no effect on spring yield, but it was highly influenced by plant genotype.

There was a difference in LER between tall fescue genotypes, regardless of endophyte infection. When grown at 25^{o}C, LER ranged from 11 to 19 mm d^{-1} (Table 2.1) with all genotypes except genotype 11, being similar. While LER for all genotypes was substantially less when grown at 4.5 vs. 25^{o}C, the differences were greater between genotypes when they were grown at 4.5^{o}C. When grown at the 4.5^{o}C temperature, the

Mediterranean hexaploid, 103-2, had the greatest LER at 4.7 mm d^{-1} followed by genotype 9 with an average LER of 3.7 mm d^{-1}. The two Continental genotypes, GA80-85 and GA80-88, did not differ with LER of 2.0 and 1.8 mm d^{-1}, respectively. The octoploid genotype 11 had the slowest LER at approximately 1.0 mm d^{-1}. When comparing the percentage of growth at 4.5°C compared to 25°C, it is quite astonishing that genotypes 11, GA80-85, and GA80-88 had an LER at 4.5°C that was only 7 to 12% of the LER at 25.0°C. In comparison, genotype 9 averaged 21% and 103-2 averaged 27% of the growth rate at 25.0°C.

Thomas and Lazenby (1968) first observed that tall fescue plants originating from Algeria in North Africa possessed exceptional growth at cold but non-freezing temperatures. We observed similar results with genotype 103-2 in this study. More recently, yield data from germplasm collected in North Africa and the Mediterranean Basin shows superior growth and two- to threefold greater yield compared to Continental tall fescue varieties in areas that have mild winter conditions (Anderson et al., 1999; Reed et al., 2004). This is the same trend observed from our data.

Proline

Neither proline levels at 25.0°C, nor the increase in proline concentration when plants were grown at 4.5°C, was affected by endophyte status, but cold stress may have affected E- plants to have higher proline concentration at 4.5°C (P = 0.1) (Table 2.1). However, it seems that GA80-85 E- is influencing the overall difference between E+ and E- plants since all other E- genotypes examined had proline levels lower than E+ genotypes. This may be a specific case where GA80-85 responds differently than other genotypes in the presence/absence of an endophyte. When averaged across endophyte

status, both Continental and Mediterranean genotypes showed approximately a 5.8 fold increase in proline concentration in response to the cold stress. However, the Continental genotypes GA80-85 and GA80-88 produced approximately twice as much proline, regardless of growing temperature, compared to all other genotypes.

Elbersen and West (1996) showed that E- plants contain higher proline concentrations than E+ when subjected to water-stress. They attributed the greater accumulation of proline to the higher osmotic stress encountered by E- plants. Elmi and West (1995) demonstrated that E+ tall fescue had superior osmotic adjustment in tiller bases and overall survival during water-stress conditions compared to E-. Like drought, cold and freezing temperatures create osmotic stress due to water crystallization. Proline is thought to counteract the osmotic stress by acting as both an osmolite (Baker et al., 1993) and a protein stabilizer that protects the structural integrity of enzymes (Rajendrakumar et al., 1994) at cold and/or freezing temperatures. At higher temperatures (i.e. > 30^{o}C) proline does not have the same protective ability of other compounds (Laurie and Stwewart, 1990). Interestingly, proline does not reduce enzyme activity (K_m) **(**Wang and Bolen, 1996). Therefore, the change in concentration of proline may not be as influential as the final concentration to function and survival under cold stress.

Mono- and Disaccharides and Fructan

The mono- and disaccharides - glucose, fructose, and sucrose - were not affected by the endophyte status of the plant at either temperature tested (Table 2.2). Additionally, endophyte status did not affect fructan levels at 25.0^{o}C. However, fructan levels at 4.5^{o}C and the change in fructan concentration was influenced ($P < 0.01$ and $P < 0.05$, respectively) by endophyte status (Table 2.1). This is attributed to the higher levels of

fructan found in endophyte-infected genotypes 9 and 103-2 at 4.5°C. This is likely due to specific genotypic interactions with the endophyte as observed with proline. The remaining genotypes (11, GA80-85, and GA80-88) had similar levels regardless of endophyte status. Overall, the range of fructan concentrations for all genotypes was 15 to 87 mg g^{-1} DM when plants were grown at 25.0°C (Table 2.1). After the plants were subjected to 4.5°C for thirty days, fructan levels increased between 2.6 and 10.4 fold. The final concentrations had values ranging from 48 to 201 mg g^{-1} DM. The hexaploid genotypes had the highest values with 103-2 assimilating the greatest amount of storage carbohydrates while the octoploid genotypes 9 and 11 the lowest concentrations.

Non-structural carbohydrates are thought to function as osmolites during environmental stresses, similar to proline. These mono- and disaccharides increase in concentration to maintain cell turgor for continued function during environmental stress periods. In previous research, an increase in both fructose and glucose due to water-stress was observed, but endophyte infected plants had a greater accumulation of those compounds while sucrose was not affected by endophyte status (Richardson et al., 1992). However, this difference due to endophyte status was not observed by Belesky et al. (1989), as well as in the present study in which genotypic differences were more prominent. Sucrose was found to make up between 60 and 80% at 25°C and 40 to 70% at 4.5°C of the total non-structural carbohydrates (Table 2.2). These values are similar to the results seen in barley, wheat, and rye (Olien and Clark, 1993), perennial ryegrass (Hisano et al., 2008) and tall fescue (Spollen and Nelson, 1994).

Although endophyte infection had no effect on mono- and disaccharide concentrations, plant genotype did. It is obvious that the three groups tested followed

unique accumulation patterns. Perhaps under cold conditions, 103-2 shuttles these sugars into growth and storage components. Supporting evidence for this hypothesis is the accelerated LER and higher fructan levels of 103-2 under cold stress. It has been shown that plants containing the M_1 and M_2 genomes from *Festuca mairei* St. Yves have greater apparent photosynthesis and RuBP carboxylase, which leads to an increase in net photosynthesis (Randal et al., 1985). Since it is proposed that Mediterranean tall fescue genotypes, like 103-2, contain the M_1 and M_2 and not the G_1 and G_2 genomes it would be plausible that this is the reason for the differences in the sugars and fructan levels.

Fructans are thought to play a major role in regrowth after a defoliation event (Morvan-Bertrand et al., 2001) as well as increase when plants are exposed to cold growing conditions (Nelson, and Spollen, 1987; Hisano et al., 2008). However, there is no information regarding the role endophyte infection plays in fructan accumulation either at optimum growth temperatures or under cold stress conditions. When grown at 4.5^{o}C, every genotype that was endophyte infected had higher levels of fructan although the differences were not always significant. This result was heavily influenced by the large concentration in 103-2 E+ although, endophyte presence can affect the saccharide concentration under other abiotic stress conditions.

Experiment II

Tiller Survival after Freezing

Endophyte had no effect on tiller survival after subjecting plants to freezing temperatures. Plant genotype had a much larger impact for survival at the temperatures examined. At -3.0 and -6.0^{o}C, there was little tiller mortality (Table 2.3) for any genotype. In fact, for some genotypes new tillers developed which resulted in greater

than 100% survival (Figures 2.2 and 2.3). At -9.0°C, however, a separation of fescue genotypes occurred with the Continental genotypes GA80-85 and GA80-88 having the greatest tiller survival at 78.5 and 94.4%, respectively, while genotypes 9 and 103-2 had the poorest survival at 7.7 and 5.8%, respectively. Tiller survival was poorest at -12.0°C with only one Continental genotype, GA80-88, having superior survivability at 89.7%. Genotype GA80-85 only had a tiller survival rate of 17.7% and genotype 11, 11.8%. The remaining genotypes were nearly zero. Casler and van Santen (2008) showed that spring yield and percent ground cover were not affected by endophyte status following winter conditions in Wisconsin, but was dependent on plant genotype. Our results support this observation.

The values reported here are similar to results reported by Thomas and Lazenby (1968) who used -4.4, -6.1 and -7.8°C to test a Continental and Mediterranean hexaploid as well as a fescue decaploid, F. *arundinacea* var. *letourneuxiana* St. Yves or var. *cirtensis* St. Yves. Taken together, the results of these experiments suggest that a gene or genes critical for freezing tolerance must be located on either the G1 or G2 genomes. These genomes are present only in the Continental hexaploid tall fescue lines GA80-85, GA80-88, and S170 along with the octoploid genotypes 9 and 11 and the syn. 2 decaploid. Additionally, this gene is either not present in all lines or there are multiple genes that must be present to give hardier plants. This is evident when looking at the Continental hexaploids and octoploids; for example line GA80-88 had greater survivability than GA80-85. Additionally, the octoploid 11 was hardier than that of octoploid genotype 9. Also, the potential for rhizomes to enhance winter hardiness may also play a factor between genotypes. This superior level of survival for GA80-88 at

-12^{o}C might be due to the rhizomatous nature of this genotype as compared to the other Continental genotype (D'Uva et al,. 1983; Bouton et al., 1993). In Johnsongrass (*Sorghum halepense* L.) biotypes from Ontario, Canada (Warwick et al, 1986) and Pennsylvania (Hartzler et al., 1991) were able to survive much cold winter conditions than those from southern latitudes in the US due to their ability to produce rhizomes at much greater depths. Therefore, rhizome production and depth may enhance winter survival in tall fescue.

The genes that allow for greater growth, as measured by LER, during low non-freezing temperatures appear to be present on the M_1 or M_2 genomes. These genomes are only present in the octoploid genotypes 9 and 11 and the Mediterranean hexaploid 103-2. Like the gene(s) responsible for freezing tolerance, they are either not present in all lines or the trait is multi-genic since genotype 11 did not have elevated growth rates compared to genotypes 9 and 103-2.

CONCLUSION

In conclusion, the plant genotype has a much greater influence on its ability to react to and survive cold and or freezing temperatures than with the presence or absence of an endophyte. This is in contrast to the enhancement to withstand other biotic and abiotic stresses commonly attributed to endophyte infection. However, we did see an impact of endophyte presence on fructan levels and proline at 4.5^{o}C. Even though endophyte status did not have an overall impact, there may be a specific genotype by endophyte response. Additional testing with greater a number of genotypes and an array of temperatures will elucidate this matter further.

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Table 2.1. Leaf extension rates (LER), proline, and fructan concentrations of tall fescue genotypes with and without endophyte infection.

Genotype	Endophyte	LER 25°C	LER 4.5°C	Proline 25°C	Proline 4.5°C	Fructan 25°C	Fructan 4.5°C
		---mm d^{-1}---		--µmol g^{-1} DW--		---mg g^{-1} DW---	
9	E+	17.7	3.8	1.7	10.4	23.9	113.8
	E-	17.2	3.5	1.6	6.4	25.7	86.9
11	E+	11.2	1.1	2.2	10.4	19.4	49.7
	E-	13.4	1.0	1.7	9.7	15.0	48.2
103-2	E+	19.0	4.5	4.1	14.5	45.3	201.1
	E-	17.6	4.9	4.7	12.5	87.2	153.9
GA-8085	E+	19.4	1.9	4.2	19.6	45.9	120.2
	E-	18.6	2.1	7.1	41.8	24.1	116.2
GA-8088	E+	17.9	1.9	3.4	22.8	70.4	149.0
	E-	18.3	1.6	3.7	21.5	59.3	144.8
LSD†		3.4	0.6	2.2	7.0	16.9	26.8
Orthogonal Contrasts							
E+ vs. E-		NS[a]	NS	NS	0.1[b]	NS	0.008
E+		17.1	2.7	3.1	15.5	41.1	128.9
E-		16.9	2.4	3.7	18.6	37.3	105.5

† Fisher's Protected LSD (0.05)
[a] NS, Non-significant
[b] *P*-value

Table 2.2. Mono- and disaccharide levels of tall fescue genotypes with and without endophyte infection.

Genotype	Endophyte	Glucose 25°C	Glucose 4.5°C	Fructose 25°C	Fructose 4.5°C	Sucrose 25°C	Sucrose 4.5°C
		----mg g^{-1} DW----		----mg g^{-1} DW----		----mg g^{-1} DW----	
9	E+	2.1	13.4	1.9	14.1	17.1	21.9
	E-	2.0	12.8	1.7	15.3	14.8	16.5
11	E+	1.3	9.7	1.8	10.6	14.5	29.5
	E-	2.0	13.1	2.3	15.5	17.8	26.1
103-2	E+	5.8	6.7	7.6	6.0	38.9	43.9
	E-	5.5	7.4	8.1	7.9	36.2	33.2
GA-8085	E+	3.7	16.7	5.2	18.4	22.3	37.2
	E-	2.3	12.0	4.3	13.0	23.8	42.8
GA-8088	E+	3.8	14.7	6.6	15.1	18.0	39.6
	E-	4.7	16.9	7.4	18.4	18.0	41.1
LSD†		1.0	2.7	1.8	3.5	4.8	7.8
Orthogonal Contrasts							
E+ vs. E-		NS	NS	NS	NS	NS	NS
E+		3.4	12.1	4.7	12.6	22.69	34.7
E-		3.1	13.0	4.4	14.7	20.55	31.8

† Fisher's Protected LSD (0.05)
[a] NS, Non-significant

Table 2.3. Survivability of tall fescue tillers grown at different cold temperatures based on endophyte infection.

	Survivability (% of Tillers)			
Endophyte	**-3.0°C**	**-6.0 °C**	**-9.0 °C**	**-12.0 °C**
E+	109.2	107.0	57.5	24.5
E-	112.0	105.7	35.3	23.3
P-value	0.48	0.88	0.33	0.95

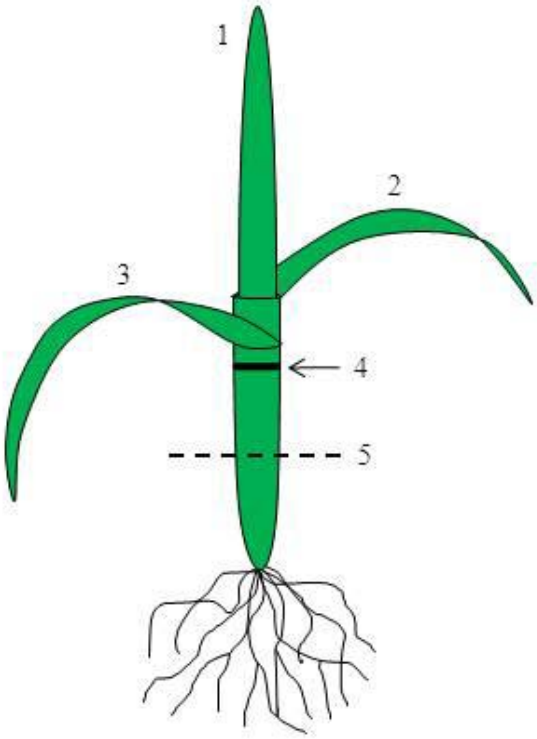

Figure 2.1. Schematic of a tall fescue tiller where 1) represents the newest emerging leaf, 2) the second newest leaf, 3) the third newest leaf, 4) the position of the reference point to measure LER, and 5) the cutting height at approximately 3.0 cm.

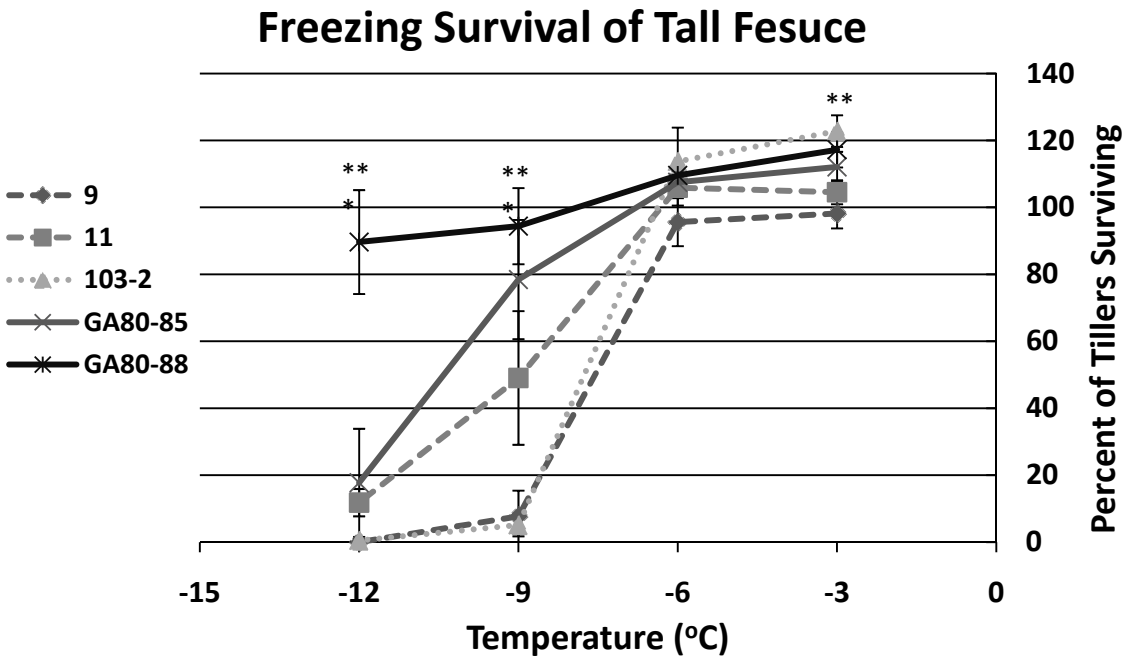

Figure 2.2. Survival of five tall fescue genotypes tested at -3, -6, -9, and -12°C. Genotypes GA80-85 and GA80-88 are Continental hexaploids, 9 and 11 are octoploids, and 103-2 is Mediterranean hexaploid. Bars represent the +/-S.E.M. n=5 or 6 plants for every genotype at each temperature.
** $P < 0.01$
*** $P < 0.001$

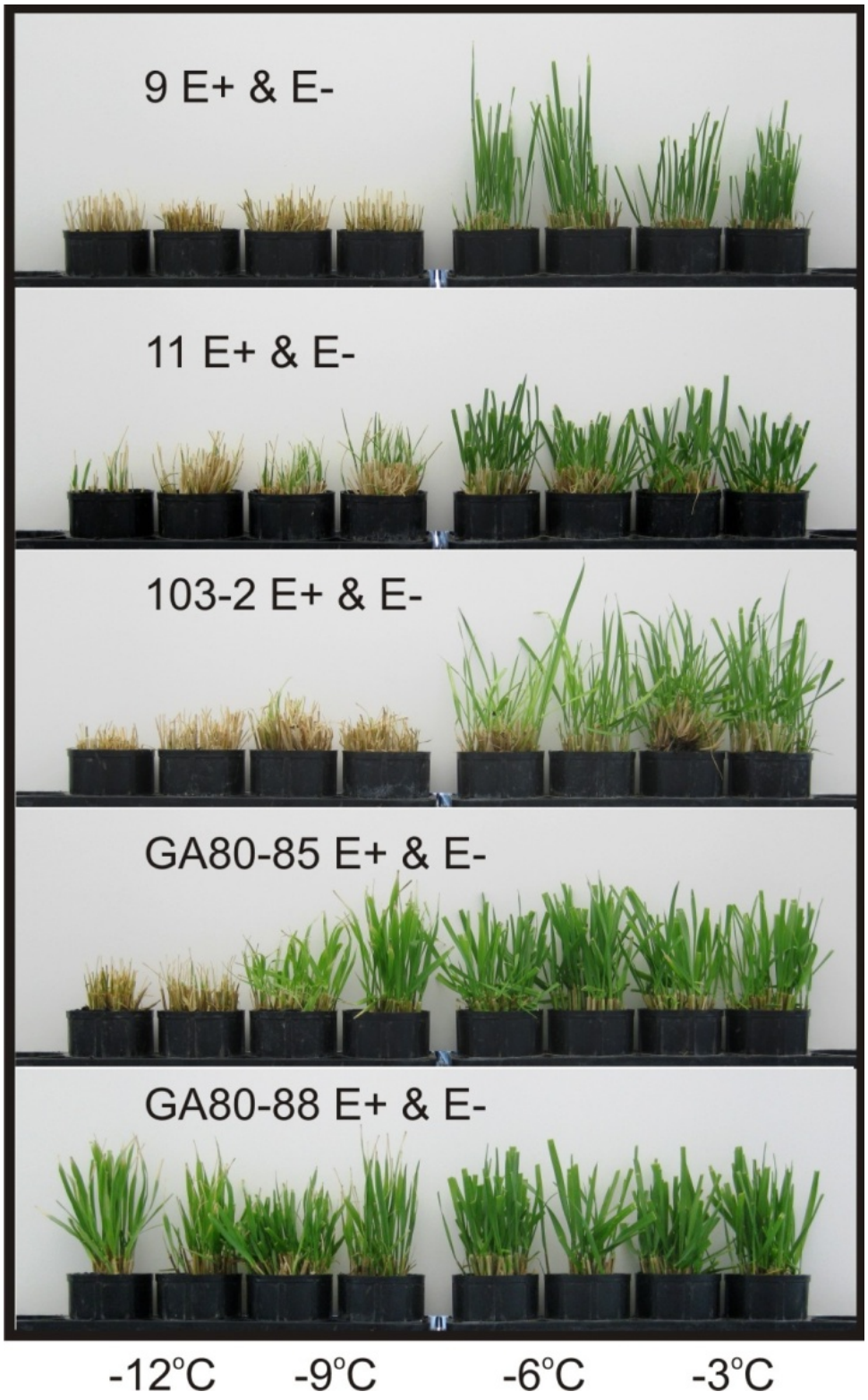

Figure 2.3. Survival of five tall fescue genotypes either infected or not infected with an endophyte. At each temperature the plant on the left is E+ and on the right E-. Picture was taken five days after the plants were removed from the freezing chambers.

EFFECTS OF COLD, NON-FREEZING TEMPERATURES ON LEAF EXTENSION, PROLINE, FRUCTAN, ABSCISIC ACID (ABA), AND CARBOHYDRATES IN CONTINENTAL AND MEDITERRANEAN HEXAPLOID TALL FESCUE

ABSTRACT

Continental tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] is the predominant forage utilized by cattle producers throughout much of the Midwest. However, another type known as Mediterranean tall fescue is gaining popularity in warmer, drier climates. One major difference is the ability of Mediterranean tall fescue to enter a semi-dormant period during hot, dry summer weather, and become active again during the fall and winter months. During this resurgence it has exceptional growth, but it is unable to survive prolonged periods of freezing weather. To determine how both Continental and Mediterranean tall fescues respond to low temperatures, the fall growth, quantified by leaf extension rate (LER), was measured along with several osmolites and abscisic acid (ABA). Three Mediterranean and four Continental accessions were tested. The LER at 4.5^{o}C for the Mediterranean genotypes averaged 4.7 mm d^{-1} while the Continental genotypes averaged only 2.0 mm d^{-1}. Between 25.0 and 4.5^{o}C proline concentrations had only an average increase of 3.7-fold for Mediterranean accessions, while Continental accessions increased an average of 15.8-fold; however, Kentucky-31 only increased 5.5-fold. Abscisic acid (ABA) levels were vastly different at 25^{o}C, with Mediterranean accessions containing approximately half the ABA concentration as the Continental accessions. While this difference persisted at 4.5^{o}C, the difference between the groups was reduced. Lastly, mono- and disaccharide concentrations were lower and fructan concentrations where greater for the Mediterranean accessions compared to the Continental types.

INTRODUCTION

One of the greatest expenses to beef cattle producers is feeding stored forages during the winter when pastures are typically dormant (May et al., 1999; Prevatt et al., 2001). One method to mitigate these winter feeding costs is to stockpile forages in autumn and use the stockpiled forage for deferred grazing through the winter. Tall fescue is an excellent choice for stockpiling because it maintains its nutritive value longer into winter than many other forages (Taylor and Templeton, 1976; Ocumpaugh and Matches, 1977).

During the late summer and autumn, typically when tall fescue is being stockpiled, day length is becoming shorter with a coincidental drop in temperature. As a result, forage accumulation rates slow as the season progresses. Therefore, development of tall fescue cultivars that have greater growth rates under colder temperatures would be an important to the livestock industry. One possibility is to screen tall fescue genotypes for the ability to maintain faster growth rates between the temperatures of 5 and 10^{o}C. By identifying genes responsible for faster growth at cool temperatures and introducing them into adapted cultivars, the autumn growing season could be more productive. This trait would achieve greater stockpiled forage for use by livestock operations during the winter.

Tall fescue's native range encompasses a wide geographical area from the Mediterranean coasts to the high altitudes of the Alps, to the northern latitudes of continental Europe. This broad geographical range has allowed tall fescue to develop specific traits unique to those environments. The tall fescue genus is comprised of two distinct morphotypes: Continental and Mediterranean. Continental plants continue to grow regardless of day length, temperature, and water status during the summer months.

They originated at the higher latitudes and/or altitudes commonly found in northern Europe and mountainous regions in west and central Asia. Because Continental types grow through the summer, they are sometimes referred to as summer-active tall fescue. On the other hand, Mediterranean tall fescue (summer-dormant) types are typically found around the Mediterranean Basin. There, temperatures rarely drop below freezing, but the plants must endure high temperatures and water shortages throughout parts of the year. During these stress conditions the plants become inactive and will not break dormancy even if watered (Norton et al., 2006). It appears that day length signals the plant to enter and emerge from this summer dormancy (Volaire and Norton, 2006). When the plant does break its dormancy in the autumn, it has excellent growth potential during the cooler winter months. This growth is in contrast to Continental tall fescue types which grow more slowly in autumn and winter (Anderson et al., 1999). Mediterranean germplasm is becoming commonly used in parts of South Australia and in parts of the U.S. with mild winters including Oklahoma and Texas where it can be a perennial option to annually sown crops, like wheat, oats, and cereal rye.

When plants are exposed to extreme abiotic stresses, osmoprotectants are known to dramatically increase in concentration. Three types of compounds that fall in this category include: (i) polyols and sugars (i.e trehalose and glucose), (ii) betaines, and (iii) amino acids like proline (Hare et al., 1998). These osmolytes are produced rapidly and are located within the cell. The rapid production of these compounds increases the osmotic potential in the cell and draws water from the apoplast into the cell cytoplasm. This action stabilizes proteins and membranes. Proline accumulation is the highest in leaf tissue during exposure to cold temperatures and is thought to be a mechanism in

establishing cold resistance (Chu et al., 1974). Simple sugars have also been shown to increase with environmental stimuli that occur before stressful conditions. These conditions initiate the process of storing food reserves for a prolonged dormancy. Grasses convert the simple sugars into long chains of carbohydrates collectively known as fructans.

Likewise, the hormone abscisic acid (ABA) increases in plants with biotic and abiotic stresses. However, the mechanism(s) that initiates this response in relation to cold stress is not completely understood. The response of ABA is likely due to not one, but several factors working in a dependent or independent manner (Gusta et al., 2005). *Arabidopsis* ABA deficient mutants accumulated proline at similar levels as wild type plants although not at the same level as when exogenous ABA was applied. This result suggests that proline accumulation has both an ABA-dependent and –independent biosynthetic pathway and may not play an important role in cold adaptation (Savouré et al., 1997).

Considering these factors we hypothesized that the Mediterranean accessions of tall fescue are either not able to perceive the cold temperatures, as measured by the production of osmolites, or are not able to synthesize sufficient concentrations of these compounds to survive the cold stress. The objectives of this study were to assess (i) the growth potential of Continental and Mediterranean tall fescue accessions subjected to cold, and (ii) the accumulation of osmoprotectants and abscisic acid (ABA) under these conditions.

METHODS AND MATERIALS

Plant Materials

An array of diverse tall fescue germplasm was obtained from the National Plant Germplasm System based on longitude, latitude, and altitude. In a preliminary experiment, 56 tall fescue accessions were selected and grown under greenhouse conditions to measure the leaf extension rate (LER) of each accession (data not shown). Of the 56 accessions screened, 5 were selected based on their LER. The accessions that were selected are PI 172423, PI 200339, PI 283297, PI 314684, PI 610956. Additionally, the cultivars 'Flecha' and 'Kentucky-31' were used as controls since each variety was of known Mediterranean and Continental origin, respectively. The cultivar Flecha (Miller, 2000), from AgResearch Grasslands of New Zealand/Pennington Seed, was derived from germplasm that originated in Tunisia and Argentina. Kentucky-31, hereafter referred to as KY-31, was from seed harvested in Missouri. From each of the accession/varieties eight individual seeds were planted to represent some of the genetic diversity within an accession. The eight genotypes were then clonally split into three replications and used as technical replicates.

Planting, Growing Conditions, and Growth Chambers

Plants were clonally split and grown in D40H (6.4 cm dia. x 25 cm tall) Deepots™ (Stuewe and Sons Inc., Tangent, OR) filled with Pro-Mix (Premier Tech Horticulture, Rivière-du-Loup, Québec, Canada) potting soil. The pots were arranged inside modified glass-topped freezers (described below) located in a greenhouse. A 12-h day length was used at 25.0 and 4.5^{o}C to ensure day length would not affect the results. Plants received supplemental light from high-pressure sodium lamps. During the 12-h

photoperiod, photosynthetic photon flux density (PPFD) ranged from 500 to 1600 μmol $m^{-2}s^{-1}$ which depended largely on sunlight. The greenhouse temperature was 25.0 +/- 5.0 ^{o}C, while that of the chambers was maintained at 4.5 +/- 0.5 ^{o}C during the plants' acclimation and growth periods. Plants received 200 +/- 25 μmol $m^{-2}s^{-1}$ of artificial light while in the chambers. This light intensity was chosen to mimic the lower light levels of winter, because increased light intensity can produce effects that may not be associated with acclimated plants at low temperatures (Ndong et al., 2001). Lastly, plants were given the equivalent of 9.0 kg ha^{-1} of nitrogen, 3.0 kg ha^{-1} of phosphorus, and 6.0 kg ha^{-1} of potassium fertilizer weekly to counteract the leaching from the potting soil.

The chambers were made from vencold freezers, model GST65-C22 (Guelph, Ontario, Canada). Low emissivity glass was used to prevent the chamber's internal temperature from becoming too warm while the lights were turned on. Sofasco (Winchester, VA) fans measuring 92x92x25 mm (model: D9225V12H) and 60x60x25 mm (model: D6025V12H), were used to circulate air within each chamber so that air would not stratify into warm and cold layers. Evercool (Taiwan) fans measuring 30x30x7 mm (model: EC3007M05CA) were used to push fresh air into each chamber through the duration of the experiment. The chamber temperature was monitored using thermocouples in each chamber placed near the crown tissue height of the plants. Each freezer had a heating tape applied to the inside of the freezer adjacent to the cooling coils. This heat tape was thermostatically controlled by a personal computer running custom LabVIEW (National Instruments, Austin, TX) software.

LER, Proline, Fructan and ABA Determination

To measure leaf extension rate (LER), one tiller was selected from each plant where the newest leaf was between 0.25 and 0.5 the length of the second newest leaf. The third newest leaf sheath had a permanent mark placed below the leaf collar (Figure 3.1). This mark was used as a reference mark each day when measuring the LER. Additionally, the leaf being evaluated was either the third, fourth, or fifth emerging leaf for that particular tiller. A clear ruler was used to measure the leaf's length each day. The measurements were completed on a 24-h period for 5 days. These measurements were averaged to calculate the LER for each individual plant at both 25.0 and 4.5^{o}C. Since LER is a measure of leaf growth and is positively correlated to potential yield and regrowth (Horst et al., 1978), it was necessary to select leaves that would have the same growth characteristics so that leaf number would have little impact on the measurement. From preliminary testing in our lab and work by Robson (1974) and Durand et al. (1999), it has been shown in tillers with three or more leaves that the third and successive leaves have nearly the same growth rate.

To determine free proline, both leaves and stems were harvested prior to placement in the cold growth chamber and again after plants had grown for 30 d at 4.5^{o}C. The tissues from each plant used to determine proline, ABA, and saccharide concentrations were harvested between 0.0-1.0 cm from the soil and immediately frozen using liquid nitrogen. Samples were then lyophilized and kept at -20^{o}C. Because of the small sample size, samples were ground using Braun (Cincinnati, OH) model KSM2 coffee grinders for approximately 30 sec. and then returned to the freezer. Free proline was determined by using the method described by Bates (1973) with some modification.

Samples harvested at 25.0^{o}C used 100 mg of tissue while samples harvested at 4.5^{o}C needed only 50 mg of tissue for proline extraction to remain within the standard curve due to their higher concentrations. Additionally, only 1 mL of filtrate was used for samples collected at 4.5^{o}C due to the high proline concentration while 2 mL was used for samples harvested at 25.0^{o}C during the reaction process. For each set of samples analyzed, a new standard curve was used at concentrations of 1, 3, 5, 7, 9, 10, and 20 μg ml^{-1}. This curve was used to calculate free proline in the tissue. Then using the equation provided by Bates (1973), the values determined by the standard curve were converted to μmol mg^{-1} dry matter (DM).

The fructan concentration of the same samples was determined using the ©Megazyme (Wicklow, Ireland) fructan assay. The procedure was performed according to the manufacturer's instructions with one exception. Since the samples contained between 0.5 and 20% fructan, a 200 mg sample was used and diluted in a 50 mL volumetric flask. The remaining protocol was followed according to the manufacturer's instructions.

Lastly, to determine ABA concentrations, ELISA kits were purchased from Agdia (Elkhart, IN, USA). For ABA extraction, 10 mg of DM was weighed into 2 mL Eppendorf (Hamburg, Germany) tubes. Once all samples were weighed, 1.0 mL of TBS buffer was added. The samples in TBS were incubated at 4°C overnight to extract the ABA. The samples were then centrifuged at 10,000 g for 10 minutes to pellet the debris. One hundred μL of supernatant was diluted into 1.9 mL (20x dilution) of TBS buffer for ABA analysis. Once all the samples were diluted for analysis the ELSIA kit was used according to the manufacturer's instructions.

Mono- and Disaccharide Determination

The same tissue used for proline, and fructan determination was used to determine glucose, fructose, and sucrose. To determine the mono- and disaccharide concentrations, a 12.5 mg sample was extracted with 1.2 mL of 40% ethanol. This solution was placed on a shaker at 200 rpm for 30 min at 50.0^{o}C. The samples were vortexed prior to their placement in the incubator and half-way through the 30 min. extraction. After the incubation period, 100 µl were filtered through a Millipore (Billerica, MA) 96 well filter plate with 45 µm filter for 2 min. at 2000g. This filtrate was then used for injection on a Agilent 1100 series high performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD) HPLC with an ESA Coulochem III detector (Agilent Technologies, Chesterfield, MO). The filtrate was separated using a Dionex Carbo Pac PA 10 analytical column (250 mm x 4 mm, 10 µm) connected to a Carbo Pac 10 guard column (50 mm x 4 mm). The mobile phase included 90 mM NaOH solution with a flow rate of 1.5 ml min^{-1}, maintained at 30^{o}C. Detection settings were: time 0, 0.1 v, time 0.41, -2.0 v, time 0.42 0.6 v, and time 0.44, -0.1 v.

Statistical Analysis

Plants were arranged in a randomized complete block design (RCBD) (Steel and Torrie 1980). Each accession was comprised of 8 genotypes where each genotype was replicated three times by clonal propagation. For the LER determination, all eight genotypes were used (n=8). For proline, ABA, fructan, and saccharide concentration only five of the eight genotypes, randomly selected, were analyzed (n=5). These genotypes were used as the replication for determining the accession variation. Main effects and all interactions were analyzed by analysis of variance using PROC GLM of SAS version 9.1

(SAS Inst. Inc., Cary, NC). Correlations to LER were performed using PROC CORR in SAS statistical software (SAS Inst. Inc.).

RESULTS AND DISCUSSION

Leaf Extension Rate

The LER differences were substantial between the seven accessions examined. When grown under cool conditions, the LER of Mediterranean genotypes was consistently higher than the Continentals ranging from 4.4 to 5.0 mm d^{-1}, while the Continental accessions ranged between 1.3 and 2.5 mm d^{-1} (Table 3.1 and Figure 3.2). There was also a difference among the Continental accessions as those from the eastern geographical range (PI 172423 PI 314684) had a slower LER than those of western origin (PI 283297 and KY-31) 1.3 and 1.9 versus 2.4 and 2.5, respectively. This result may be due to the elevation at which these accessions are adapted, since the tall fescue accession from Turkey and Kazakhstan are from mountainous regions with elevations of over 1500m, compared to those collected in Western Europe where elevation is less than 800m.

These data are similar to those from other laboratory and field experiments in which Mediterranean lines of tall fescue had two to three times more growth or yield compared to Continental lines at low temperatures (Robson and Jewiss 1968; Thomas and Lazenby 1968a; Nelson et al., 1978; Anderson et al., 1999; Reed et al., 2004; Chapter 2). It seems that a majority of the highest yielding material originates from North Africa rather than southern Europe (Reed et al., 2004). To obtain this yield benefit, these accessions must be grown in a location that rarely experiences temperatures below

freezing, since they have been shown to be susceptible to cold stress (Robson, 1967; Thomas and Lazenby, 1968a; Chapter 2).

Survival of Mediterranean tall fescue in continental climates is likely influenced by managerial practices as cutting height and repeated freeze-thaw cycles have a profound influence on the plant's ability to survive (Thomas and Lazenby, 1968a, 1968b). From our observations, Mediterranean plants that are not cut in the late autumn had between 30 and 70% winter survival when measured in March, whereas winter survival was 0 to 10% if plants were harvested to a 5-cm height in late autumn (unpublished data). An alternative to not harvesting the forage in autumn would be to identify specific genotypes from Continental germplasm that have superior growth under cold conditions or Mediterranean genotypes that survive periods of freezing weather. We identified one potential genotype from accession PI 172423 that had superior LER while maintaining high concentration of proline (Figure 3.3). Therefore, it may be possible to select for both traits simultaneously as they do not appear to be controlled by the same genetic mechanism. However, these traits appear to be genetically linked but this linkage can be broken to obtain plants with superior fall growth and winter survival.

Proline and ABA

The proline concentration at 25^{o}C did not differ between the two tall fescue types, although there were differences between individual accessions. Proline at 25.0^{o}C had a narrow range between 2.77 and 7.33 μmol g^{-1}DM (Table 3.1). However, at 4.5^{o}C there was a difference in proline concentrations, with Continental accessions averaging 50.76 μmol g^{-1} DM while the Mediterranean accessions averaged only 11.31 μmol g^{-1} DM. Within the Continental accessions, PI 283297 and PI 314684 contained the highest

proline concentrations at 63 and 62 μmol g^{-1} DM, respectively. Accession PI 172423 had an intermediate concentration at 47 μmol g^{-1} DM, and KY-31 had the lowest concentration at 30 μmol g^{-1} DM. The Continental lines showed a 15-fold increase in proline concentrations between the two temperatures compared to just 3.6 for the Mediterranean accessions. Interestingly, the increase in proline for KY-31 was not different from the Mediterranean accessions (Table 3.1).

There were unexpected differences in ABA levels at 25.0°C. Continental accessions contained 982 pmol g^{-1} DW of ABA while those of Mediterranean origin averaged just 548 pmol g^{-1} DW (Table 3.1). The difference between the two fescue types was not as great after the cold stress, with concentrations increasing to 1,426 and 1,231 pmol g^{-1} DW, for Continental and Mediterranean types, respectively. The difference between types of tall fescue ABA concentrations at 4.5°C was unexpected since Mediterranean accessions PI 200339 and PI 610956 had similar or greater ABA concentrations at 4.5°C than some Continental accessions. This is observed by the change in ABA concentration after cold stress where Mediterranean accessions averaged a 2.6-fold increase compared to only 1.6 for the Continental lines. Additionally, ABA concentrations at 25°C were correlated to both LER and proline concentrations at 4.5°C, r=0.77 and 0.67, respectively (Figures 3.4 and 3.5).

This paper is the first to report the response of proline and ABA in either Continental or Mediterranean tall fescue after plants had been subjected to cold stress. The proline values for KY-31 are similar to the clones GA80-85 and GA80-88, which are selections from KY-31, and had values ranging from 20 to 42 μmol g^{-1} DM (Chapter 2). The values obtained from the Continental genotypes after subjecting plants to cold

treatment are consistent with values from tall fescue and other forages during water-stress events. However, proline concentrations of the Mediterranean accessions were 3 to 7 times lower than for the Continental accessions at 4.5^{o}C (Bokhari and Trent, 1985; Abernethy and McManus, 1998).

Winter wheat lines that over accumulate proline compared to the wildtype, due to mutagenesis, have superior frost tolerance in conjunction with elevated concentrations of glucose, fructose, and ABA (Dörffling et al., 2009). This is in conflict with results seen in ABA deficient *Arabidopsis* mutants, as well as wild type plants, where proline concentrations increased only slightly in response to cold stress (Savouré et al., 1997). The results of this work in combination with Dierking and Kallenbach (Chapter 2) validate those of Dörffling et al. (2009), where an increase in proline was observed in conjunction with superior freezing tolerance of the Continental accessions.

The initial concentrations of ABA at 25.0^{o}C were similar to other reports in tall fescue (Abernethy and McManus, 1998) and rice ranging from 125 to 300 ng g^{-1} DM (Dingkuhn et al., 1991). Although, ABA did accumulate in response to the cold stress, it did not have the same final concentration as those reported by Dingkuhn et al. (1991) and Aberethy and McManus (1998) in response to water stress. Both authors report final concentrations of 1000-1200 ng g^{-1} DM, while our results showed a final concentration of only 270 to 415 ng g^{-1} DM. Additionally, Abernethy and McManus (1998) reported a 10-fold increase in ABA, while Joost and Holder (1994) reported a 16-fold increase in response to water stress, which is a greater response than what we observed during cold stress. Even though the increase we found was not as large as those reported above, there was a difference observed between the two tall fescue types for ABA accumulation. One

possibility for the greater ABA increase for Mediterranean tall fescue is that the surge of ABA may be an early indicator hormone, cueing the plant to initiate summer dormancy from the previous winter conditions (Ofir and Kigel, 1998). However, the accumulation of ABA in response to a cold stress has not been shown to be as responsive as that for other abiotic stresses. In wheat, ABA levels only increased by two-fold in both controlled and field experiments to cold stress (Dörffling et al., 2009) - a result similar to ours. Therefore, ABA may only play a small role in the plant's mechanisms to manage cold stress.

Fructan

Fructan concentrations at 25.0^{o}C were similar among the tall fescue types and accessions within types. Plant introduction 172423 contained the lowest fructan concentration (9 mg g^{-1} DM) while KY-31 contained the greatest concentration (21 mg g^{-1} DM) (Table 3.1). Fructan levels increased 8 to 18-fold when the tall fescue plants were exposed to 4.5^{o}C for 30 days. Additionally, the Mediterranean accessions had a greater concentration of fructan when compared to the Continental accessions at 4.5^{o}C. When comparing Mediterranean and Continental types, the final fructan concentration of Mediterranean germplasm averaged 115 mg g^{-1} DM while Continental accessions averaged 95 mg g^{-1} DM.

These results are slightly lower than those we reported earlier (Chapter 2), although the difference observed between 25 and 4.5^{o}C was greater during this experiment. Fructan accumulation has been reported (Hisano et al., 2008) in both leaf and crown tissue in perennial ryegrass, with a marked difference among short, medium, and long fructan chain lengths. Although we did not evaluate fructan accumulation rates

during the trial, Hisano et al. (2008) showed it took 7 to 14 days for accumulation to begin when plants were exposed to low temperatures. This delay might be a result of photosystem I and II adjusting to use light at low temperatures without damaging the reaction centers through the use of non-photochemical quenching (Savitch et al., 2002; Sveshnikov et al., 2006), or more likely, having higher photochemical quenching (Huner et al., 1998) which results in higher sugar concentrations.

Interestingly, the leaves of winter wheat cultivars exposed to cold temperatures had fructan concentrations that were three times greater than those of spring wheat cultivars (Savitch et al., 2002). However the difference in survival could be due to the shorter fructan chains types found in wheat in contrast to the longer types of fructan molecules found in tall fescue (Nelson and Spollen, 1987). Therefore, based on fructan alone, it would seem that the Mediterranean genotypes should be just as or even more cold hardy than the Continental genotypes. However, this is not the case. Perhaps Mediterranean genotypes have a more efficient photoreceptor mechanism at low temperatures resulting in greater glucose and fructose concentrations. These molecules could then be used to accumulate fructan as well as enhance LER even though fructan does not have an impact on plant survival at cold temperatures.

Mono- and Disaccharides

At 25.0^{o}C, neither the type of tall fescue nor the accessions within a type differed for any of the sugar concentrations measured (Table 3.2). This was not the case when comparing the tall fescue accessions and types at 4.5^{o}C. The concentration of all sugars examined were lower ($P < 0.06$) for the Mediterranean compared to the Continental accessions at this temperature. Glucose and myo-inositol showed only slight increases

from the levels found at 25.0°C. Fructose, however, showed a slightly different response. Fructose concentrations for the Continental accessions did not change while concentrations for the Mediterranean lines actually decreased. Sucrose had the greatest response to the cold temperatures with Continentals averaging 58 mg g^{-1} DM while Mediterranean genotypes had concentrations of 52 mg g^{-1} DM, which is about a 3-fold increase compared to the concentrations at 25°C.

The trends of glucose and sucrose are similar to those we reported earlier (Chapter 2); although, here we observed a larger increase in sucrose and a smaller increase for glucose. This difference between the results in Chapter 2 and this experiment may be due to the difference in cutting height of the tillers (3.0 vs. 0.5cm). It has been shown that the mono- and disaccharides along with oligo- and polyfructans have varying concentrations along the length of the tiller/leaf (Schnyder and Nelson, 1987 and 1989). Thus, the concentrations differ depending on how much of the lower-basal tissue is sampled.

The large difference in fructose concentration between the Mediterranean and Continental genotypes here and in the Chapter 2 may be related to the process by which the two different tall fescue types are able to produce fructan under cold conditions. Nelson and Spollen (1987) describe the process of converting mono- and disaccharides into fructan. It is thought that two sucrose (glucose-fructose) molecules are brought together to form the trisaccharide 1-kestose (glucose-fructose-fructose). The 1-kestose then transfers the last fructose to extend a fructan molecule. Our research supports that idea. At cold temperatures, we have shown elevated fructan concentrations and a

simultaneous reduction in glucose, fructose, and sucrose concentrations in Mediterranean tall fescue compared to Continental tall fescue.

The difference observed between the mono- and disaccharides and fructan is likely due to the ability of Mediterranean germplasm to photosynthesize at a higher rate. This greater rate has been shown in both tall fescue and perennial ryegrass of Mediterranean origin (Chatterjee 1961; Robson 1967; McFarlane et al. 2001). Tall fescue's wild relatives with tetraploid genomes ($M_1M_1M_2M_2$) from *Festuca mairei* had increased rates of apparent photosynthesis (Randal et al., 1985). It may be that not having to adapt to harsh winter conditions allowed these Mediterranean genotypes to partition more of the photosynthate into foliage production (Robson and Jewiss, 1968; Blacklow and McGuire, 1971). This idea seems consistent with our LER data. Additionally, due to the potentially higher photosynthetic rates, Mediterranean accessions may be using the larger pool of photosynthate to create slightly higher fructan concentrations, which is why the mono- and disaccharides measured were lower compared to the Continental lines. This is consistent with Robson and Jewiss (1968) where a Continental genotype accumulated greater soluble carbohydrates during the winter months of December and January compared to a Mediterranean genotype with a faster LER.

In several plant species, the accumulation of mono- and disaccharides enhances freezing tolerance (Gusta et al., 1996). Working with winter wheat, Fowler et al. (1981) reported a correlation coefficient of 0.88 between sucrose concentrations in crown tissue and winter hardiness of the plant. Additionally, glucose has been shown to have extensive control over ABA synthesis as well as expression of transcription factors of ABA. It has also been suggested that sucrose may influence ABA production (Arroyo et al., 2003;

Leon and Sheen, 2003). Possibly, the slightly higher sugar concentrations in the Continental accessions led to an increase in ABA concentrations and that was adequate to achieve a higher level of cold tolerance. However, two of the three Mediterranean accessions had ABA concentrations that were similar to the Continental lines. Therefore, it seems that for tall fescue the interaction between sugar and ABA is insignificant. In any case, it appears that the sugars, whether they are interacting with ABA or other compounds, play a small role for enhancing the overall winter survivability, and that the differences between the two types of tall fescue are a consequence of the differences in their photosynthetic apparatus.

CONCLUSION

Initially, we hypothesized that the Mediterranean accessions are either not able to sense the cold temperatures, or are not able to fully respond to the near freezing conditions. From this work it appears that the later hypothesis satisfies the experimental results. If the plants were unable to sense the environmental conditions, the fructan levels would have been lower than in the Continentals and there would not have been a rapid increase in ABA. However, the Mediterranean genotypes exhibited a response in ABA, fructan, and mono- and disaccharides. Therefore, as indicated with the proline levels, it appears they lack the necessary machinery (i.e. not able to fully respond to the climatic conditions) needed for protection in regions with long periods of freezing temperatures.

Lastly, to determine if all Mediterranean hexaploids possess greater LER, but lack persistence to cold and/or freezing conditions a greater number of accessions will need to be studied. As indicated by the correlation analysis particular genotypes can possess both high LER and the potential for survival at freezing temperatures. It may be that the

proline levels, ABA, and/or simple sugars can be uncoupled/linked to accessions that have low LER and high survival. Additionally, by achieving structured crosses between the Continental and Mediterranean hexaploid plant groups, the genomes, and more precisely the gene(s), responsible for these effects might be elucidated.

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Table 3.1. Leaf Extension Rate (LER), proline, abscisic acid (ABA), and fructan measures taken at 25.0 and 4.5°C for 7 accessions of tall fescue originating from either Continental (summer-active) or Mediterranean (summer-dormant) germplasm.

Accession	Origin	LER 4.5 °C	Proline 25 °C	Proline 4.5 °C	ABA 25 °C	ABA 4.5 °C	Fructan 25 °C	Fructan 4.5 °C
		mm d^{-1}	---µmol g^{-1} DM---		--pmol g^{-1} DW--		----mg g^{-1} DW----	
PI 172423	**Turkey**	1.8	2.9	47.5	866	1416	9.2	89.1
PI 283297	**Sweden**	2.3	6.6	63.4	917	1431	13.0	104.8
PI 314684	**Kazakhstan**	1.2	3.5	61.8	1014	1568	10.1	83.3
Kentucky-31	**Missouri**	2.5	7.3	30.4	1134	1292	20.7	105.3
PI 200339	**Israel**	4.4	5.1	12.5	574	1431	11.3	105.2
PI 610956	**Tunisia**	5.0	3.3	12.7	596	1238	10.8	113.9
Flecha	**Tunisia**	4.8	2.8	8.7	475	1025	11.0	126.9
	LSD†	0.63	3.5	11.0	177	244	NS	NS
Orthogonal Contrast		0.0001[a]	NS[b]	0.0001	0.0001	0.005	NS	0.034
Continental		2.00	5.1	50.8	983	1427	13.6	95.4
Mediterranean		4.70	3.7	11.3	549	1231	11.0	115.3

† Fisher's Protected LSD (0.05)
[a] P-value
[b] NS, Non-significant

Table 3.2. Mono- and disaccharide concentrations taken at 25.0 and 4.5°C for 7 accessions of tall fescue originating from either Continental (summer-active) or Mediterranean (summer-dormant) germplasm.

Accession	Origin	Glucose 25 °C	Glucose 4.5 °C	Fructose 25 °C	Fructose 4.5 °C	Myo-inositol 25 °C	Myo-inositol 4.5 °C	Sucrose 25 °C	Sucrose 4.5 °C
		----------mg g^{-1} DW----------							
PI 172423	**Turkey**	3.3	4.7	3.7	3.9	1.9	2.2	17.1	62.3
PI 283297	**Sweden**	4.3	4.9	5.3	4.6	2.0	2.9	19.3	59.9
PI 314684	**Kazakhstan**	3.8	5.1	3.7	4.2	1.4	2.2	17.2	52.3
Kentucky-31	**Missouri**	4.2	5.2	5.1	5.2	1.8	1.9	28.8	58.4
PI 200339	**Israel**	4.4	4.4	4.0	3.8	1.8	1.8	16.0	55.3
PI 610956	**Tunisia**	4.2	4.0	5.2	3.2	1.9	2.1	16.6	48.7
Flecha	**Tunisia/Argentina**	3.9	4.7	3.8	3.4	1.8	1.9	25.4	52.1
	LSD†	NS[a]	NS	NS	0.9	NS	NS	NS	NS
Orthogonal Contrast		NS	0.076[b]	NS	0.0005	NS	0.053	NS	0.053
Continental		3.9	5.0	4.4	4.4	1.8	2.3	20.6	58.2
Mediterranean		4.1	4.4	4.3	3.6	1.8	1.9	19.3	52.0

† Fisher's Protected LSD (0.05)
[a] NS, Non-significant
[b] P-value

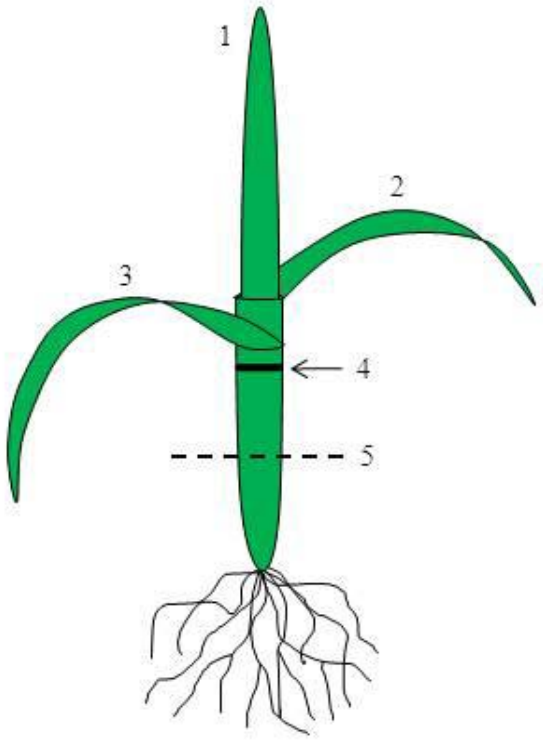

Figure 3.1. Schematic of a tall fescue tiller where 1) represents the newest emerging leaf, 2) the second newest leaf, 3) the third newest leaf, 4) the position of the reference point to measure LER, and 5) the cutting height at approximately 0 to 1 cm.

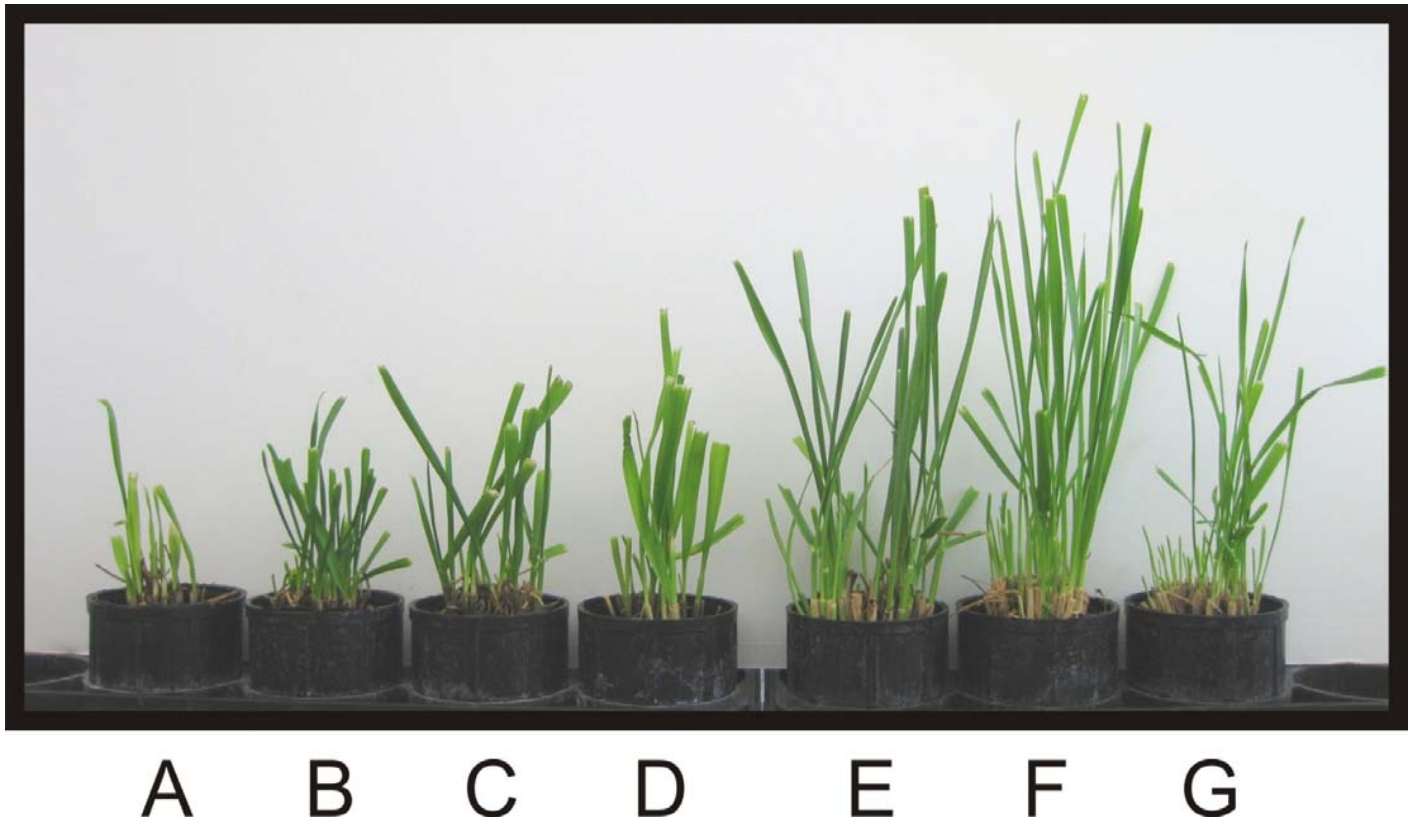

Figure 3.2. Regrowth after 30 days at 4.5°C. A is PI 314684, B is PI 172423, C is PI 283297, D is Kentucky-31, E is PI 200339, F is Flecha, G is PI 610956. The accessions A-D are Continental (summer-active) and E-G are Mediterranean (summer-dormant).

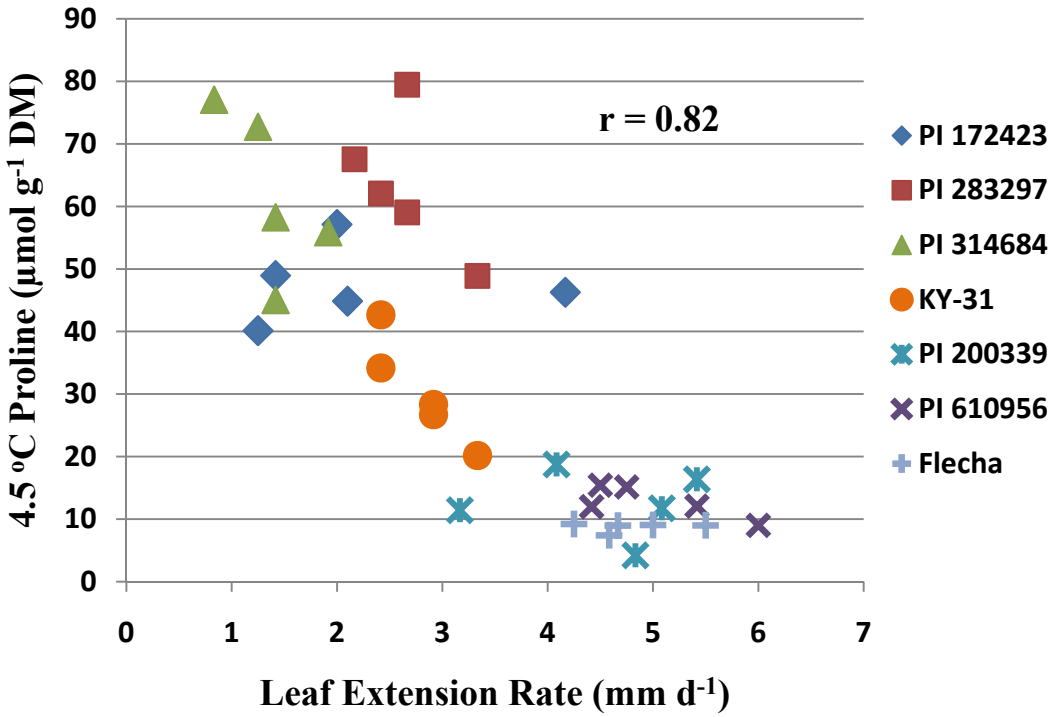

Figure 3.3. Correlation between Leaf Extension Rate (LER) and proline levels taken at 4.5°C for all accessions examined. Accessions PI 172423, PI 283297, PI 314684, and KY-31 are Continental. Accessions PI 200339, PI 610956, and Flecha are Mediterranean.

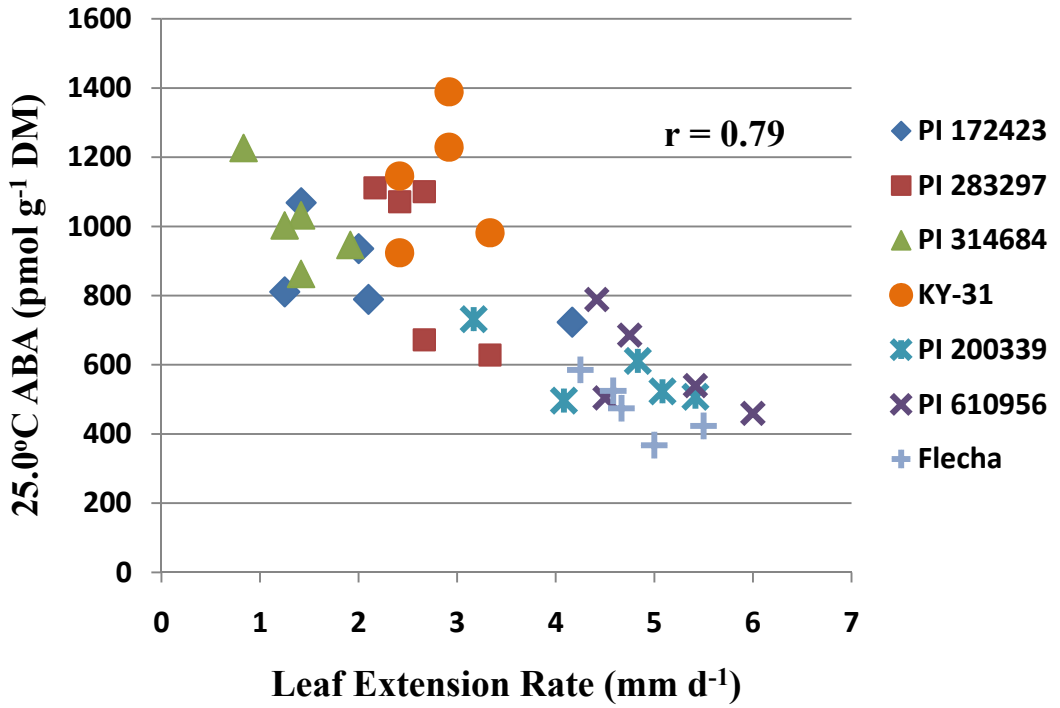

Figure 3.4. Correlation between Leaf Extension Rate (LER) and abscisic acid (ABA) concentrations at 25.0°C for all accessions examined. Accessions PI 172423, PI 283297, PI 314684, and KY-31 are Continental. Accessions PI 200339, PI 610956, and Flecha are Mediterranean.

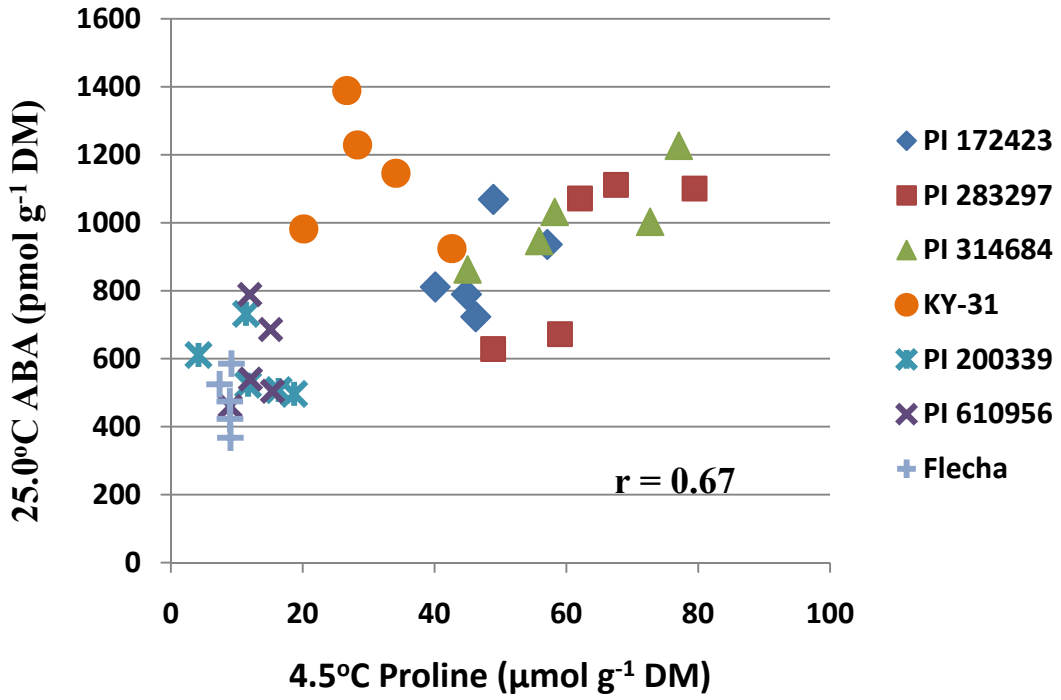

Figure 3.5. Correlation between proline concentration taken at 4.5°C and ABA concentrations at 25.0°C for all accessions examined. Accessions PI 172423, PI 283297, PI 314684, and KY-31 are Continental. Accessions PI 200339, PI 610956, and Flecha are Mediterranean.

DEVELOPMENT OF A LINKAGE MAP AND IDENTIFICATION OF QTLS FOR FALL GROWTH AND WINTER SURVIVAL IN A MEDITERRANEAN BY CONTINENTAL TALL FESCUE POPULATION

ABSTRACT

Tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] has two distinct morphotypes, Continental (summer-active) and Mediterranean (summer-dormant). Continental tall fescue originates from Continental Europe while the Mediterranean type is found around the Mediterranean basin. Here we report for the first time a genetic map of tall fescue from a Continental by Mediterranean cross. The Noble Foundation mapping population developed from a Continental type tall fescue (R43-64) crossed onto a Mediterranean type tall fescue (103-2). The objective of this study was to construct a genetic linkage map and identify QTLs relating to fall growth and winter survivability. PCR and DArTFest markers were used to construct parental linkage maps. Parent R43-64 contained 20 linkage groups (LG) with an average of 94.2 cM per LG while parent 103-2 comprised of 23 LG averaging 58.8 cM. Only 7 LGs were shared between both parental maps. The parental linkage map of R43-64 had 563 markers while the linkage map of 103-2 only contained 191 markers due to large amount of segregation distortions. Marker densities for R43-64 and 103-2 were 3.4 and 7.1 cM marker^{-1}, respectively. For QTL traits all plants were phenotyped under field conditions in Wisconsin and two locations in Missouri. Fall growth, measured by leaf extension rate was assessed during October and November. Winter survival was assessed in March and April at each location. QTLs specific to location and year were identified as well as across years and locations. QTL for fall growth and winter survival were located at similar positions on the same LGs for 103-2 and R43-64. These LG were found to be homologous and most likely represent the shared P genome between both Mediterranean and Continental types. Based on linkage maps and other evidences these two types of tall fescue appear to be two distinctive species.

INTRODUCTION

Tall fescue [*Lolium arundinacea* (Schreb.) S.J. Darbysh. = *Lolium arundinaceum* (Schreb.) S.J. Darbysh.] belongs to the *Poaceae* family and is closely related to the ryegrasses. It has been proposed by Darbyshire (1993) that the *Festuca* subgenus *Schedonorus* should be merged with the *Lolium* genus. However, it is clear that *Lolium* and *Festuca* fall into discrete taxa based on morphological traits as well as genetic differences (Kolliker et al., 1999; Catalan et al., 2004; Mian et al., 2005). Tall fescue, like nearly all the species found in the *Festuca-Lolium* complex, is predominately a cross-pollinated species which stems from the high degree of self-incompatibility; this results in high levels of heterozygosity. Due to obligate out-crossing, nearly all progenies are genetically unique allowing populations to have large amounts of genetic variations for multiple traits. Tall fescue, an allohexaploid (2n = 6x = 42), is part of a larger group of related plants that range from diploid (e.g. *F. pratensis*; 2n = 2x = 14) to decaploid (*F. arundinacea* var. letourneuxiana and cirtensis; 2n = 10x = 70) species. However, tall fescue is the most economically important (Sleper, 1985). Tall fescue is comprised of two distinctive morphotypes, or germplasm pools, Continental (summer-active) and Mediterranean (summer-dormant). The Continental germplasm originates from Continental Europe, Central Asia and Siberia; the Mediterranean types are typically found in the Mediterranean Basin of Southern Europe, North Africa, and the Middle East.

The genomic constitution for Continental tall fescue is $PPG_1G_1G_2G_2$ (Sleper and West, 1996). The P genome is thought to have derived from meadow fescue (*Lolium pratensis* [Huds.] Darbysh) while the G genomes are supposed to be from the tetraploid fescue (*F. arundinacea* var. glaucescens Boiss) (Humphreys et al., 1995; Xu et al., 1991).

Although Mediterranean tall fescue is known to share the P genome with Continental tall fescue, the remaining genomes have not been defined. It has been hypothesized that Mediterranean tall fescue is constituted from the putative M_1 and M_2 genomes from the *F. mairei* St.-Yves. (Saha et al., 2010). These two types of tall fescue, Continental and Mediterranean, can hybridize but essentially all F_1 progenies are sterile. It is common to have meiotic irregularities among the progeny even though both parents have a uniform ploidy level (Malik, 1967; Evans et al., 1973; Hunt and Sleper, 1981). These meiotic irregularities are thought to stem from a breakdown in control of the chromosome pairing that is derived from the different genomic constitutions (Jauhar, 1975; 1991). However, fertility can be restored to progenies from a Continental by Mediterranean cross when the chromosome number is doubled (Hopkins et al., 2009).

Development of a linkage map has great utility in the detection of important agronomic traits such as resistance to fungal infections (Curley et al., 2005), fiber digestibility (Cardinal et al., 2003), and tolerance to abiotic stresses like cold/freezing (Francia et al., 2004). The genetic linkage maps are highly valuable in that they provide the framework to identify where genes of interest are located (Tondelli et al., 2006). The quantitative trait loci (QTL) can then be ascertained from the genetic linkage maps and associated markers used in marker-assisted selection for economically valuable traits.

Prior to PCR technologies, the first tall fescue map was produced from restricted fragment length polymorphisms (RFLPs) which resulted in a low resolution map covering only 1,274 cM (Xu et al., 1995). However, with the advent of PCR based molecular markers, marker density has greatly increased and updated linkage maps have been developed (Saha et al., 2005). The use of PCR markers, particularly simple

sequence repeats (SSRs), is popular due to their locus specific nature, being highly informative and their high throughput capabilities (Yu et al., 2002). However, developing SSR markers can be labor-intensive and costly (Squirrell et al., 2003). One alternative is to use expressed sequence tags (ESTs) to develop SSRs (Theil et al., 2003; Eujayl et al., 2004). These EST derived markers allow for the construction of comparative maps of expressed genes within and across species (Kantety et al., 2002). This allows for identification of putative homeologous linkage groups. However, Theil et al. (2003) found that genomic-SSR markers had a greater polymorphism rate than that of EST-SSRs. Recently, the advent of arrays and chip technology is leading to high-throughput genome profiling in many crops, and are based on single nucleotide or single feature polymorphisms (SNP and SFP, respectively) (Gupta et al., 2008). However, knowledge of the crops DNA sequence is needed and therefore can only be used with success when some minimum level of sequencing is completed. A relatively new technology called diversity array technology DArT (Jaccoud et al., 2001) which does not require any prior sequence knowledge has emerged as a tool for large scale genotyping. This technology has only recently become available for the *Festuca-Lolium* complex (Kopecký et al., 2009a).

Tall fescue's genomic complexity, self-incompatibility, and the small number of known morphological genetic markers, make genetic progress difficult (Xu et al., 1991). In particular, self-incompatibility makes creating traditional (F_2, RILs, or BC) mapping populations and constructing linkage maps difficult or almost impossible. To overcome this issue, a two-way pseudo-test cross strategy is implemented for the construction of marker-based linkage maps for outcrossing species (Ritter et al., 1990). This procedure

utilizes highly heterozygous parental lines which are crossed to create an F_1 population. Due to the parental heterozygosity, the markers can be dominant or co-dominant with the linkage phases (coupling or repulsion) commonly unknown (Maliepaard et al., 1997).

The objectives of this study were to: i) construct a genetic linkage map of a pseudo-testcross between Mediterranean and Continental tall fescue germplasm ii) to identify QTL regions conferring greater fall growth (FG) and winter survivability (WS) and iii) identify markers associated with these traits for potential use in marker-assisted breeding programs.

MATERIALS AND METHODS

Plant Materials

The tall fescue mapping population used for this study was a pseudo F_1 test-cross derived from a ♀Mediterranean by ♂Continental cross. The female parent, 103-2, is a selection from the cultivar Flecha (Miller, 2000) from AgResearch Grasslands of New Zealand/Pennington Seed. The male parent, R43-64, originates from a selection of 97TF1 which was collected in 1997 from Woodward Co., in northwestern Oklahoma. Two-hundred F_1 progenies collected from 103-2 were planted for trait evaluation of which 195 were used for mapping. These F_1 progenies were confirmed by the SSR markers used for linkage map construction. All plants derived from this cross were maintained as clonally propagated tillers.

Field Locations and Planting

The tall fescue populations were planted at Arlington, WI, USA (43.30N, -89.34W; elevation 321 m), Columbia, MO, USA (38.89N, -92.20W; elevation 271 m), and Mt. Vernon, MO, USA (37.07N, -93.88W; elevation 373 m). These locations are

hereafter referred to as WI, MO1 and MO2, respectively. The clones were planted in July and August each season, in a randomized complete block design. Due to limited plant material, the WI and MO2 locations contained only three replications while the MO1 location had four. The plants were space planted with 0.3 m between plants in each row at WI and 0.6 m at MO1 and MO2. At all locations, space between rows was 0.9 m and 1.8 m between blocks. For the first year (2008-2009) the population was only planted at WI and MO1. This was due to a severe winter and 0% survival at the WI location, the entire population was replanted in July 2009. In the second year (2009-2010) all three locations were replanted from the material at MO1. The MO1 location was also replanted to ensure the measurements were taken from plants of the same age at all locations. The study years 2008-2009 and 2009-2010 will be referred to as YR1 and YR2, respectively.

Trait Evaluation

Parent R43-64 has superior WS, while parent 103-2 has exceptional FG. Prior to the FG measurement, plants were cut back to 5.0-10.0 cm in early September to simulate a grazing event which was followed by a growth period of 8-12 weeks. When the day/night temperatures averaged 4 to 8^{o}C, two mature tillers were selected from each plant that had a newly emerging leaf between 5.0 and 10.0 cm in length. The third newest leaf sheath was marked and used as a reference point to measure the leaf extension rate (LER) of the emerging leaf (Figure 4.1). This leaf was re-measured 48h later in the sequence in which they were previously measured. After measurement, the plants were again cut to 5.0-10.0 cm to simulate the grazing of the stockpiled forage.

Winter survivability (WS) was determined by the percentage of the crown that survived from the previous fall; therefore, the measure was specific to each crown size.

These were visually scored by three or four trained researchers. Each researcher's values were averaged together into a single value for each plant/clone for all locations. The values were scored in increments of 10% with 0% being a completely dead plant and 100% indicating survival of the entire crown. Additionally, 1% was included as some individuals had only three or fewer tillers surviving. This measurement was taken in late April at all locations.

DNA Extraction

From each progeny, young leaf tissue from a clonal copy kept in the greenhouse was collected and placed in a 2-ml tube and immediately frozen in liquid nitrogen. Each sample was ground to fine powder using a Mixer Mill Type MM 300 (Retsch, Hann, Germany). The DNA was extracted using a DNeasy® Plant Mini-prep DNA Extraction Kit (QIAGEN Inc. Valencia, CA) according to manufacturer's instructions with the following modifications. The extraction solution used was: 500 µl AP1 lysis buffer, 5 µl RNase A, 165 µl AP2 buffer and 120 µl AE. DNA concentrations were quantified using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

High throughput SSRs and STS genotyping

Tall fescue TF-EST-SSRs (NFFA), tall fescue Genomic SSRs (NFFG), conserved grass EST-SSRs (CNL) and meadow fescue MF-EST-SSRs (NFMF) were used to screen the parental lines for polymorphism. Additionally, STS (sequence-tagged site) markers were developed from tall fescue ESTs that had high similarity to orthologous CBF genes from wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*), and perennial ryegrass (*Lolium perenne*); these are termed CTG (Table 4.1). TF-EST-SSRs, Genomic SSRs and MF-EST-SSRs were developed at the Noble

Foundation. Forward primers were modified by M13 tail at the 5' end. This allows concurrent fluorescence labeling of all PCR products by a third primer with an incorporated fluorophore (Schuelke, 2000). The final reaction volume of the PCR was 10 µl, which contained 15 ng of template DNA, 0.1 µl of 10 µM reverse primer, 0.25 µl of 10 µM forward primer, 3.0 mM $MgCl_2$, 2.5 mM of each dNTPs, 1 µM M13 fluorescent dye (Applied Biosystems, Foster City, CA) and 0.09 units of *Taq* DNA polymerase with 1x PCR Buffer (GeneScript corp. Piscataway, NJ). All of the primer combinations were first screened on the parents to identify the polymorphic primer pairs (PPs). The selected PPs were then screened on a subset of six to ten F_1 progenies from the population to select markers for mapping the whole population. Some of the polymorphic PPs with non-specific amplifications and/or too faint products as well as markers that amplified in all or none of the progenies were discarded from the final population assay.

PCR reactions were completed using an Applied Biosystems (Foster City, CA) Geneamp 9700 thermocycler. The following touch-down PCR program was used for all primer sets; 95^{o}C (180 s) followed by 6 cycles of 94^{o}C (45 s), 68^{o}C (5 min), and 72^{o}C (60 s) with the annealing temperature reduced by 2^{o}C per cycle then 8 cycles of 94^{o}C (45 s), 58^{o}C (120 s) and 72^{o}C (60 s) with the annealing temperature reduced by 1^{o}C per cycle, followed by 25 cycles of 94.0^{o}C (45 s), 50^{o}C (120 s) and 72^{o}C (60 s) with a final extension of 72^{o}C (7 min). The PCR products were read by an Applied Biosciences (ABI) 3730 fluorescence analyzer using the size standard LIZ®™ 500 (Applied Biosystems). The amplified PCR products were analyzed using ABI Prism Gene Scan Analysis (ver. 2.1, Applied Biosystems). The Genescan tracefiles were imported and analyzed by GeneMapper 3.0 and 3.5 software (Applied Biosytems).

DArTFest Genotyping

Diversity Array Technology (DArT) http://www.diversityarrays.com/ markers were assayed in the mapping population by DArT. The DArTFest array, which are allele specific markers, were created from five species from the *Festuca-Lolium* complex. These species include: *F. pratensis, F. arundinacea, F. glaucescens, L. perenne and L. multiflorum* (Kopecký et al., 2009a). These markers are designated by "loPt" followed by the clone ID. A total of 108 progenies were targeted for DArTFest genotyping. The DNA of progenies (10 µg) was shipped to DArT 1 Wilf Crane Cr., Yarralumla, ACT 2600, Australia and the genotyping scores (1 and 0) were received.

Linkage Analysis and Map Construction

The SSR and STS markers with alleles segregating in one or both parents were analyzed in 189 F_1 progenies. The DArTFest marker genotypic data from 108 progenies were then integrated. The DArtFest array analyzed six additional progeny which were not present in the SSR and STS marker screen. With these maker data combined a total of 195 progeny were used for linkage map analysis and construction. Both of the parents were used to define their respective coupling LGs using JOINMAP 4.0 (van Ooijen, 2006). Construction of the linkage map was accomplished by treating the segregating data as a cross pollinator (CP). After constructing the LOD grouping trees, LGs were selected with a LOD grouping threshold of 3.0 and above. The calculation of the linkage maps utilized all pair-wise recombination estimates of less than 0.499 and a LOD score greater than 0.01 (ripple value = 1, jump threshold = 5, triple threshold = 7). Map distances were calculated using the Kosambi mapping function (Kosambi, 1944). Two parental maps were first constructed with markers segregating in a 1:1 ratio. Markers

present in both parents that segregated in a 3:1 ratio were later included to find the homologous LGs in the two parental maps. The homologous group nodes of two parental maps were selected and the integrated LGs were constructed using the map integration command under the MAP tab.

QTL Analysis

Fall growth and WS data were used to map QTL on the constructed male and female linkage maps using MapQTL Version 5.0 (van Ooijen, 2004). Trait and map data used for QTL analysis were as described previously. Kruskal-Wallis, Interval mapping and Multiple QTL Mapping (MQM) were executed. Automatic co-factor selection option was used with the backward elimination procedure to adjust for other segregating QTL in the genetic effect x residual variance to identify significant ($P < 0.02$) cofactors to use during the MQM procedure (Jansen and Stam, 1994). Logarithm of the odds (LOD) threshold values ($\alpha = 0.05$) were determined by the average LG length and the average marker density (van Ooijen, 1999). These values were compared to empirically derived LOD scores in MapQTL using 1,000 permutations of the mapping and used to declare the QTLs. The additive allele effect and the percentage of variance explained by each QTL were obtained from the MQM model. QTLs were named according to the nomenclature proposed by McCouch et al. (1997) in the form of q-trait-year-female/male map linkage group.

Statistical Analysis

The population of 200 plants was arranged at each location in a randomized complete block design (RCBD) (Steel and Torrie, 1980). To ensure all trait data was normal each trait from each year and location was analyzed using PROC UNIVARIATE

using SAS statistical software (SAS Version 9.2, SAS Inst. Inc.). Some of the trait data (WI-WS-YR2 and MO1-WS-YR2) were unable to be normalized and the raw data was used to determine significance. To determine if locations and years were different all trait data, both normalized and raw data, were analyzed using PROC GLM.

RESULTS and DISCUSSION

Molecular markers

Altogether four sets of SSR markers (CNL, NFFA, NFFG, and NFMF) were used to construct the parental linkage maps. The SSR markers NFFA, NFFG, and NFMF were the primary SSRs used for creating both parental linkage maps. The female parent, 103-2, had 56, 29, and 31 markers of NFFA, NFFG, and NFMF scored, respectively, that segregated in a 1:1 or 3:1 ratio. Of those scored markers, only 35, 21, and 15 could be mapped for each respective marker group (Table 4.2). The NFFG marker class provided the most loci per PP with an average of 1.5 loci per PP while NFFA and NFMF averaged 1.2 and 1.3 loci per PP, respectively. The male parent, R43-64, was able to map substantially more SSRs than that of the female parent. Like 103-2, the NFFA marker group was the largest with 163 scored markers followed by NFFG group with 137 scored markers. The NFMF class was the smallest group which only consisted of 65 scored markers. However, only 151, 132, and 55 markers of NFFA, NFFG, and NFMR, respectively, were used to create the parental map for R43-64 (Table 4.3). Again, NFFG marker type provided the most loci per PP with an average of 2.0 followed by NFFA with 1.9 and NFMF with 1.6 loci per PP. Additionally, two CNL markers were used in the linkage map construction with an average of 1.0 loci per PP. If all loci, which include 1:1, 3:1, and distorted markers, are considered for both parents NFFG markers would average

3.6 loci per PP while NFFA and NFMF had mean values of 3.3 and 2.6 loci per PP, respectively.

The STS-CTG class of markers was developed from orthologous CBF sequences from other grass species. Although these markers were gene based they were not constructed to specifically amplify a single product. Therefore, these markers may not only amplify CBF homologues but genomic DNA that has similar sequence properties. A total of nine CTG markers were scored on one or both parental genotypes with an average of 2.4 loci per PP. However, of those nine, only six segregated in a 1:1 or 3:1 ratio and were used for mapping purposes. Between the parental genotypes, six loci were mapped in the female parent and seven mapped to the male parent. The female and male linkage maps averaged 1.5 and 1.6 loci per PP, respectively.

The remaining class of markers was from the DArTFest. These markers formed the largest group in both parental genotypes. The female parent contained 169 unique DArTFest markers that segregated in either a 1:1 or 3:1 ratio. Of those 169 markers 103 were mapped to 20 linkage groups. The male parent contained 267 markers that segregated in either a 1:1 or 3:1 ratio with 207 mapped to 20 linkage groups. An additional 261 DArTFest markers were identified; however, these were distorted from the normal 1:1 or 3:1 ratio and were not used for mapping in either parent.

Here we have shown, for the first time, the use and mapping of DArTFest markers in tall fescue. This platform has been used successfully to genotype individuals as well as for generating genetic maps of across various species of monocots (Akbari et al., 2006; Mace et al., 2008; Wenzl et al., 2004; Wenzl et al., 2006; White et al., 2008). For the creation of this array, five species were used and each species was comprised of 40

accessions from mapping populations, cultivars, and ecotypes (Kopecký et al., 2009a). The primary advantage of this DArTFest array is the number of markers that can be generated with a limited amount of tissue as well as the rapid speed at which the data can be generated. A total of 7,680 probes were created during the development of this array. However, among the 40 tall fescue genotypes only 2638 probes could be scored with only 512 markers that appeared to be polymorphic. One drawback to using this array is the probes are not always consistent between runs with some probes working while others do not. This was the case between our analysis and that of Kopecký et al. (2009a). In our population 1066 unique probes were identified and 805 probes were in common between our study and that of Kopecký et al. (2009a).

Additionally, this is the third tall fescue map after Xu et al. (1995) and Saha et al. (2005) using PCR markers and the second time the construction of a parental map using SSRs for R43-64. The first time a map of R43-64 was constructed, only 153 SSRs were used with a majority of the markers being amplified fragment length polymorphisms (AFLPs) (Saha et al., 2005). However, their percentage of SSRs mapped was much higher (84%), while due to the high number of distorted markers, we only mapped 60% of the polymorphic SSR markers. The use of SSRs is advantageous due to their ability to transfer across both species and even genera (Peakall et al., 1998; Roa et al., 2000; Gaita´ n-Solı´s et al., 2002), This is also is evident in this study by mapped SSRs from meadow fescue (*Lolium pratensis* [Huds.] Darbysh = *Festuca pratensis* [Huds.]) or DArTFest markers from other *Festuca* and *Lolium* species. This has allowed the identification of homeologous regions or even entire chromosomes across species.

Marker segregation, Parental Map Construction and Integration

Due to tall fescue's allohexaploid nature, alleles are inherited in a disomic manner. Based on Mendelian inheritance marker products will segregate in either a 1:1 (testcross) or a 3:1 (dominant) pattern. All markers that segregated from either one or both parents were scored and used to construct a linkage map. A total of 1,522 markers were scored between the two parents. Of the 1,522 markers, 1,215 (80%) were scored as segregating from either 103-2 or R43-64 (Aa x aa or aa x Aa); the remaining 20% were segregating in both parental genotypes (Aa x Aa). Parental genotype R43-64 had more markers segregating in the population than 103-2; 714 (59%) vs. 501 (41%), respectively (Table 4.4). From each category, every marker locus was confirmed to segregate in either a 1:1 or 3:1 using a chi-square test ($P < 0.05$). Only 683 (45%) of the total markers scored segregated in a 1:1 ratio. Of those 683 markers, R43-64 had the largest contribution with 517 (76%) while 103-2 only had 166 (24%). There were only 125 (8%) markers that segregated from both parents in a 3:1 ratio. Together, markers segregating in a 1:1 and 3:1 only comprised 53% of the total markers scored. The remaining 714 (47%) markers showed segregation distortions. A majority of the distorted markers originated from 103-2 which had 335 markers (47%) while R43-64 had only 197 (28%) of the 714 markers. The parental genotypes shared 182 (25%) of the skewed markers. None of the distorted markers were included in the construction of the parental maps due to improper LG assembly.

For the construction of the map for each parental genotype only the markers that segregated in a 1:1 ratio were used. Later, the addition of the markers segregating in a 3:1 ratio was used to construct the final linkage map. In total, only 82 SSRs and 109

DArTFest/STS based markers segregating in a 1:1 or 3:1 were used out of the 808 scored markers for the 103-2 parental map (Table 4.4). The remaining 100 markers were not able to be mapped potentially due to scoring errors, proximity to other markers, or absence of a segregation pattern. A total of 517 markers were unused due to having a distorted segregation pattern; 299 were SSRs and 218 DArTFest/STS based markers. The 191 markers that were mapped created 23 linkage groups ranging in size from 1.5 to 132.8 cM with an average of 58.8 cM (Figure 4.2). However, the total map length was only 1,373 cM. The marker density was quite low averaging only 8.8 markers per LG with 7.19 cM marker^{-1}.

In contrast to the Mediterranean (103-2) linkage map, the Continental (R43-64) parental linkage map contained 349 SSRs and 214 DArTFest/STS based markers out of the total 1,021scored markers. This left only 79 (12%) of the markers unmapped (Table 4.5). Also, R43-64 did not produce the magnitude of distorted markers as was seen with 103-2. R43-64 only had 379 (37%) distorted markers of the total markers scored of which 212 were SSR and 167 were DArTFest/STS based markers. The 563 mapped markers generated 20 LGs. The size of the LGs ranged from 44.6 to 134.6 with an average of 97 cM (Figure 4.3). The entire map length for R43-64 was 1,939 cM. Unlike the 103-2 linkage map, the marker density was much higher with an average of 28.2 markers per LG and a density of 3.44 cM marker^{-1} (Table 4.5).

The distribution of each marker class used for the linkage map creation overall is homogenous. The DArTFest markers have the greatest distribution covering 20 LGs for 103-2 and all LGs for R43-64 (Table 4.6). The NFFA, NFFG, and NFMF markers covered 18, 19, and 16 LGs for R43-64 map, respectively. For 103-2 NFFA markers were

identified in 15 out of 23 LGs while only 11 and 10 LG had NFFG and NFMF markers, respectively. The CTG markers in both maps clustered together with 2 groups found on LGs 1 and 19 for 103-2 and 2 groups on LGs 5 and 9 for R43-64. However, there are some LGs for each parental genotype that are dominated by one marker class. The DArTFest markers were the largest group having more than 66% of markers in the LGs 1, 3, 5, 15, 21 and 22 for 103-2. This was also observed on LGs 9, 12, 17, and 20 for R43-64 where DArTFest markers composed 58% or more of the markers. Additionally, the NFMF markers were observed to have high densities on LGs 5 and 15 for R43-64 and 8 and 9 for 103-2.

To construct the integrated linkage map, markers that segregated in a 3:1 ratio and mapped in both parental lines were used to identify and join complementary LGs. A minimum of two markers are necessary to join homologous LGs. Surprisingly, out of the 125 markers that segregated in a 3:1 ratio only 28 markers were mapped in both parental genotypes. Out of the total 43 LGs from both parental genotypes, only 14 LG, 7 LGs from each parent, could be integrated into 7 consensus LGs (Figure 4.4). The remaining LGs from the parental genotypes either had no markers that segregated in a 3:1 ratio, or only one of the parents contained a 3:1 marker. In the integrated map the DArTFest had the largest portion of markers (40%), followed by NFFA, NFFG, NFMF, CTG and CNL. (Table 4.7). On LGs 2 and 4 there were a large number of NFMF class markers however, the other LGs contained only a small portion of these markers. The lengths of the seven integrated LGs varied between 47.7 and 145.7 cM, which is comparable to that of the first reported PCR tall fescue map (Saha et al., 2005). However, the overall length was only 632.2 cM due to each parents remaining LGs inability to be integrated. Lastly, the

marker density was similar to that of Saha et al. (2005) with an average of 3.0 cM marker^{-1}.

The abundance of markers that segregate in a 1:1 ratio or were only donated from one of the parental lines indicate that those regions are in a heterozygous state while being homozygous recessive or absent in the other parent. The markers that are heterozygous in both parental genotypes (3:1) are typically used to identify the homologous linkage groups from each of the parents (Maliepaard et al., 1998). In the previous tall fescue linkage map of Continental x Continental cross, 20% of the markers segregated from both parents (Saha et al., 2005), which was similar to this study at 18%. However, we were only able to map 28 of those loci while Saha et al. (2005) mapped 99. This allowed them to identify 37 LGs from both parental maps, and construct 17 integrated LGs. However, from our 28 common markers only 14 LGs from both parental maps were identified resulting in the construction of 7 integrated LGs. This indicates that these parental genotypes may be divergent and share few markers.

The distorted marker class was a large portion of our total markers scored. In general, the distorted markers make up only a small percentage of the total markers, however, in polyploidy this number can be higher. For instance in the first PCR marker based tall fescue linkage map, the distorted markers comprised only 20 to 24% of the total markers (Saha et al., 2005). This is similar to the first tall fescue linkage map (Xu et al., 1995). In maize, it has been observed that distorted markers could comprise up to 36% of the total markers, although the average was 17% (Lu et al., 2002). Interestingly, in an interspecific cross between tomato species *Lycopersicon esculentum* × *L. chmielewskii*, the distorted markers reached 68% (Paterson et al., 1988). Our results

appear similar to Paterson et al. (1988) where nearly 64% of all markers were distorted for 103-2 and 37% of those for R43-64. This discrepancy between parents has also been observed in maize where one parent had 55% of the total distorted markers while the other parent had 36% (Lu et al., 2002). In this tall fescue population these distorted markers would usually be present or absent in nearly all 200 progenies with typically fewer than 20 showing segregation. Additionally, evidence suggests that segregation distortion and restricted recombination are often found in the genomes of interspecific hybrid populations (Paterson et al., 1988; Arnold, 1997). This can be due to the accumulation of mutations by the individual species prior to their hybridization (Rieseberg et al., 1995). This high proportion of distorted markers may indicate that this cross between Mediterranean and Continental tall fescue genotypes is an interspecific cross like that of the wide tomato cross.

It is assumed if a gene that causes segregation distortion is segregating in the mapping population, then markers that map close to its location would also display distorted ratios (Zamir and Tadmor, 1986). Other reasons thought to cause these distorted markers are the result of gametic selection and/or faulty chromosome pairing, an association between heterozygosity and plant vigor or the selection of one parental type (Xu et al., 1995). When we attempted to add the distorted markers to the framework map, all distorted makers were grouped into one large linkage group for both parents. Typically, when distorted markers are added to a linkage map with the informative markers (1:1) correctly ordered, the addition of distorted markers is negligible. However if the marker loci are not arranged correctly on our linkage map, the following QTL analysis will not be valid (De-qiang et al., 2007). Because of this issue we did not map

any of the distorted markers. It is observed that when distorted markers are mapped they usually are grouped together (De-qiang et al., 2007; Lu et al., 2002; Saha et al., 2005 and 2009). Interestingly, the 3:1 markers grouped as clusters which would normally be found for distorted markers. This occurred primarily in the parental genotype 103-2 where entire LGs (13, 15, 18, 21) were composed of markers segregating in a 3:1 ratio.

Regardless of the distorted markers, the map length and marker densities were quite good for R43-64. The map create for R43-64 in this study had a total length of 1,939 cM which was similar to that of Saha et al. (2005) where they reported a total length of 1,722 cM for R43-64 in a different mapping population. However, they observed a higher marker density at 2.97 cM marker^{-1} compared to ours at 3.44 cM marker^{-1}. Although it appears that there are differences between these two maps, it is not uncommon for linkage maps to have different overall lengths. This can be attributed to the variation in the number of recombination events that occurred between these populations. Also, the number of markers used for mapping can influence the overall length.

In contrast to the Continental parent, the overall length of 103-2 map was smaller by 566 cM which was similar to the first tall fescue map constructed by Xu et al. (1995). This difference in length between the parental lines cannot be accounted for by recombination events alone. There were 372 fewer markers used in the construction of 103-2 linkage map which is likely one of the causative factors. The majority of these markers were unusable due to their segregation distortion as mentioned earlier. As suggested earlier, this may be due to faulty chromosome pairing as a result of different genome structure between the Mediterranean and Continental genotypes. This faulty

paring might be a consequence of the unequal representation of the G_1 and G_2 genomes or possibly a different genetic constitution. It was observed by Kopecký et al. (2009b) that the G genomes regularly paired when hexaploid *F. arundinacea* was crossed with diploid species. Therefore, a pairing control system might be located on one of the *F. glaucescens* genomes.

Another indication that these two types of tall fescue are distinct species is the fact that only seven common linkage groups were identified. This could be the result of these two types sharing only the P genomes. Evidence of this lies in the marker data provided by the NFMF SSR and DArTFest markers. The NFMF markers derived from the meadow fescue were found in higher proportions on particular linkage groups of both parental genotypes. These LGs may be the P genomes. For R43-64 the two linkage groups that contained the highest proportion of NFMF markers were used in the creation of the integrated linkage map. Also, the LG that contained the greatest number of NFMF markers for 103-2 was also integrated into the consensus map. The addition of more NFMF markers should elucidate this matter further. Additionally, when looking at the DArTFest marker data of the genotype Fa-35 from Morocco http://bioinf.scri.ac.uk/germinate_grasses/, a potential Mediterranean tall fescue genotype, it contained 78 unique markers identified for the *F. arundinacea* probes that appear to be unique to this accession only when compared to the other 39 genotypes (Kopecký et al., 2009a). These unique markers were associated 10 times higher with the Mediterranean parent 103-2 markers than for R43-64. Lastly, the Mediterranean tall fescue sequence variations of nuclear and chloroplast genomes were more similar to *F. mairei* than *F. glaucescens* (Saha, personal communication).

QTLs for Fall Growth and Winter Survival

The phenotypic traits used for QTL analysis had a wide range at all locations each year. Fall growth, as measured by LER, for the fastest growing genotype was approximately three times greater than that for the slowest growing genotype at all locations for each year (Table 4.8; Figures 4.5 and 4.6). At all locations, R43-64 was always in the bottom 5 individuals for FG while 103-2 was consistently in the top half, except for YR1 at the WI location. Depending on the daily temperatures when the measurements were taken R43-64 had a range in FG of 1.5 to 4.0 mm d^{-1} across years and locations. 103-2 also had the same variability during the same time with values ranging from 2.9 to 10.1 mm d^{-1}. For the FG trait several individuals in the population appeared to have transgressive segregation with FG rates greater than 103-2, however this was not observed on the low end where individuals were consistently lower than R43-64.

Winter survival was greatly influenced by the environmental conditions unique to each location. For instance, at the WI location during YR1, the winter was unusually snow free and colder than normal with soil temperatures dropping to -13^{o}C. The combination of these conditions killed all the plants. However, at this same location in YR2, continuous snow cover resulted in a moderation of the soil temperatures as well as the temperature surrounding the plants. This resulted in F_1 progeny survival rates ranging from 32% to 100%. Winter survival at the MO1 location was not as dynamic between years but ranged from 0 to 90% in YR1 and 0 to 97% in YR2 for the F_1 progeny. The MO2 location was only present during YR2 and had the most moderate winter temperatures of all three locations, however this location had no snow cover resulting in

poorer WS than that at the WI location. At MO2 location, the range for WS was from 10 to 97%. For WS, 103-2 always had the lowest survival rate while R43-64 always had an average survivability of 96% or greater. These results showed that the Continental genotype had a better WS than the Mediterranean. Unlike the FG values no individuals were significantly greater than the best parent R43-64 or worse than the worst parent 103-2. This may be due to the fact that in nearly all locations and years 103-2 had nearly 0% survival while R43-64 was almost always at 100%.

Nearly all of the QTL for FG and WS between years and locations were located at the same positions on the same LGs between parental maps. The Mediterranean map, 103-2, had few QTLs for the FG and WS traits. For FG, plants grown at the MO1 location did not have any QTLs for either year (Table 4.9). At both the WI location for YR1 and the MO2 location for YR2 only one QTL was identified on LG1. Additionally, the MO2 location identified two QTL for WS which were both located on LG1. The LOD values were 3.9 to 10.6 with the percent variation ranging from 33 to 43%. For the R43-64 parental line, QTL were identified for FG and WS primarily on LG13 and on LG20 (Table 4.10). One additional QTL was located on LG3 for WS at the MO2 location. These QTL had LOD scores ranging from 3.7 to 10.5. The percent variation these QTL accounted for had a wide range, 2.6 to 45.4%, but the majority was around 30%.

It was quite surprising that only in YR1 at the WI location and YR2 at the MO2 location were significant QTLs on 103-2 LG1 for FG observed. However, due to a low marker density linkage map for 103-2, it may be that not all QTLs were identified, since some genomic regions are not yet defined. This may be the reason as to why no QTL

were detected at the MO1 location in both years of the study. Although 103-2 has higher FG, as measured by LER, the reason may not be completely based on the nuclear genome and may lie in part with the chloroplast's genome and its ability to function at lower temperatures. It has been observed that plants containing the M_1 and M_2 genomes had a higher apparent photosynthetic rate (Randal et al., 1985). Recently, the chloroplast genome of Mediterranean tall fescue 103-2 was found to be more similar to the *F. mairei* than the *F. glaucescens* chloroplast genome (Saha personal communication).

Many more QTL were identified for the R43-64 parental genotype. Both FG and WS were located predominately on LG13 of R43-64 (Figure 4.3) which is homologous to LG1 of 103-2 (Figure 4.2). These homologous LGs formed the integrated LG1 (Figure 4.4). Since only 7 LG were found in common, it seems that these traits may be on the P genomes which these fescue types share. Similar results were observed in an annual × perennial ryegrass interspecific hybrid population where the traits FG and WS were mapped to the same genomic regions on the same homologous LGs of the parental maps across years (Xiong et al., 2007). Similarly, it seems that the same genomic regions controlling FG are also responsible for WS in our population as well.

Although we were able to map the CTG markers, which were developed from homologous CBF sequence data, none of our traits mapped to those regions. It was shown in wheat and barley that major QTL controlling WS is located on chromosome 5H (Galiba et al., 1995; Francia et al., 2004). Francia et al. (2004) reported that the WS QTL explained between 21.5 to 36.6% of the variation in barley. Additionally, in barley winter survival and freezing tolerance QTLs were consistently found near the locations of CBF genes and other cold tolerance related genes (Skinner et al., 2006; Tondelli et al., 2006).

However, this was not observed in this study. Therefore, the CTG loci we identified are not responsible for enhancing WS because they may not be the CBF genes of tall fescue or they are CBF genes, but do not play a role in WS.

CONCLUSIONS

We present for the first time a linkage map of Continental × Mediterranean tall fescue with SSR, STS and DArTFest markers. The map coverage and marker density was encouraging in the Continental parent. However, the Mediterranean parental map needs further improvement. In this study FG and WS QTLs were identified on homologous LGs from the female (103-2) and male (R43-64) maps across years and locations. These QTLs appear to be stable over a wide range of environmental conditions and would be desirable for marker-assisted selection (MAS). Therefore, these QTL have the potential to assist future tall fescue breeding programs in both Continental and Mediterranean germplasm. The construction of this linkage map and the detection of these QTL responsible for FG and WS traits impart unique information regarding the genomics and genetics of these tall fescue types.

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Table 4.1. STS markers termed CTG used for mapping in parental lines 103-2 and R43-64. STS markers were developed from homologous CBF sequences from related monocot species.

Parental Genotype[a]	STS Marker[b]	Tall Fescue EST	Forward[c] and Reverse Primer Sequences
103-2 and R43-64	CTG009	DT688788F	TGTAAAACGACGGCCAGTGGAGAACATCCTCCTTGTCAGC
		DT688788R	GCTCTGTCGACCTGATACACTCG
R43-64	CTG010	DT695008F	TGTAAAACGACGGCCAGTTCGACAAGAGGAGGAGGGAAGG
		DT695008R	GAAGGTGCCCAGCCAGATGC
103-2 and R43-64	CTG012	DT696484F	TGTAAAACGACGGCCAGTCACGGAAAGCAGAGGATATGG
		DT696484R	CATGACCATGGGGAAGTAAGG
103-2 and R43-64	CTG019	DT708315F	TGTAAAACGACGGCCAGTGCCGAGAAGAAGAAGATCATGG
		DT708315R	CTTCAGCTCATTGTTCTGAGTCG
103-2 and R43-64	CTG023	DT712249F	TGTAAAACGACGGCCAGTCGCTGGTTGTTTTGTACCTAAGC
		DT712249R	CCAGAAAGAGGAAAGTTGACAGC
R43-64	CTG035	TC4337F	TGTAAAACGACGGCCAGTCATCTTCCGGTACAAGGACACC
		TC4337R	CTAGACTCTGGACGCACACAGC

[a] The parental genotypes where the primers amplified genomic sequence and used in the construction of the parental linkage maps

[b] The STS marker name given to the specific tall fescue EST sequence

[c] The forward primers have a 18 bp M-13 tail at the 5' end

Table 4.2. SSR, STS, and DArTFest array primers used for the construction of the Mediterranean parent (103-2, ♀) map of the tall fescue population.

SSRs			DArTFest Array Markers[d]	STS Based Markers[e]
Tall Fescue EST-SSRs[a]	Tall Fescue Genomic-SSRs[b]	Meadow Fescue EST-SSRs[c]		
nffa030, nffa034	nffg042	nfmf016	loPt-355747, loPt-355767, loPt-355843, loPt-355880	ctg009
nffa059, nffa073	nffg043	nfmf027	loPt-355943, loPt-355984, loPt-356006, loPt-356011	ctg012
nffa075, nffa134	nffg079	nfmf060	loPt-356024, loPt-356051, loPt-356077, loPt-356149	ctg019
nffa155, nffa169	nffg106	nfmf068	loPt-356289, loPt-356316, loPt-356369, loPt-356404	ctg023
nffa206, nffa346	nffg158	nfmf075	loPt-555068, loPt-555127, loPt-555201, loPt-555211	
nffa386, nffa387	nffg170	nfmf088	loPt-555249, loPt-555301, loPt-555630, loPt-555867	
nffa605, nffa632	nffg171	nfmf093	loPt-555889, loPt-556014, loPt-556018, loPt-556073	
nffa634, nffa635	nffg197	nfmf120	loPt-556292, loPt-556391, loPt-556516, loPt-556552	
nffa649, nffa660	nffg240	nfmf171	loPt-556564, loPt-556628, loPt-556644, loPt-556698	
nffa666, nffa684	nffg253	nfmf178	loPt-556792, loPt-556801, loPt-556897, loPt-556964	
nffa711, nffa721	nffg273	nfmf189	loPt-556981, loPt-556985, loPt-557119, loPt-557120	
nffa722, nffa723	nffg324	nfmf191	loPt-557126, loPt-557292, loPt-557371, loPt-557383	
nffa743, nffa772	nffg391	nfmf192	loPt-557411, loPt-557434, loPt-557444, loPt-557538	
nffa773, nffa777	nffg423	nfmf194	loPt-557699, loPt-557711, loPt-557734, loPt-557878	
nffa785, nffa787	nffg464	nfmf210	loPt-557979, loPt-558034, loPt-558220, loPt-558264	
nffa791, nffa802	nffg483	nfmf218	loPt-558444, loPt-558624, loPt-558690, loPt-558731	
nffa870		nfmf219	loPt-559712, loPt-559748, loPt-559777, loPt-559778	
		nfmf227	loPt-559780, loPt-559978, loPt-560015, loPt-560349	
			loPt-560491, loPt-560493, loPt-560563, loPt-560680	
			loPt-560941, loPt-561039, loPt-561062, loPt-561075	
			loPt-561082, loPt-561210, loPt-561212, loPt-561224	
			loPt-561248, loPt-561279, loPt-561295, loPt-561361	
			loPt-561364, loPt-561497, loPt-561622, loPt-561752	
			loPt-561844, loPt-562235, loPt-562269, loPt-562316	
			loPt-562318, loPt-562343, loPt-562376, loPt-562495	
			loPt-562625, loPt-562648, loPt-562685	

[a] Tall fescue TF-EST-SSRs; Noble Foundation, USA

[b] Tall fescue (Genomic-SSRs); Noble Foundation, USA

[c] Meadow fescue EST-SSRs; Noble Foundation, USA

[d] DArtFest array markers

[e] STS markers developed from tall fescue ESTs homologous to grass specific CBF genes

Table 4.3. SSR, STS, and DArtFest array based primers used for the construction of the Continental parent (R43-64, ♂) map of the tall fescue population.

SSRs				DArTFest Array Markers[e]	STS Based Markers[f]
Conserved grass EST-SSRs[a]	Tall Fescue EST-SSRs[b]	Tall Fescue Genomic-SSRs[c]	Meadow Fescue EST-SSRs[d]		
cnl039	nffa002, nffa015	nffg006, nffg009	nfmf002	loPt-355729, loPt-355738, loPt-355814, loPt-355852	ctg010
cnl151	nffa030, nffa031	nffg017, nffg025	nfmf016	loPt-355937, loPt-355938, loPt-356038, loPt-356044	ctg012
	nffa034, nffa040	nffg026, nffg042	nfmf029	loPt-356082, loPt-356084, loPt-356086, loPt-356369	ctg019
	nffa043, nffa059	nffg043, nffg079	nfmf032	loPt-356378, loPt-356383, loPt-555080, loPt-555085	ctg023
	nffa088, nffa112	nffg101, nffg106	nfmf041	loPt-555092, loPt-555101, loPt-555104, loPt-555122	ctg035
	nffa129, nffa155	nffg107, nffg111	nfmf050	loPt-555127, loPt-555144, loPt-555159, loPt-555171	
	nffa205, nffa206	nffg135, nffg140	nfmf055	loPt-555201, loPt-555210, loPt-555249, loPt-555270	
	nffa233, nffa234	nffg147, nffg156	nfmf064	loPt-555298, loPt-555301, loPt-555346, loPt-555435	
	nffa247, nffa289	nffg157, nffg158	nfmf080	loPt-555472, loPt-555477, loPt-555517, loPt-555551	
	nffa322, nffa346	nffg163, nffg164	nfmf087	loPt-555584, loPt-555590, loPt-555608, loPt-555623	
	nffa366, nffa376	nffg167, nffg170	nfmf091	loPt-555630, loPt-555641, loPt-555672, loPt-555690	
	nffa383, nffa386	nffg171, nffg175	nfmf094	loPt-555703, loPt-555707, loPt-555746, loPt-555749	
	nffa387, nffa599	nffg182, nffg197	nfmf099	loPt-555753, loPt-555780, loPt-555787, loPt-555797	
	nffa602, nffa607	nffg202, nffg203	nfmf100	loPt-555804, loPt-555827, loPt-555839, loPt-555864	
	nffa610, nffa611	nffg209, nffg219	nfmf103	loPt-555865, loPt-555874, loPt-555879, loPt-555899	
	nffa617, nffa619	nffg236, nffg239	nfmf106	loPt-555905, loPt-555946, loPt-555955, loPt-555957	
	nffa621, nffa622	nffg240, nffg253	nfmf120	loPt-555972, loPt-555982, loPt-556002, loPt-556018	
	nffa623, nffa628	nffg258, nffg260	nfmf130	loPt-556032, loPt-556047, loPt-556091, loPt-556114	
	nffa632, nffa634	nffg264, nffg273	nfmf131	loPt-556127, loPt-556134, loPt-556147, loPt-556160	
	nffa635, nffa648	nffg282, nffg305	nfmf144	loPt-556183, loPt-556196, loPt-556198, loPt-556215	
	nffa649, nffa650	nffg324, nffg325	nfmf171	loPt-556233, loPt-556241, loPt-556277, loPt-556305	
	nffa654, nffa658	nffg330, nffg331	nfmf172	loPt-556313, loPt-556333, loPt-556340, loPt-556358	
	nffa660, nffa663	nffg354, nffg377	nfmf176	loPt-556404, loPt-556405, loPt-556410, loPt-556441	
	nffa681, nffa688	nffg389, nffg391	nfmf178	loPt-556461, loPt-556468, loPt-556480, loPt-556516	
	nffa689, nffa691	nffg392, nffg405	nfmf186	loPt-556542, loPt-556552, loPt-556566, loPt-556580	
	nffa692, nffa694	nffg413, nffg416	nfmf191	loPt-556628, loPt-556644, loPt-556658, loPt-556697	
	nffa695, nffa698	nffg423, nffg457	nfmf194	loPt-556698, loPt-556700, loPt-556728, loPt-556778	
	nffa709, nffa715	nffg464, nffg480	nfmf200	loPt-556835, loPt-556840, loPt-556847, loPt-556848	
	nffa723, nffa730	nffg482, nffg483	nfmf202	loPt-556897, loPt-556936, loPt-556939, loPt-557071	
	nffa731, nffa738	nffg493, nffg497	nfmf209	loPt-557168, loPt-557204, loPt-557209, loPt-557228	
	nffa739, nffa743	nffg505, nffg506	nfmf210	loPt-557263, loPt-557280, loPt-557288, loPt-557330	
	nffa762, nffa774	nffg668, nffg672	nfmf214	loPt-557383, loPt-557385, loPt-557434, loPt-557464	
	nffa786, nffa787	nffg673, nffg673	nfmf220	loPt-557486, loPt-557515, loPt-557522, loPt-557559	
	nffa788, nffa789	nffg676, nffg677	nfmf228	loPt-557595, loPt-557645, loPt-557783, loPt-557828	
	nffa800, nffa802	nffg679	nfmf229	loPt-557878, loPt-557880, loPt-557916, loPt-557982	
	nffa803, nffa809		nfmf235	loPt-557989, loPt-558071, loPt-558269, loPt-558539	
	nffa812, nffa815		nfmf238	loPt-558568, loPt-558576, loPt-558584, loPt-558599	
	nffa821, nffa822			loPt-558622, loPt-558623, loPt-558624, loPt-558676	
	nffa826, nffa855			loPt-558690, loPt-558694, loPt-558710, loPt-558721	
	nffa859, nffa862			loPt-558725, loPt-558745, loPt-558841, loPt-558845	
	nffa862, nffa864			loPt-558878, loPt-558889, loPt-558891, loPt-559712	
	nffa868, nffa869			loPt-559750, loPt-560141, loPt-560316, loPt-560399	
	nffa875, nffa877			loPt-560491, loPt-560563, loPt-560565, loPt-560592	
				loPt-560758, loPt-560783, loPt-560869, loPt-560941	
				loPt-561089, loPt-561133, loPt-561188, loPt-561228	
				loPt-561275, loPt-561361, loPt-561366, loPt-561473	
				loPt-561671, loPt-561700, loPt-561740, loPt-561835	
				loPt-561853, loPt-561879, loPt-561926, loPt-562019	
				loPt-562032, loPt-562084, loPt-562125, loPt-562199	
				loPt-562235, loPt-562301, loPt-562342, loPt-562376	
				loPt-562401, loPt-562440, loPt-562512, loPt-562516	
				loPt-562648, loPt-562657, loPt-562691	

[a] Conserved grass EST-SSRs; Cornell University, USA

[b] Tall fescue TF-EST-SSRs; Noble Foundation, USA

[c] Tall fescue (Genomic SSRs); Noble Foundation, USA

[d] Meadow fescue EST-SSRs; Noble Foundation, USA

[e] DArtFest array markers

[f] STS markers developed from tall fescue ESTs homologous to grass specific CBF genes

Table 4.4. Summary of the Mediterranean tall fescue parent 103-2♀ and the Continental tall fescue parent R43-64♂ markers used to create parental linkage maps. Data shown for markers that segregate in a 1:1 or 3:1 ratio or that were distorted but were present in one (1:1) or both (3:1) parental genotypes.

Segregation	103-2	R43-64	Total
Non-Distorted			
1:1	166	517	683
3:1	125	125	125
Distorted			
1:1	335	197	532
3:1	182	182	182
Total	808	1021	1522

Table 4.5. Summary of the Mediterranean tall fescue parent 103-2♀ and the Continental tall fescue parent R43-64♂ markers used to create parental linkage maps. Data shown for SSRs, DArT/STS, and total markers used for mapping are markers that segregate in a 1:1 or 3:1 ratio. Data shown for total markers scored include all distorted makers in addition to the 1:1 and 3:1 markers.

Parameters	103-2	R43-64
SSRs		
	116	
Scored	[28][a]	367 [63]
Mapped	82 (71)[b]	349 (95)
Unlinked	34 (29)	18 (5)
DArT Array/STS		
Scored	175 [45]	275 [62]
Mapped	109 (62)	214 (78)
Unlinked	66 (38)	61 (22)
Total Markers Used for Mapping		
Scored	291 [36]	642 [63]
Mapped	191 (66)	563 (88)
Unlinked	100 (34)	79 (12)
Distorted Markers		
SSRs	299 [72]	212 [37]
DArT Array/Gene	218 [55]	167 [38]
Total	517 [64]	379 [37]
Total Markers Scored		
SSRs	415	579
DArT Array/Gene	393	442
Total	808	1021
Linkage analysis		
Linkage groups	23	20
Map Length (cM)	1373	1939
Marker density (cM/marker)	7.19	3.44

[a] Numbers in brackets indicate the percentage of the total markers scored for that specific category (i.e SSR or DArT)

[b] Numbers in parenthesis indicate the percentage of the markers mapped within that specific category

Table 4.6. Distribution of tall fescue (NFFA, NFFG), conserved grass (CNL), meadow fescue (NFMF) EST-SSR and array (DArT) and gene based (CTG) marker loci in different linkage groups of the two parental maps.

Linkage group	Parental Maps													
	103-2							R43-64						
	CNL[a]	NFFA[b]	NFFG[c]	NFMF[d]	DArT[e]	CTG[f]	Total	CNL	NFFA	NFFG	NFMF	DArT	CTG	Total
1	0	2	1	1	27	2	33	0	19	19	0	19	0	57
2	0	5	1	1	5	0	12	0	7	11	1	6	0	25
3	0	1	1	1	6	0	9	0	6	12	4	12	0	34
4	0	5	4	1	0	0	10	0	8	10	1	6	0	25
5	0	1	0	0	5	0	6	0	20	6	16	10	2	54
6	0	1	0	0	1	0	2	0	15	5	3	19	0	42
7	0	0	0	2	2	0	4	0	16	7	2	13	0	38
8	0	2	1	6	5	1	15	1	8	6	2	18	1	36
9	0	0	3	4	7	0	14	0	0	1	1	12	3	17
10	0	5	3	0	6	0	14	0	2	6	2	6	0	16
11	0	4	0	0	2	0	6	0	6	13	0	4	0	23
12	0	4	0	0	0	0	4	0	2	3	3	11	0	19
13	0	0	0	0	3	0	3	1	12	13	1	15	0	42
14	0	1	5	0	5	0	11	0	9	5	3	3	0	20
15	0	0	1	0	7	0	8	0	4	2	10	14	0	30
16	0	3	0	1	1	0	5	0	4	4	5	7	0	20
17	0	0	0	1	2	0	3	0	0	2	5	13	0	20
18	0	0	0	0	3	0	3	0	7	4	1	7	0	19
19	0	1	1	0	3	3	8	0	6	5	0	6	1	18
20	0	0	2	1	0	0	3	0	1	0	0	7	0	8
21	0	0	0	0	6	0	6	-	-	-	-	-	-	-
22	0	1	0	0	4	0	5	-	-	-	-	-	-	-
23	0	4	0	1	2	0	7	-	-	-	-	-	-	-
Total	0	40	23	20	102	6	191	2	152	134	60	208	7	563

[a] Conserved grass EST-SSRs; Cornell University, USA

[b] Tall fescue TF-EST-SSRs; Noble Foundation, USA

[c] Tall fescue (Genomic SSRs); Noble Foundation, USA

[d] Meadow fescue EST-SSRs; Noble Foundation, USA

[e] DArtFest array markers

[f] STS markers developed from EST sequence data that were homologous to CBF genes in grasses

Table 4.7. Distribution of the marker types, map length and marker density for the integrated LGs. Integrated LGs and parental origin are shown.

Linkage Groups	Parental Groups		Origin of Marker Loci							Map Length (cM)	Marker Density (cM marker^{-1})
	103-2	R43-64	CNL[a]	NFFA[b]	NFFG[c]	NFMF[d]	DArT[e]	CTG[f]	Total		
1	1	13	1	14	14	2	33	2	66	145.7	2.2
2	8	5	0	21	7	19	14	3	64	114.5	1.8
3	6	8	1	9	6	2	18	1	37	115.6	3.1
4	15	15	0	4	2	10	14	0	30	73.6	2.5
5	22	1	0	20	19	0	19	0	58	135.1	2.3
6	18	20	0	1	0	0	7	0	8	47.7	6.0
7[g]	7	16	-	-	-	-	-	-	-	-	-
Total/Aver.	7	7	2	69	48	33	105	6	263	632.2	3.0

[a] Conserved grass EST-SSRs; Cornell University, USA

[b] Tall fescue TF-EST-SSRs; Noble Foundation, USA

[c] Tall fescue (Genomic SSRs); Noble Foundation, USA

[d] Meadow fescue EST-SSRs; Noble Foundation, USA

[e] DArtFest array Markers

[f] STS markers developed from EST sequence data that were homologous to CBF genes in grasses

[g] Linkage Group 7 from 103-2 and 16 from R43-64 share one marker in common which is not enough to join these LG into 1 consensus group therefore no data is presented.

Table 4.8. Mean and range of fall growth (FG) and winter survival (WS) for the parents and progenies in the Mediterranean and Continental mapping population in the two years of the study across all locations (WI, MO1, and MO2).

Source/ Location	YR1 (2008-2009)		YR2 (2009-2010)	
	FG (mm d^{-1})	WS (%)	FG (mm d^{-1})	WS (%)
R43-64				
WI [a]	1.5		4.0	97.0
MO1	1.1	100.0	3.8	98.8
MO2			1.8	96.7
103-2				
WI	3.4		8.7	44.0
MO1	2.9	0.0	10.1	0.0
MO2			4.1	7.3
Mean[b]				
WI	4.9		8.2	87.3
MO1	3.0	30.3	7.5	50.8
MO2			3.1	69.4
Range[c]				
WI	0.5-8.5		4.7-12.3	31.7-100
MO1	1.1-4.8	0.0-90.0	4.6-12.0	0.0-96.9
MO2			1.7-5.0	10.3-96.7

[a] Arlington, WI (WI), Columbia, MO (MO1), Mt. Vernon, MO (MO2)
[b] This is the mean of the population with the parental genotypes excluded
[c] This is the range of the population with the parental genotypes excluded unless their values are within the range

Table 4.9. Putative QTL controlling fall growth (FG) and winter survival (WS) detected on the female map of 103-2 by multiple interval mapping (MQM) in the Mediterranean × Continental tall fescue population in YR1 (2008-2009) and YR2 (2009-2010) and combined over both years and all locations.

Name of QTL[a]	Linkage group	Support interval[b]	Nearest marker	Maximum LOD score	(%) Variation explained[c]
FG YR1 WI					
q-FG-YR1-f1	1	31.3-35.1	loPt-556698%	3.9	32.5
FG YR1 MO1					
None identified					
FG YR2 MO1					
None identified					
FG YR2 MO2					
q-FG-YR2-f1	1	34.29-37.13	loPt-557711	4.39	37.2
WS YR2 MO2					
q-FG-YR2-f1.1	1	26.00-33.33	loPt-559777	7.68	43.3
q-FG-YR2-f1.2	1	40.11-41.08	loPt-558624%	10.59	40.7

[a] The QTL nomenclature followed the rules described by McCouch et al. (1997) with the form of q-trait-year-female/male map linkage group. When there were more than one QTL for the same trait in the same map and same year, then different numbers were added as suffix at the end of QTL name to distinguish the QTL. For QTL detected on the traits by combining a 2-year data across all locations, the name was given by the form of q-trait-female/male map linkage group

[b] Support interval was determined by the interval that correspond to a LOD drop of 1.0 on each side of the maximum likelihood position

[c] Percentage of phenotypic variation explained by individual QTL

Table 4.10. Putative QTL controlling fall growth (FG) and winter survival (WS) detected on the male map of R43-64 by multiple interval mapping (MQM) in the Mediterranean x Continental tall fescue population in YR1 (2008-2009) and YR2 (2009-2010) and combined over both years and all locations.

Name of QTL[a]	Linkage group	Support interval[b]	Nearest marker	Maximum LOD score	(%) Variation explained[c]
FG YR1 WI					
q-FG-WI-YR1-m13	13	76.5-77.2	loPt-560941%	4.28	13.7
FG YR2 WI					
q-FG-WI-YR2-m13	13	63.2-68.5	loPt-355938	3.72	31.3
FG YR1 MO1					
q-FG-MO1-YR1-m13	13	72.3-75.8	nffg147-129	4.53	31.2
FG YR2 MO1					
q-FG-MO1-YR2-m13.1	13	12.4-20.3	nffg219-264	4.55	2.6
q-FG-MO1-YR2-m13.2	13	25.3-33.4	nffa715-471	5.26	2.9
q-FG-MO1-YR2-m20	20	33.6-35.6	loPt-561228	8.48	40.5
FG all locations YR1 and Y2					
q-FG-m13.1	13	63.2-66.2	loPt-355938	6.09	32.6
q-FG-m13.2	13	74.8	nffg147-129	8.21	28.6
q-FG-m20.1	20	6.0-14.0	loPt-555753	10.5	45.4
q-FG-m20.1	20	39.9-41.7	loPt-562235%	5.32	29.8
WS YR2 MO2					
q-WS-MO2-YR2-m3	3	40.7-42.7	nffa698-522	5.01	14.5
q-WS-MO2-YR2-m13	13	76.5-77.2	loPt-556018%	7.03	13.9

[a] The QTL nomenclature followed the rules described by McCouch et al. (1997) with the form of q-trait-year-female/male map linkage group. When there were more than one QTL for the same trait in the same map and same year, then different numbers were added as suffix at the end of QTL name to distinguish the QTL. For QTL detected on the traits by combining a 2-year data across all locations, the name was given by the form of q-trait-female/male map linkage group

[b] Support interval was determined by the interval that correspond to a LOD drop of 1.0 on each side of the maximum likelihood position

[c] Percentage of phenotypic variation explained by individual QTL

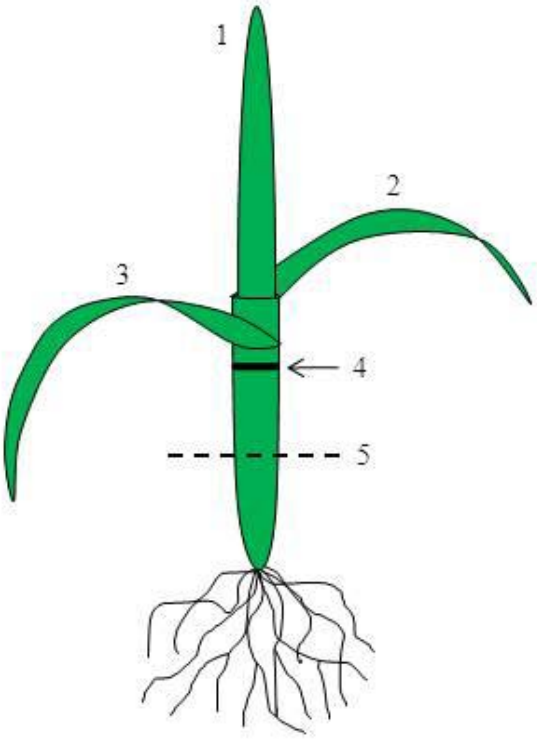
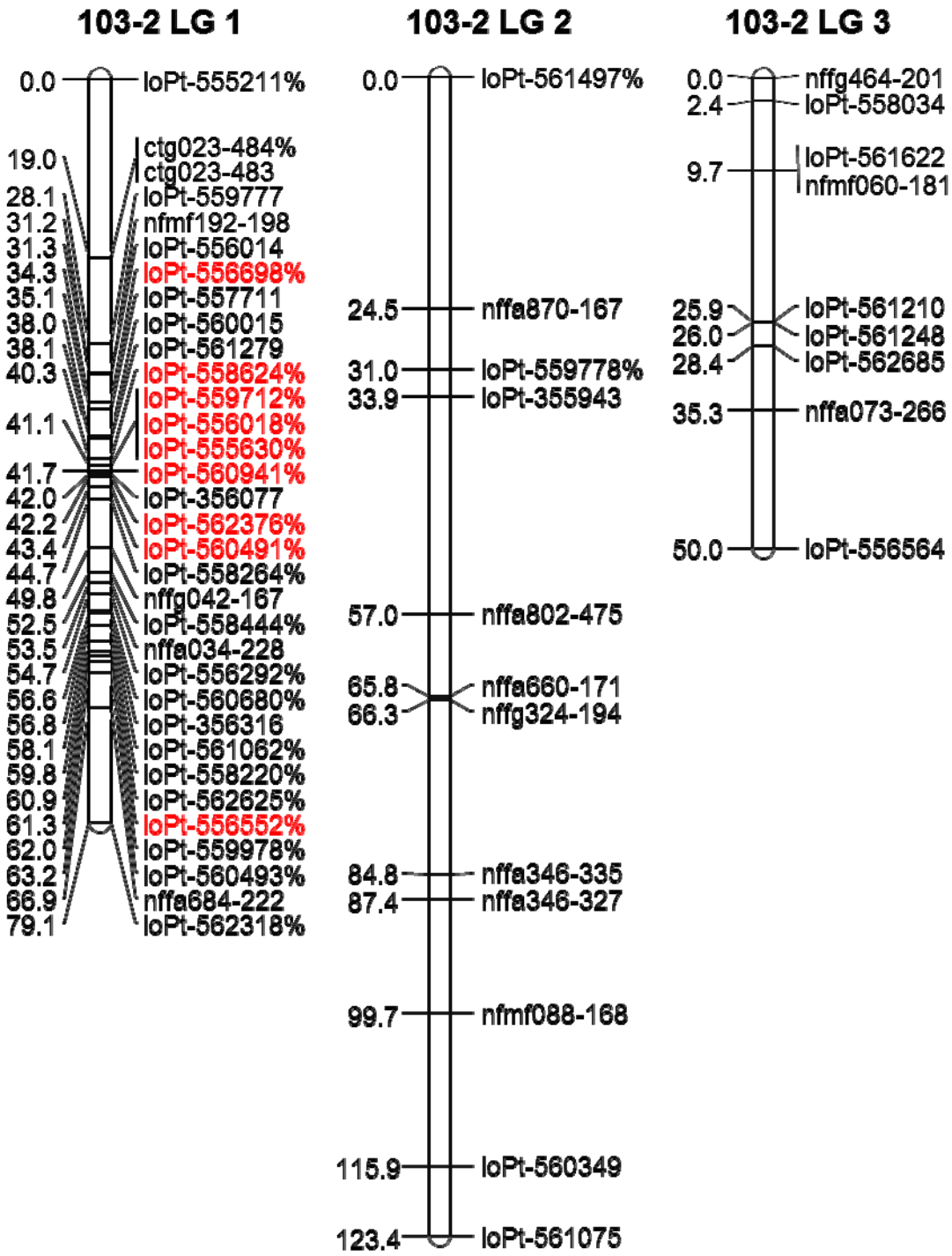
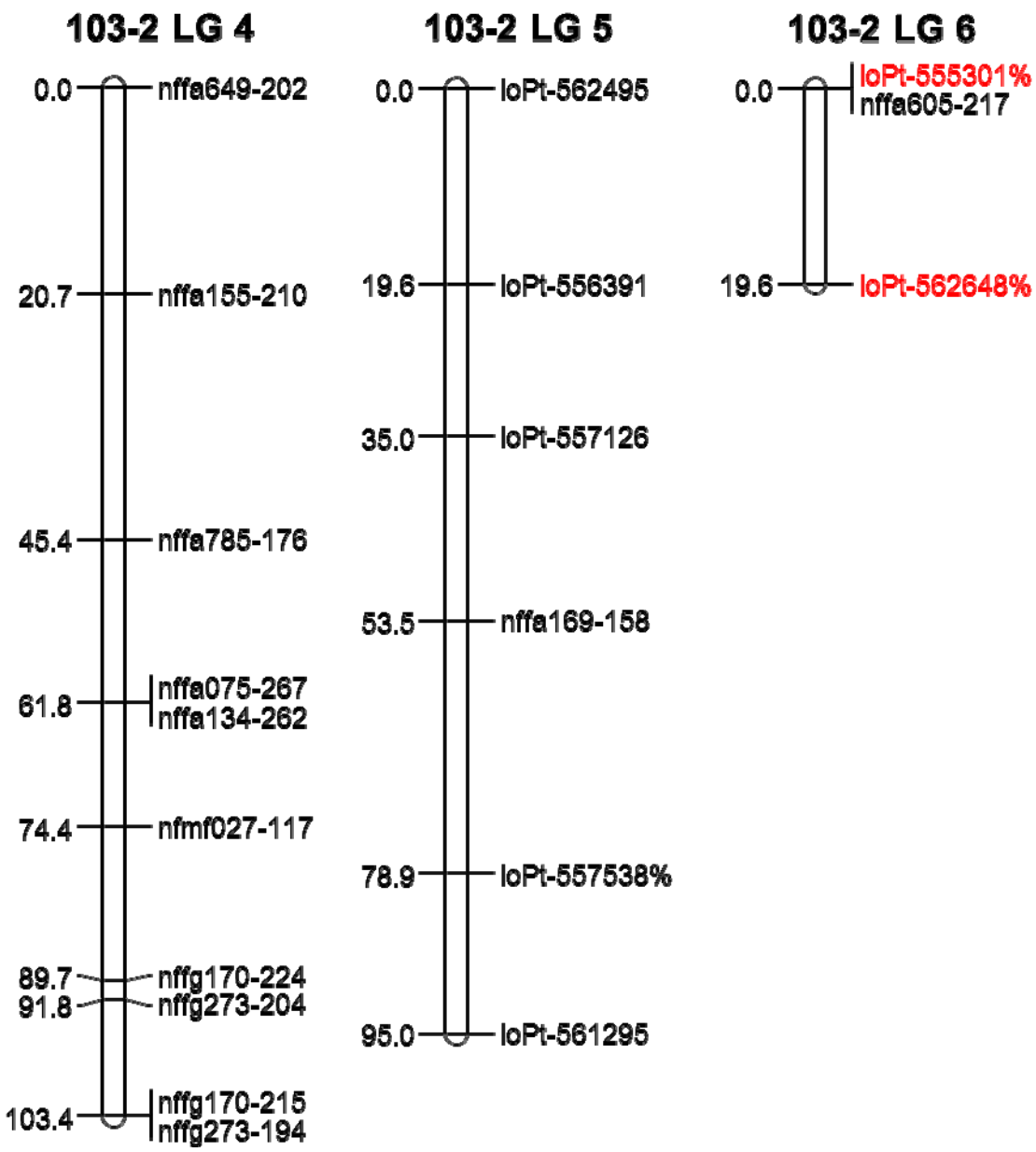
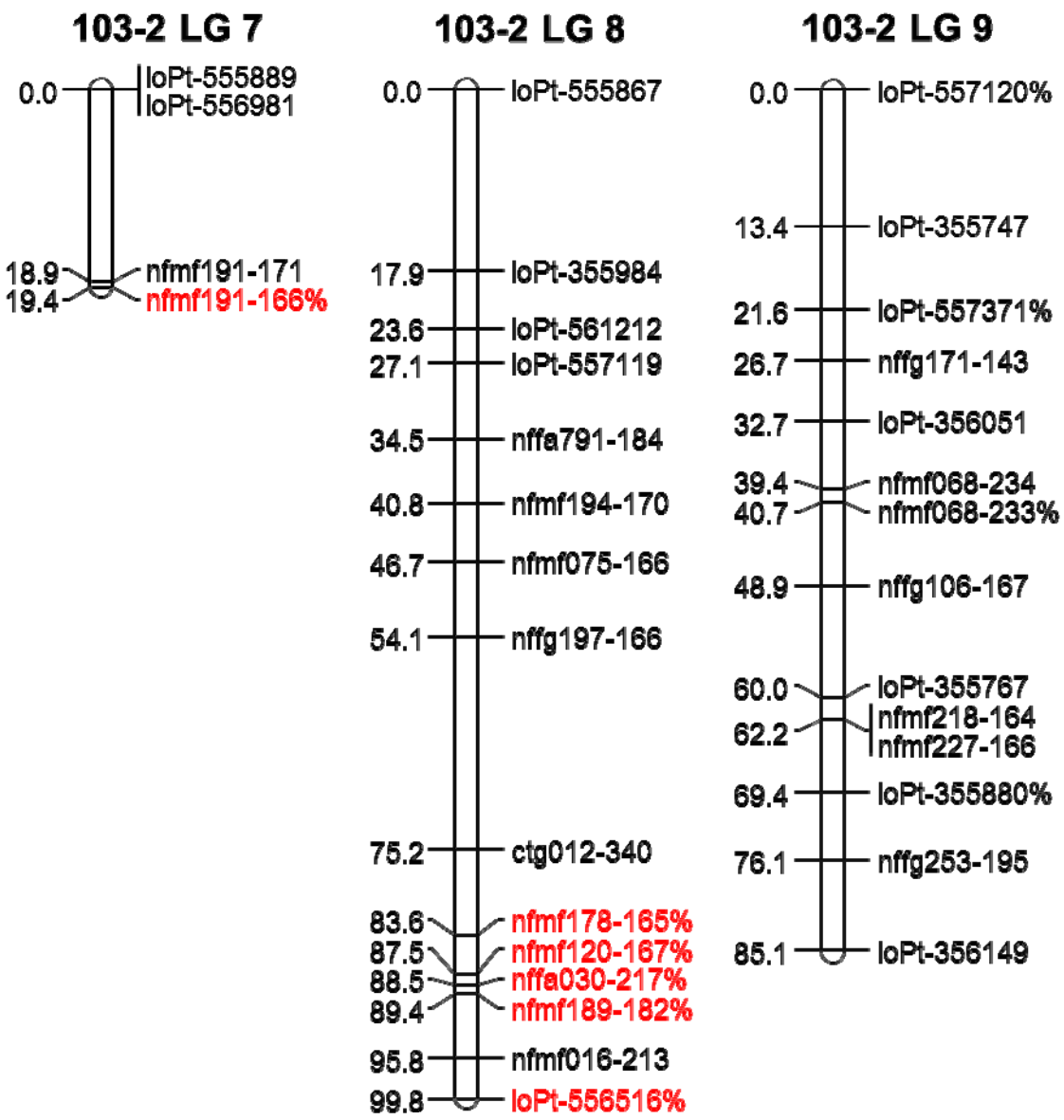
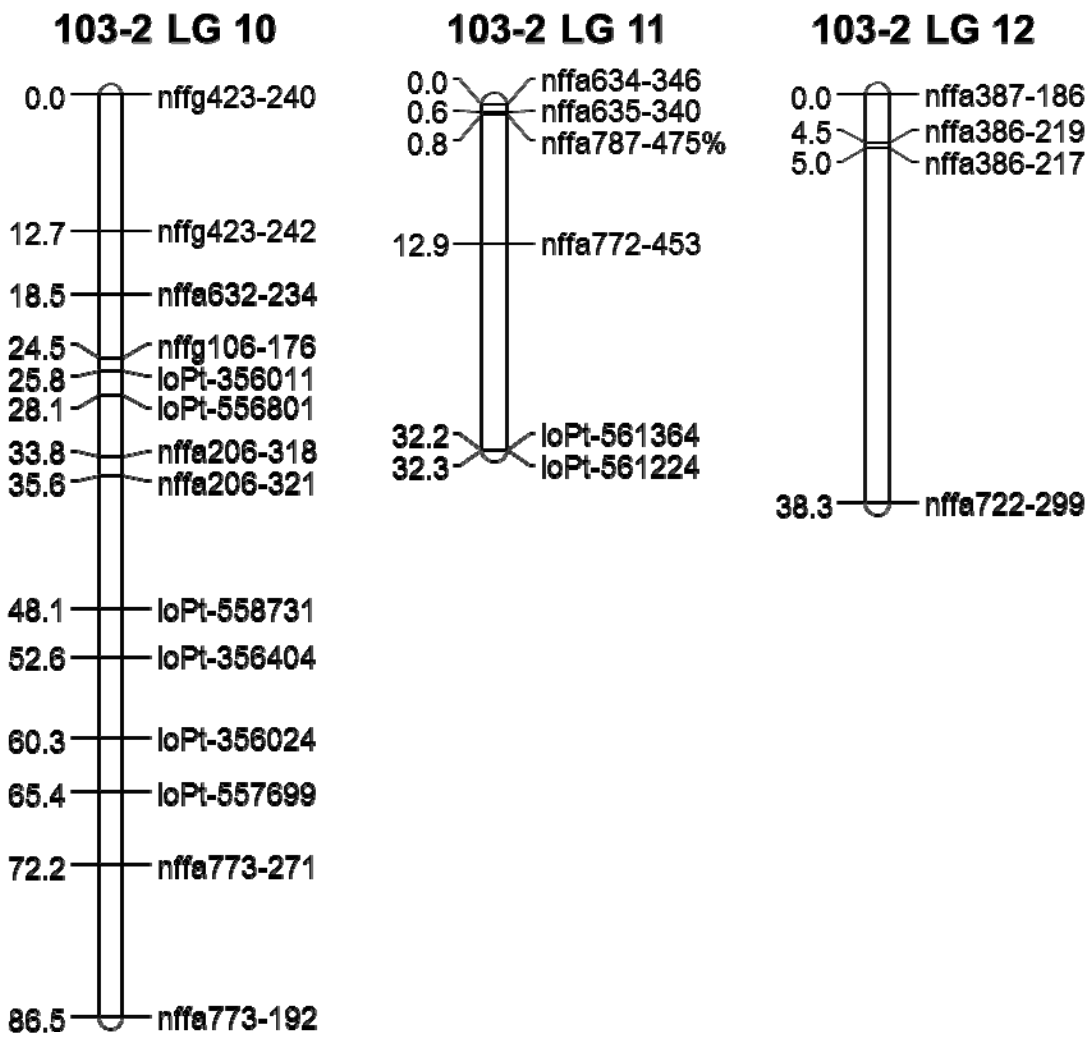


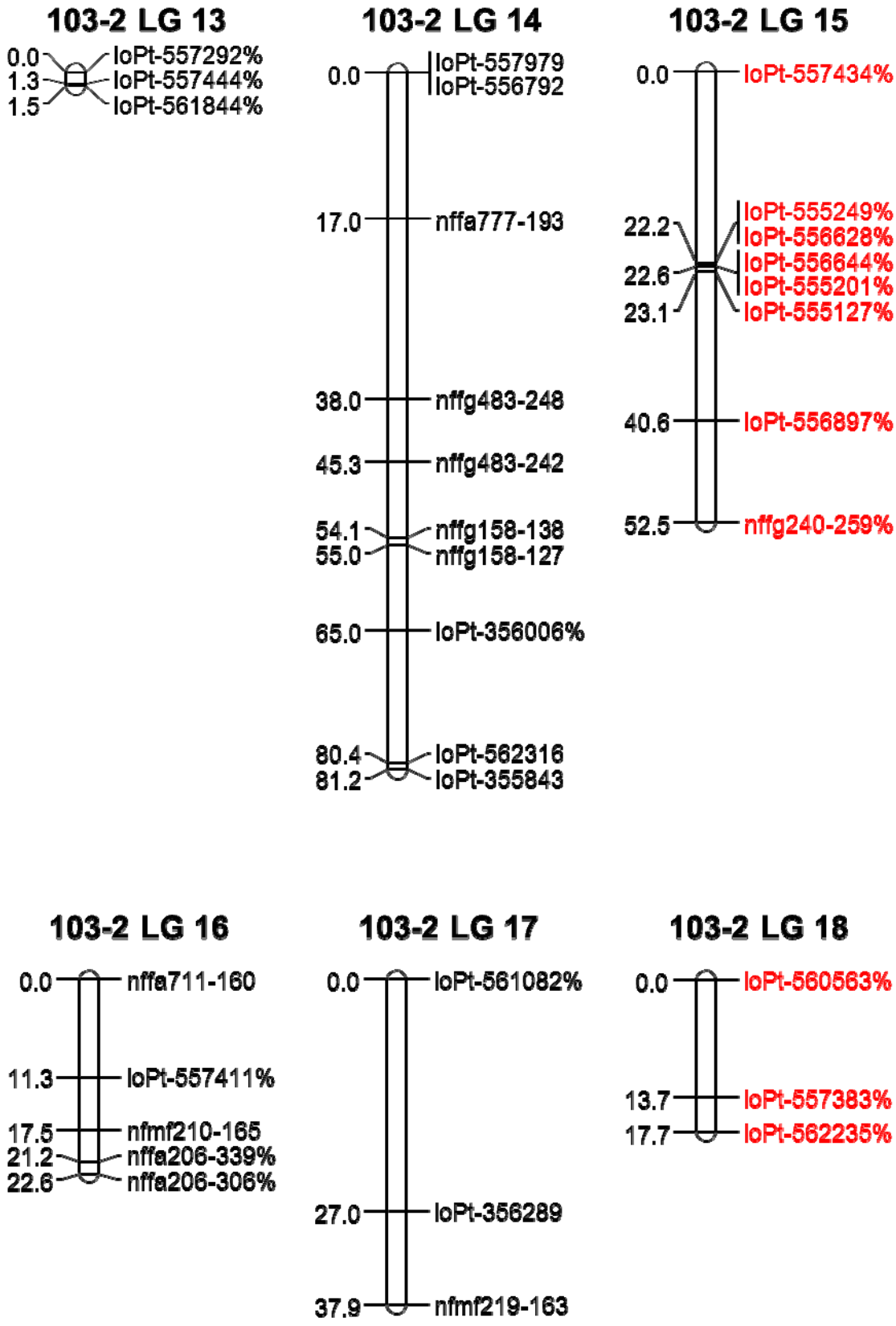
Figure 4.1. Schematic of a tall fescue tiller where 1) represents the newest emerging leaf, 2) the second newest leaf, 3) the third newest leaf, 4) the position of the reference point to measure LER, and 5) the cutting height.

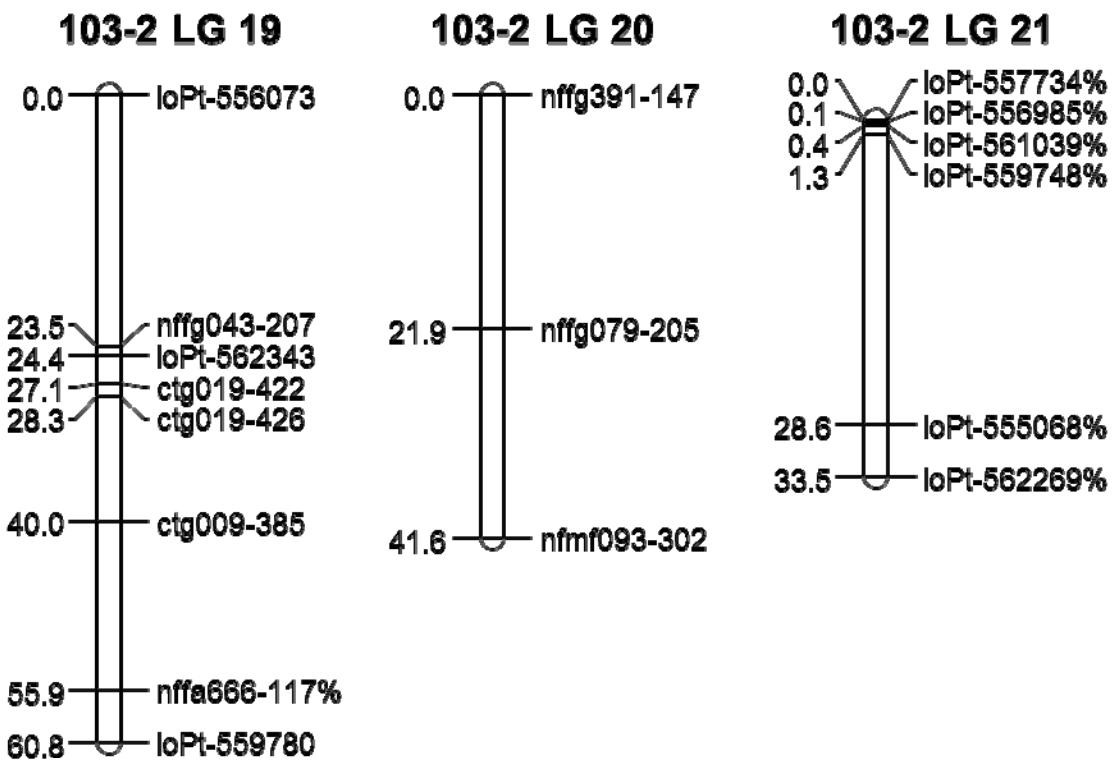

103-2 LG 1
0.0 loPt-555211%
19.0 ctg023-484%
ctg023-483
28.1 loPt-559777
31.2 nfmf192-198
31.3 loPt-556014
34.3 loPt-556698%
35.1 loPt-557711
38.0 loPt-560015
38.1 loPt-561279
40.3 loPt-558624%
41.1 loPt-559712%
loPt-556018%
loPt-555630%
41.7 loPt-560941%
42.0 loPt-356077
42.2 loPt-562376%
43.4 loPt-560491%
44.7 loPt-558264%
49.8 nffg042-167
52.5 loPt-558444%
53.5 nffa034-228
54.7 loPt-556292%
56.6 loPt-560680%
56.8 loPt-356316
58.1 loPt-561062%
59.8 loPt-558220%
60.9 loPt-562625%
61.3 loPt-556552%
62.0 loPt-559978%
63.2 loPt-560493%
66.9 nffa684-222
79.1 loPt-562318%
103-2 LG 2
0.0 loPt-561497%
24.5 nffa870-167
31.0 loPt-559778%
33.9 loPt-355943
57.0 nffa802-475
65.8 nffa660-171
66.3 nffg324-194
84.8 nffa346-335
87.4 nffa346-327
99.7 nfmf088-168
115.9 loPt-560349
123.4 loPt-561075
103-2 LG 3
0.0 nffg464-201
2.4 loPt-558034
9.7 loPt-561622
nfmf060-181
25.9 loPt-561210
26.0 loPt-561248
28.4 loPt-562685
35.3 nffa073-266
50.0 loPt-556564


103-2 LG 4
0.0
nffa649-202
20.7
nffa155-210
45.4
nffa785-176
61.8
nffa075-267
nffa134-262
74.4
nfmf027-117
89.7
nffg170-224
91.8
nffg273-204
103.4
nffg170-215
nffg273-194
103-2 LG 5
0.0
loPt-562495
19.6
loPt-556391
35.0
loPt-557126
53.5
nffa169-158
78.9
loPt-557538%
95.0
loPt-561295
103-2 LG 6
0.0
loPt-555301%
nffa605-217
19.6
loPt-562648%


103-2 LG 7
0.0
loPt-555889
loPt-556981
18.9
19.4
nfmf191-171
nfmf191-166%
103-2 LG 8
0.0
loPt-555867
17.9
loPt-355984
23.6
loPt-561212
27.1
loPt-557119
34.5
nffa791-184
40.8
nfmf194-170
46.7
nfmf075-166
54.1
nffg197-166
75.2
ctg012-340
83.6
87.5
88.5
89.4
nfmf178-165%
nfmf120-167%
nffa030-217%
nfmf189-182%
95.8
nfmf016-213
99.8
loPt-556516%
103-2 LG 9
0.0
loPt-557120%
13.4
loPt-355747
21.6
loPt-557371%
26.7
nffg171-143
32.7
loPt-356051
39.4
40.7
nfmf068-234
nfmf068-233%
48.9
nffg106-167
60.0
62.2
loPt-355767
nfmf218-164
nfmf227-166
69.4
loPt-355880%
76.1
nffg253-195
85.1
loPt-356149


103-2 LG 10
0.0 nffg423-240
12.7 nffg423-242
18.5 nffa632-234
24.5 nffg106-176
25.8 loPt-356011
28.1 loPt-556801
33.8 nffa206-318
35.6 nffa206-321
48.1 loPt-558731
52.6 loPt-356404
60.3 loPt-356024
65.4 loPt-557699
72.2 nffa773-271
86.5 nffa773-192
103-2 LG 11
0.0 nffa634-346
0.6 nffa635-340
0.8 nffa787-475%
12.9 nffa772-453
32.2 loPt-561364
32.3 loPt-561224
103-2 LG 12
0.0 nffa387-186
4.5 nffa386-219
5.0 nffa386-217
38.3 nffa722-299


103-2 LG 13
0.0
1.3
1.5
loPt-557292%
loPt-557444%
loPt-561844%
103-2 LG 14
0.0
17.0
38.0
45.3
54.1
55.0
65.0
80.4
81.2
loPt-557979
loPt-556792
nffa777-193
nffg483-248
nffg483-242
nffg158-138
nffg158-127
loPt-356006%
loPt-562316
loPt-355843
103-2 LG 15
0.0
22.2
22.6
23.1
40.6
52.5
loPt-557434%
loPt-555249%
loPt-556628%
loPt-556644%
loPt-555201%
loPt-555127%
loPt-556897%
nffg240-259%
103-2 LG 16
0.0
11.3
17.5
21.2
22.6
nffa711-160
loPt-557411%
nfmf210-165
nffa206-339%
nffa206-306%
103-2 LG 17
0.0
27.0
37.9
loPt-561082%
loPt-356289
nfmf219-163
103-2 LG 18
0.0
13.7
17.7
loPt-560563%
loPt-557383%
loPt-562235%


103-2 LG 19
0.0
loPt-556073
23.5
nffg043-207
24.4
loPt-562343
27.1
ctg019-422
28.3
ctg019-426
40.0
ctg009-385
55.9
nffa666-117%
60.8
loPt-559780
103-2 LG 20
0.0
nffg391-147
21.9
nffg079-205
41.6
nfmf093-302
103-2 LG 21
0.0
loPt-557734%
0.1
loPt-556985%
0.4
loPt-561039%
1.3
loPt-559748%
28.6
loPt-555068%
33.5
loPt-562269%

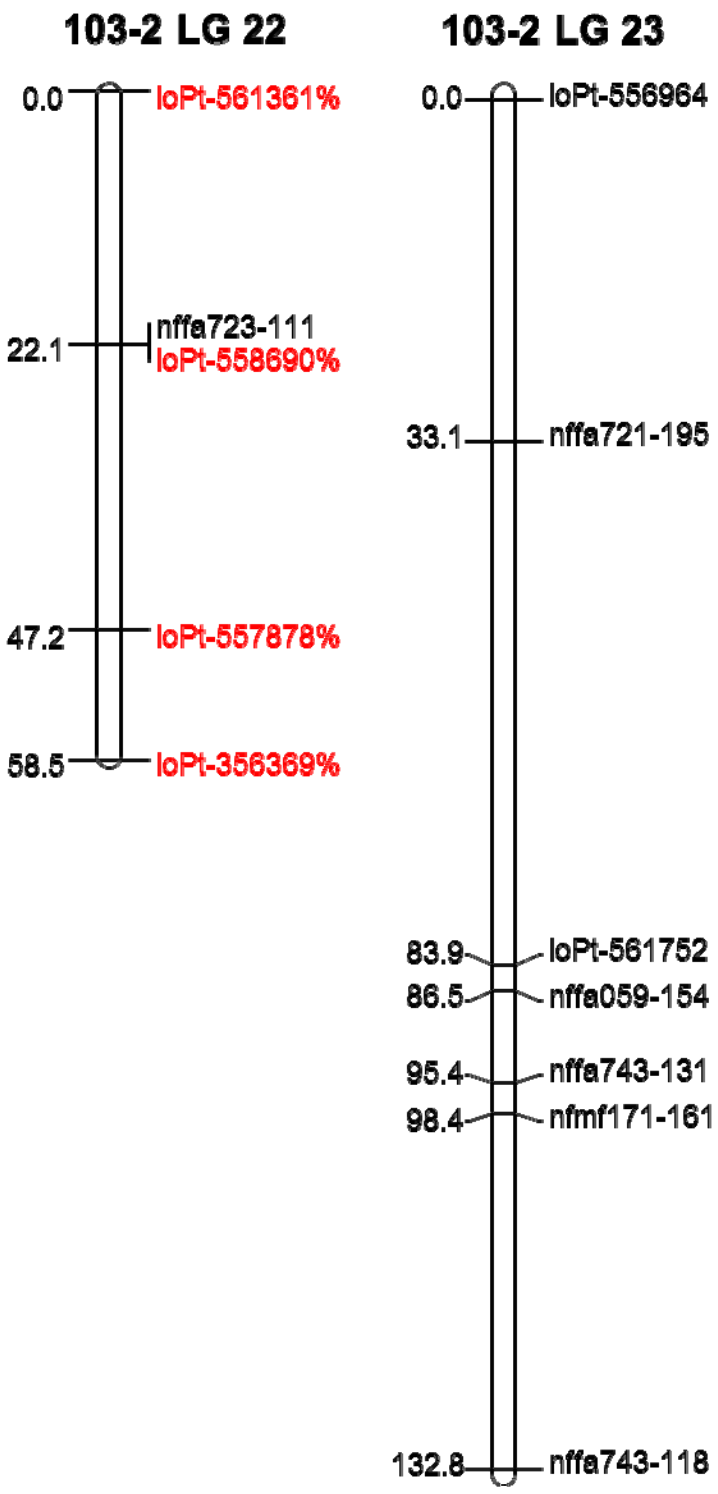
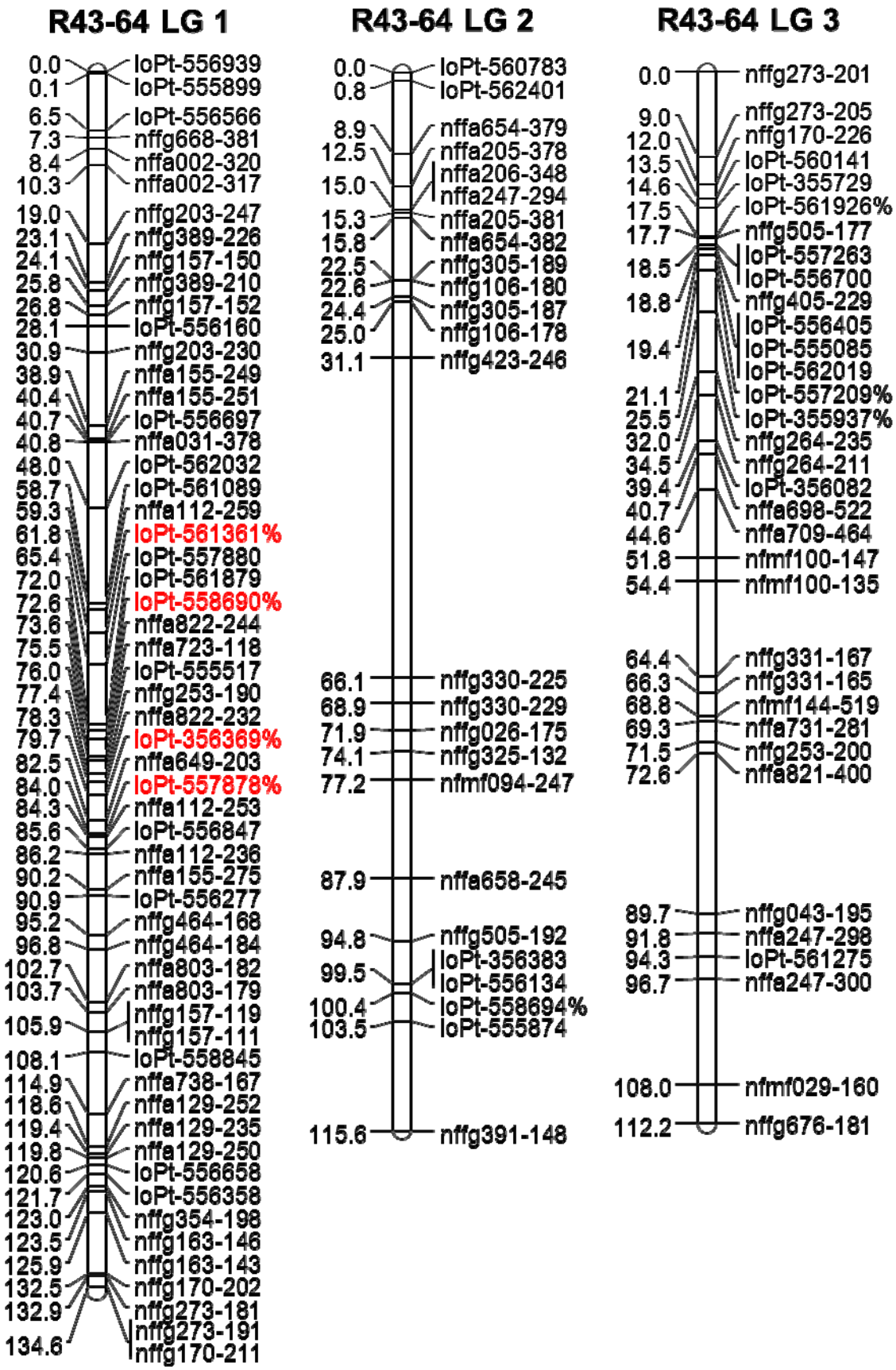
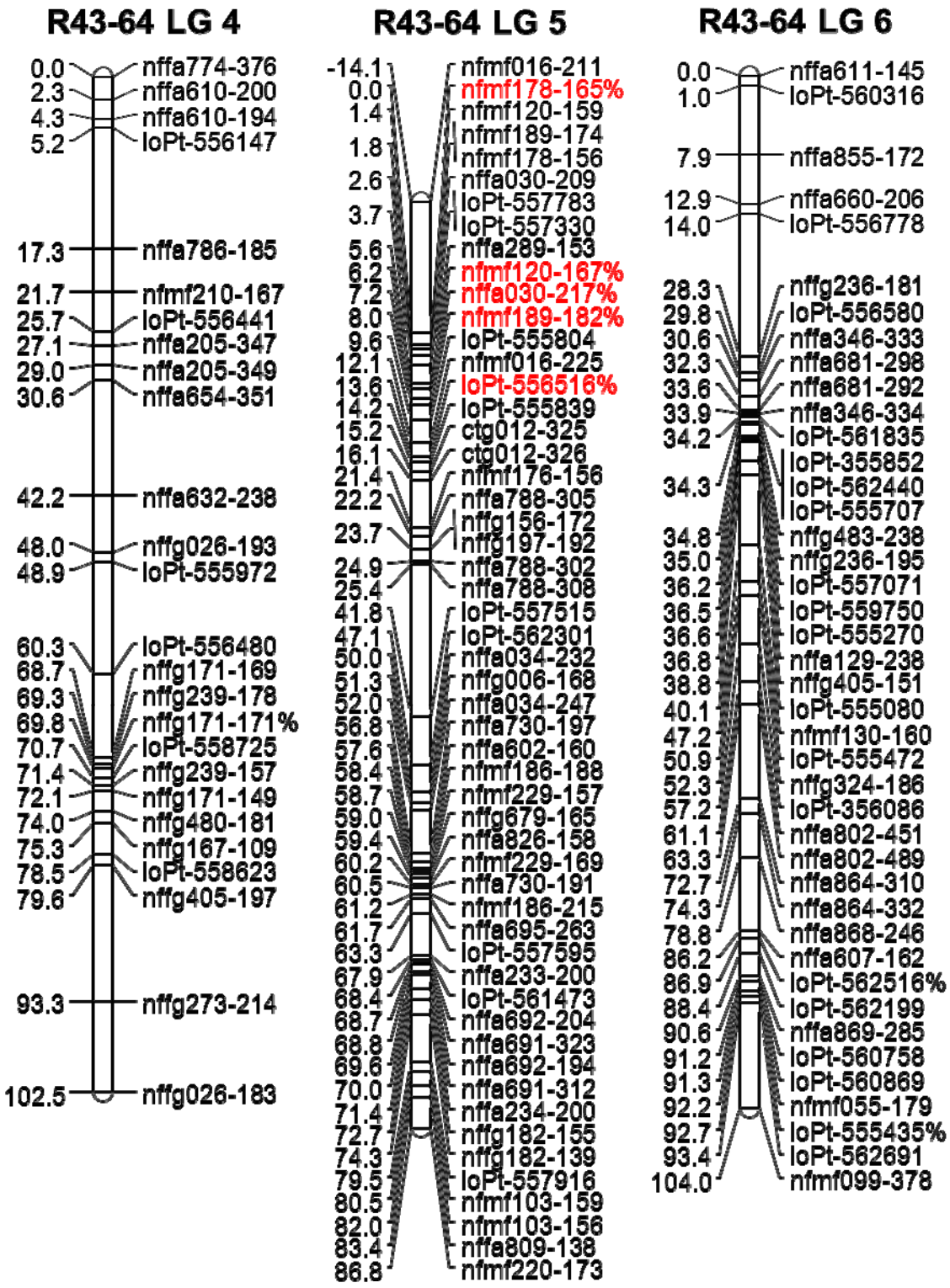
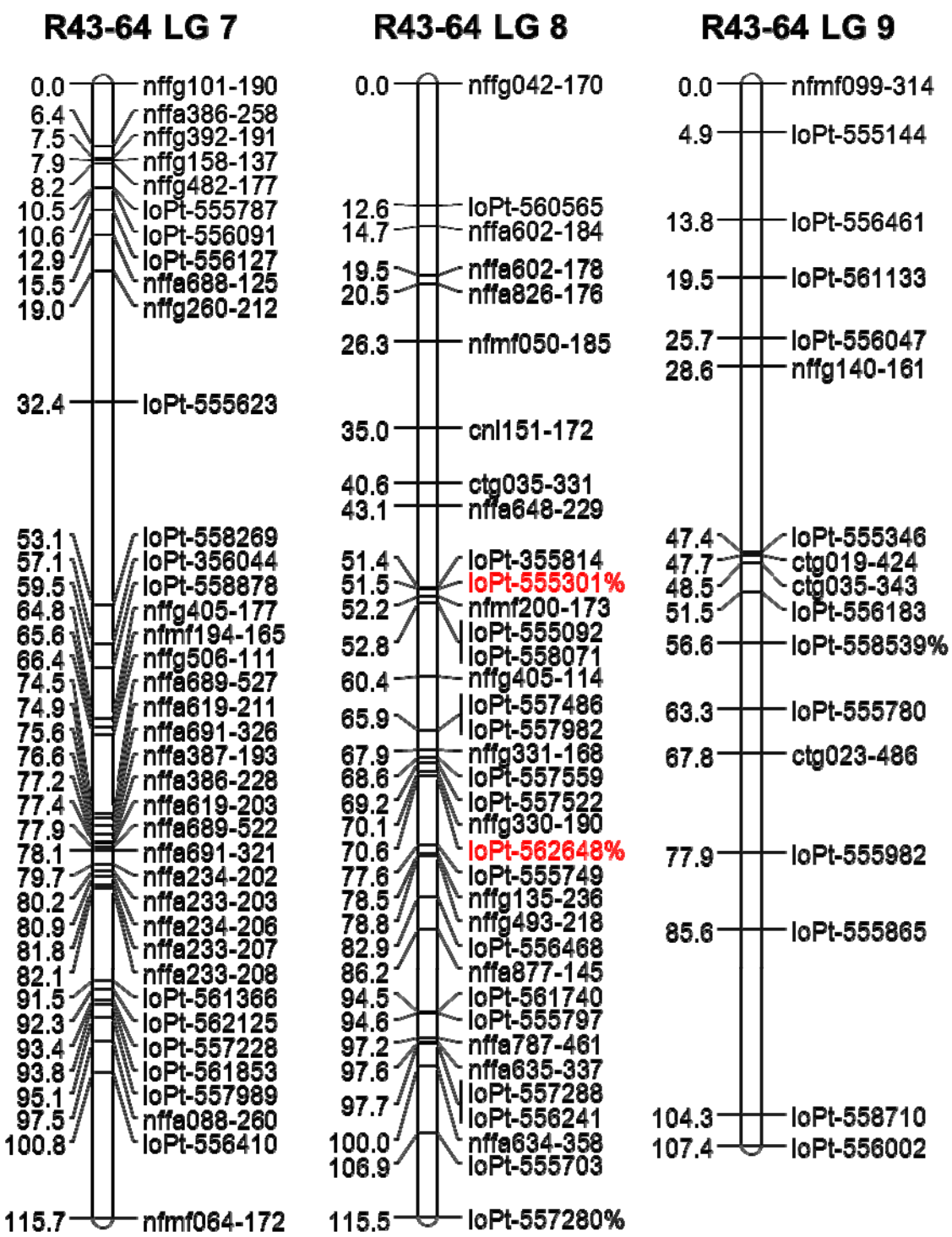
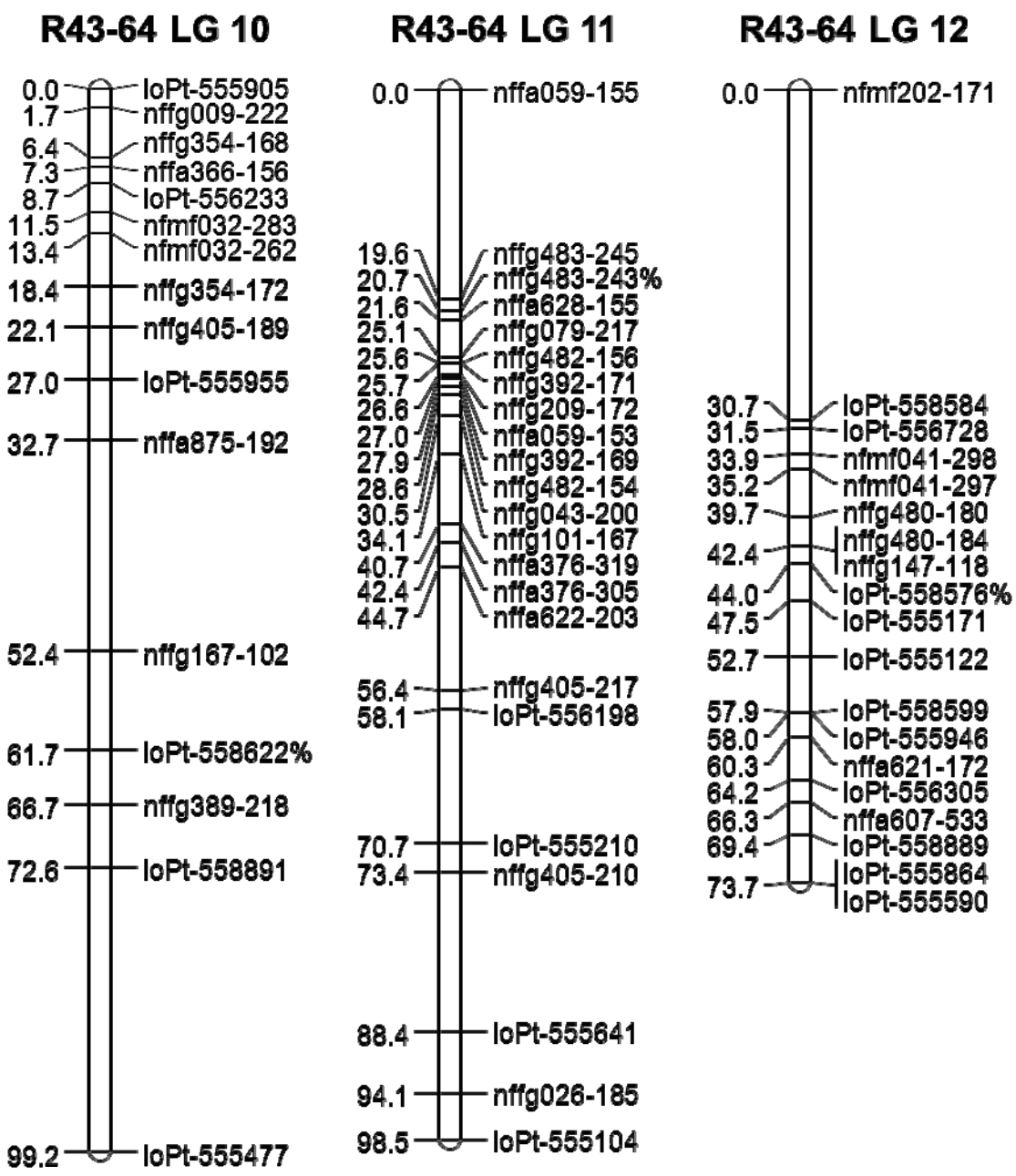


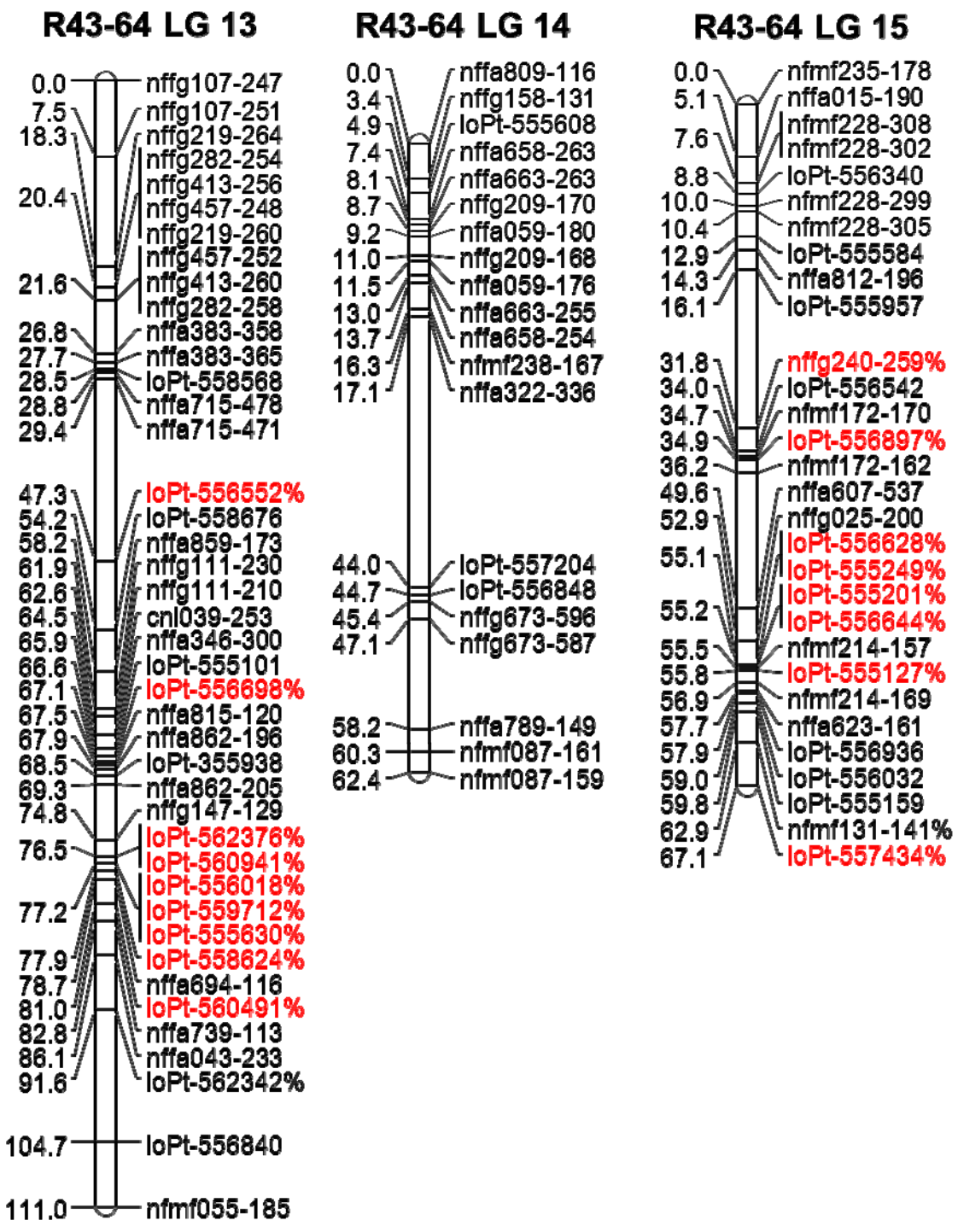
Figure 4.2. Linkage map of the 23 LGs created from the female parent 103-2. Numbers at left of each bar indicates the centiMorghan (cM) distance. Markers with % symbol segregated in a 3:1 ratio. Markers highlighted in red are common between the parental lines and were used to make a consensus map.

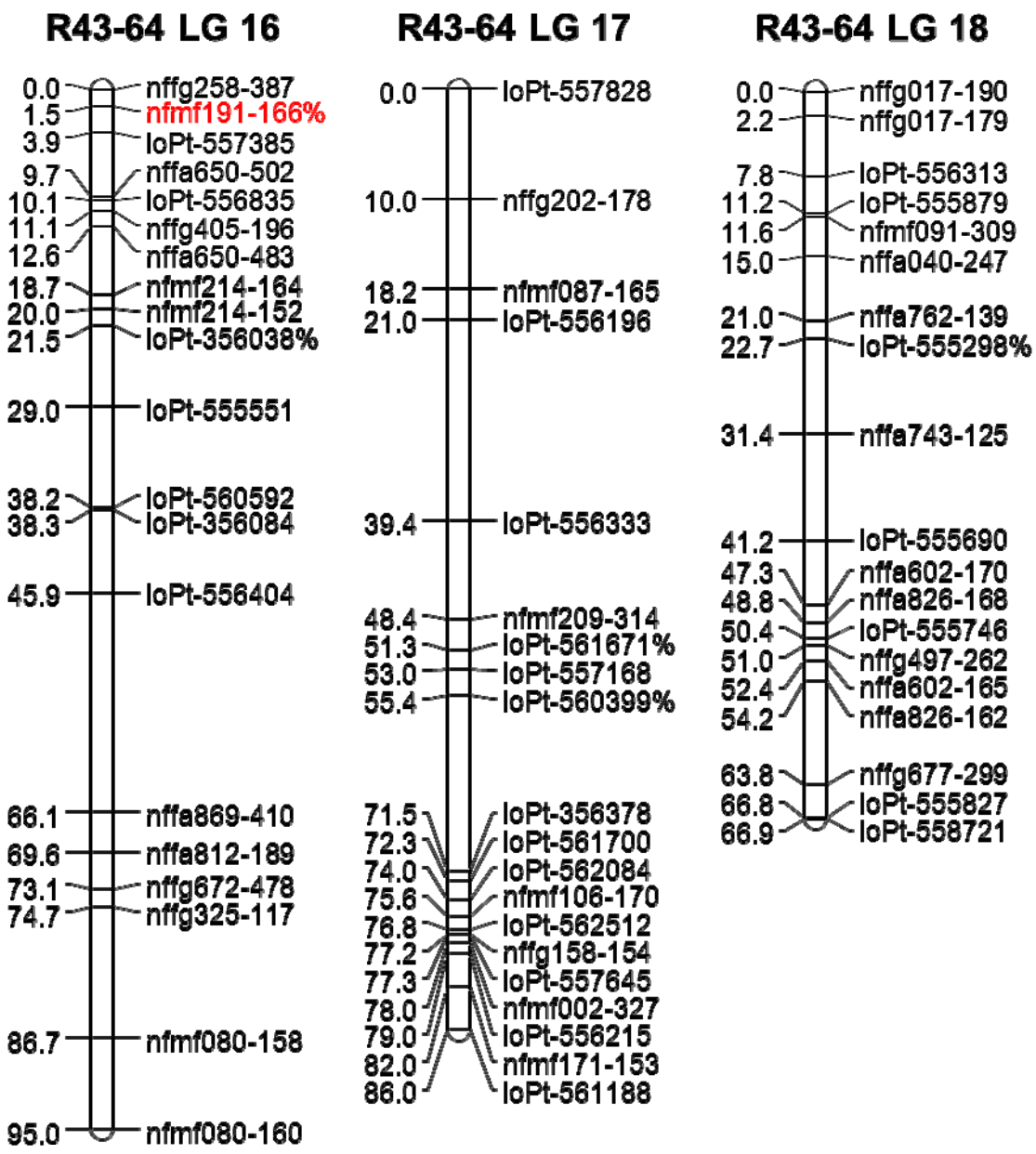

R43-64 LG 1
0.0 loPt-556939
0.1 loPt-555899
6.5 loPt-556566
7.3 nffg668-381
8.4 nffa002-320
10.3 nffa002-317
19.0 nffg203-247
23.1 nffg389-226
24.1 nffg157-150
25.8 nffg389-210
26.8 nffg157-152
28.1 loPt-556160
30.9 nffg203-230
38.9 nffa155-249
40.4 nffa155-251
40.7 loPt-556697
40.8 nffa031-378
48.0 loPt-562032
58.7 loPt-561089
59.3 nffa112-259
61.8 loPt-561361%
65.4 loPt-557880
72.0 loPt-561879
72.6 loPt-558690%
73.6 nffa822-244
75.5 nffa723-118
76.0 loPt-555517
77.4 nffg253-190
78.3 nffa822-232
79.7 loPt-356369%
82.5 nffa649-203
84.0 loPt-557878%
84.3 nffa112-253
85.6 loPt-556847
86.2 nffa112-236
90.2 nffa155-275
90.9 loPt-556277
95.2 nffg464-168
96.8 nffg464-184
102.7 nffa803-182
103.7 nffa803-179
105.9 nffg157-119
nffg157-111
108.1 loPt-558845
114.9 nffa738-167
118.6 nffa129-252
119.4 nffa129-235
119.8 nffa129-250
120.6 loPt-556658
121.7 loPt-556358
123.0 nffg354-198
123.5 nffg163-146
125.9 nffg163-143
132.5 nffg170-202
132.9 nffg273-181
134.6 nffg273-191
nffg170-211
R43-64 LG 2
0.0 loPt-560783
0.8 loPt-562401
8.9 nffa654-379
12.5 nffa205-378
15.0 nffa206-348
nffa247-294
15.3 nffa205-381
15.8 nffa654-382
22.5 nffg305-189
22.6 nffg106-180
24.4 nffg305-187
25.0 nffg106-178
31.1 nffg423-246
66.1 nffg330-225
68.9 nffg330-229
71.9 nffg026-175
74.1 nffg325-132
77.2 nfmf094-247
87.9 nffa658-245
94.8 nffg505-192
99.5 loPt-356383
loPt-556134
100.4 loPt-558694%
103.5 loPt-555874
115.6 nffg391-148
R43-64 LG 3
0.0 nffg273-201
9.0 nffg273-205
12.0 nffg170-226
13.5 loPt-560141
14.6 loPt-355729
17.5 loPt-561926%
17.7 nffg505-177
18.5 loPt-557263
loPt-556700
18.8 nffg405-229
19.4 loPt-556405
loPt-555085
loPt-562019
21.1 loPt-557209%
25.5 loPt-355937%
32.0 nffg264-235
34.5 nffg264-211
39.4 loPt-356082
40.7 nffa698-522
44.6 nffa709-464
51.8 nfmf100-147
54.4 nfmf100-135
64.4 nffg331-167
66.3 nffg331-165
68.8 nfmf144-519
69.3 nffa731-281
71.5 nffg253-200
72.6 nffa821-400
89.7 nffg043-195
91.8 nffa247-298
94.3 loPt-561275
96.7 nffa247-300
108.0 nfmf029-160
112.2 nffg676-181


R43-64 LG 4
0.0 nffa774-376
2.3 nffa610-200
4.3 nffa610-194
5.2 loPt-556147
17.3 nffa786-185
21.7 nfmf210-167
25.7 loPt-556441
27.1 nffa205-347
29.0 nffa205-349
30.6 nffa654-351
42.2 nffa632-238
48.0 nffg026-193
48.9 loPt-555972
60.3 loPt-556480
68.7 nffg171-169
69.3 nffg239-178
69.8 nffg171-171%
70.7 loPt-558725
71.4 nffg239-157
72.1 nffg171-149
74.0 nffg480-181
75.3 nffg167-109
78.5 loPt-558623
79.6 nffg405-197
93.3 nffg273-214
102.5 nffg026-183
R43-64 LG 5
-14.1 nfmf016-211
0.0 nfmf178-165%
1.4 nfmf120-159
1.8 nfmf189-174
2.6 nfmf178-156
3.7 nffa030-209
5.6 loPt-557783
6.2 loPt-557330
7.2 nffa289-153
8.0 nfmf120-167%
9.6 nffa030-217%
12.1 nfmf189-182%
13.6 loPt-555804
14.2 nfmf016-225
15.2 loPt-556516%
16.1 loPt-555839
21.4 ctg012-325
22.2 ctg012-326
23.7 nfmf176-156
24.9 nffa788-305
25.4 nffg156-172
41.8 nffg197-192
47.1 nffa788-302
50.0 nffa788-308
51.3 loPt-557515
52.0 loPt-562301
56.8 nffa034-232
57.6 nffg006-168
58.4 nffa034-247
58.7 nffa730-197
59.0 nffa602-160
59.4 nfmf186-188
60.2 nfmf229-157
60.5 nffg679-165
61.2 nffa826-158
61.7 nfmf229-169
63.3 nffa730-191
67.9 nfmf186-215
68.4 nffa695-263
68.7 loPt-557595
68.8 nffa233-200
69.6 loPt-561473
70.0 nffa692-204
71.4 nffa691-323
72.7 nffa692-194
74.3 nffa691-312
79.5 nffa234-200
80.5 nffg182-155
82.0 nffg182-139
83.4 loPt-557916
86.8 nfmf103-159
nfmf103-156
nffa809-138
nfmf220-173
R43-64 LG 6
0.0 nffa611-145
1.0 loPt-560316
7.9 nffa855-172
12.9 nffa660-206
14.0 loPt-556778
28.3 nffg236-181
29.8 loPt-556580
30.6 nffa346-333
32.3 nffa681-298
33.6 nffa681-292
33.9 nffa346-334
34.2 loPt-561835
loPt-355852
34.3 loPt-562440
loPt-555707
34.8 nffg483-238
35.0 nffg236-195
36.2 loPt-557071
36.5 loPt-559750
36.6 loPt-555270
36.8 nffa129-238
38.8 nffg405-151
40.1 loPt-555080
47.2 nfmf130-160
50.9 loPt-555472
52.3 nffg324-186
57.2 loPt-356086
61.1 nffa802-451
63.3 nffa802-489
72.7 nffa864-310
74.3 nffa864-332
78.8 nffa868-246
86.2 nffa607-162
86.9 loPt-562516%
88.4 loPt-562199
90.6 nffa869-285
91.2 loPt-560758
91.3 loPt-560869
92.2 nfmf055-179
92.7 loPt-555435%
93.4 loPt-562691
104.0 nfmf099-378


R43-64 LG 7
0.0 nffg101-190
6.4 nffa386-258
7.5 nffg392-191
7.9 nffg158-137
8.2 nffg482-177
10.5 loPt-555787
10.6 loPt-556091
12.9 loPt-556127
15.5 nffa688-125
19.0 nffg260-212
32.4 loPt-555623
53.1 loPt-558269
57.1 loPt-356044
59.5 loPt-558878
64.8 nffg405-177
65.6 nfmf194-165
66.4 nffg506-111
74.5 nffa689-527
74.9 nffa619-211
75.6 nffa691-326
76.6 nffa387-193
77.2 nffa386-228
77.4 nffa619-203
77.9 nffa689-522
78.1 nffa691-321
79.7 nffa234-202
80.2 nffa233-203
80.9 nffa234-206
81.8 nffa233-207
82.1 nffa233-208
91.5 loPt-561366
92.3 loPt-562125
93.4 loPt-557228
93.8 loPt-561853
95.1 loPt-557989
97.5 nffa088-260
100.8 loPt-556410
115.7 nfmf064-172
R43-64 LG 8
0.0 nffg042-170
12.6 loPt-560565
14.7 nffa602-184
19.5 nffa602-178
20.5 nffa826-176
26.3 nfmf050-185
35.0 cnl151-172
40.6 ctg035-331
43.1 nffa648-229
51.4 loPt-355814
51.5 loPt-555301%
52.2 nfmf200-173
52.8 loPt-555092
loPt-558071
60.4 nffg405-114
65.9 loPt-557486
loPt-557982
67.9 nffg331-168
68.6 loPt-557559
69.2 loPt-557522
70.1 nffg330-190
70.6 loPt-562648%
77.6 loPt-555749
78.5 nffg135-236
78.8 nffg493-218
82.9 loPt-556468
86.2 nffa877-145
94.5 loPt-561740
94.6 loPt-555797
97.2 nffa787-461
97.6 nffa635-337
97.7 loPt-557288
loPt-556241
100.0 nffa634-358
106.9 loPt-555703
115.5 loPt-557280%
R43-64 LG 9
0.0 nfmf099-314
4.9 loPt-555144
13.8 loPt-556461
19.5 loPt-561133
25.7 loPt-556047
28.6 nffg140-161
47.4 loPt-555346
47.7 ctg019-424
48.5 ctg035-343
51.5 loPt-556183
56.6 loPt-558539%
63.3 loPt-555780
67.8 ctg023-486
77.9 loPt-555982
85.6 loPt-555865
104.3 loPt-558710
107.4 loPt-556002


R43-64 LG 10
0.0 loPt-555905
1.7 nffg009-222
6.4 nffg354-168
7.3 nffa366-156
8.7 loPt-556233
11.5 nfmf032-283
13.4 nfmf032-262
18.4 nffg354-172
22.1 nffg405-189
27.0 loPt-555955
32.7 nffa875-192
52.4 nffg167-102
61.7 loPt-558622%
66.7 nffg389-218
72.6 loPt-558891
99.2 loPt-555477
R43-64 LG 11
0.0 nffa059-155
19.6 nffg483-245
20.7 nffg483-243%
21.6 nffa628-155
25.1 nffg079-217
25.6 nffg482-156
25.7 nffg392-171
26.6 nffg209-172
27.0 nffa059-153
27.9 nffg392-169
28.6 nffg482-154
30.5 nffg043-200
34.1 nffg101-167
40.7 nffa376-319
42.4 nffa376-305
44.7 nffa622-203
56.4 nffg405-217
58.1 loPt-556198
70.7 loPt-555210
73.4 nffg405-210
88.4 loPt-555641
94.1 nffg026-185
98.5 loPt-555104
R43-64 LG 12
0.0 nfmf202-171
30.7 loPt-558584
31.5 loPt-556728
33.9 nfmf041-298
35.2 nfmf041-297
39.7 nffg480-180
42.4 nffg480-184
nffg147-118
44.0 loPt-558576%
47.5 loPt-555171
52.7 loPt-555122
57.9 loPt-558599
58.0 loPt-555946
60.3 nffa621-172
64.2 loPt-556305
66.3 nffa607-533
69.4 loPt-558889
73.7 loPt-555864
loPt-555590


R43-64 LG 13
0.0 nffg107-247
7.5 nffg107-251
18.3 nffg219-264
nffg282-254
20.4 nffg413-256
nffg457-248
nffg219-260
nffg457-252
21.6 nffg413-260
nffg282-258
26.8 nffa383-358
27.7 nffa383-365
28.5 loPt-558568
28.8 nffa715-478
29.4 nffa715-471
47.3 loPt-556552%
54.2 loPt-558676
58.2 nffa859-173
61.9 nffg111-230
62.6 nffg111-210
64.5 cnl039-253
65.9 nffa346-300
66.6 loPt-555101
67.1 loPt-556698%
67.5 nffa815-120
67.9 nffa862-196
68.5 loPt-355938
69.3 nffa862-205
74.8 nffg147-129
76.5 loPt-562376%
loPt-560941%
loPt-556018%
77.2 loPt-559712%
loPt-555630%
77.9 loPt-558624%
78.7 nffa694-116
81.0 loPt-560491%
82.8 nffa739-113
86.1 nffa043-233
91.6 loPt-562342%
104.7 loPt-556840
111.0 nfmf055-185
R43-64 LG 14
0.0 nffa809-116
3.4 nffg158-131
4.9 loPt-555608
7.4 nffa658-263
8.1 nffa663-263
8.7 nffg209-170
9.2 nffa059-180
11.0 nffg209-168
11.5 nffa059-176
13.0 nffa663-255
13.7 nffa658-254
16.3 nfmf238-167
17.1 nffa322-336
44.0 loPt-557204
44.7 loPt-556848
45.4 nffg673-596
47.1 nffg673-587
58.2 nffa789-149
60.3 nfmf087-161
62.4 nfmf087-159
R43-64 LG 15
0.0 nfmf235-178
5.1 nffa015-190
7.6 nfmf228-308
nfmf228-302
8.8 loPt-556340
10.0 nfmf228-299
10.4 nfmf228-305
12.9 loPt-555584
14.3 nffa812-196
16.1 loPt-555957
31.8 nffg240-259%
34.0 loPt-556542
34.7 nfmf172-170
34.9 loPt-556897%
36.2 nfmf172-162
49.6 nffa607-537
52.9 nffg025-200
55.1 loPt-556628%
loPt-555249%
loPt-555201%
55.2 loPt-556644%
55.5 nfmf214-157
55.8 loPt-555127%
56.9 nfmf214-169
57.7 nffa623-161
57.9 loPt-556936
59.0 loPt-556032
59.8 loPt-555159
62.9 nfmf131-141%
67.1 loPt-557434%


R43-64 LG 16
0.0 nffg258-387
1.5 nfmf191-166%
3.9 loPt-557385
9.7 nffa650-502
10.1 loPt-556835
11.1 nffg405-196
12.6 nffa650-483
18.7 nfmf214-164
20.0 nfmf214-152
21.5 loPt-356038%
29.0 loPt-555551
38.2 loPt-560592
38.3 loPt-356084
45.9 loPt-556404
66.1 nffa869-410
69.6 nffa812-189
73.1 nffg672-478
74.7 nffg325-117
86.7 nfmf080-158
95.0 nfmf080-160
R43-64 LG 17
0.0 loPt-557828
10.0 nffg202-178
18.2 nfmf087-165
21.0 loPt-556196
39.4 loPt-556333
48.4 nfmf209-314
51.3 loPt-561671%
53.0 loPt-557168
55.4 loPt-560399%
71.5 loPt-356378
72.3 loPt-561700
74.0 loPt-562084
75.6 nfmf106-170
76.8 loPt-562512
77.2 nffg158-154
77.3 loPt-557645
78.0 nfmf002-327
79.0 loPt-556215
82.0 nfmf171-153
86.0 loPt-561188
R43-64 LG 18
0.0 nffg017-190
2.2 nffg017-179
7.8 loPt-556313
11.2 loPt-555879
11.6 nfmf091-309
15.0 nffa040-247
21.0 nffa762-139
22.7 loPt-555298%
31.4 nffa743-125
41.2 loPt-555690
47.3 nffa602-170
48.8 nffa826-168
50.4 loPt-555746
51.0 nffg497-262
52.4 nffa602-165
54.2 nffa826-162
63.8 nffg677-299
66.8 loPt-555827
66.9 loPt-558721

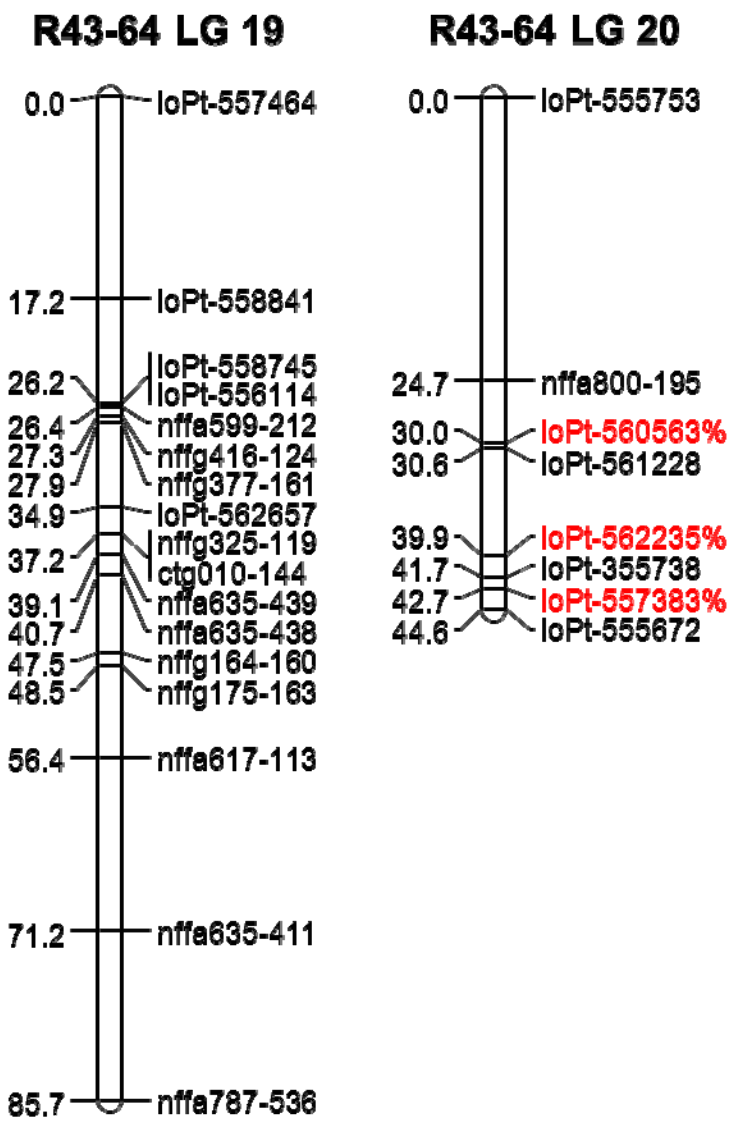
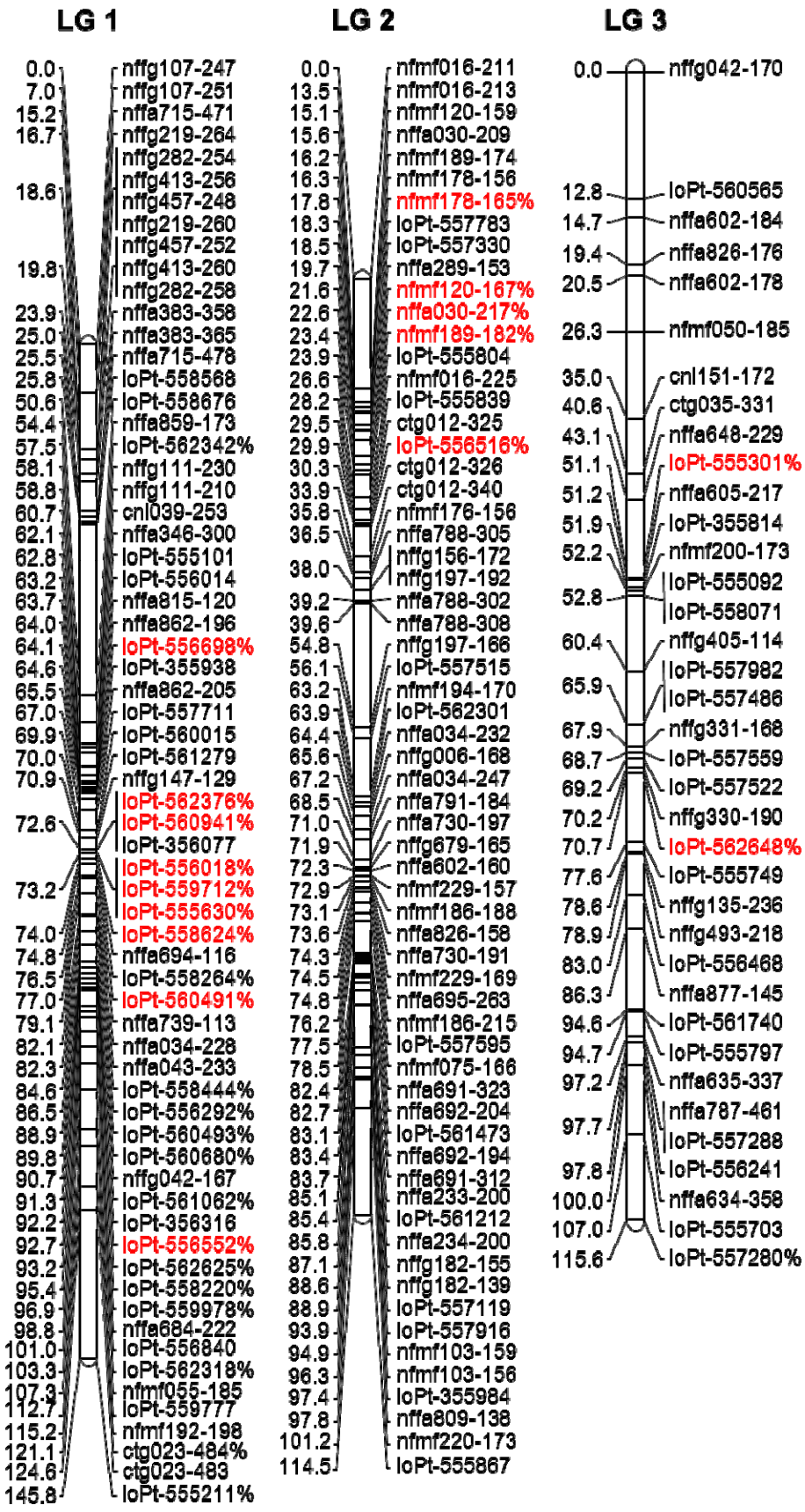
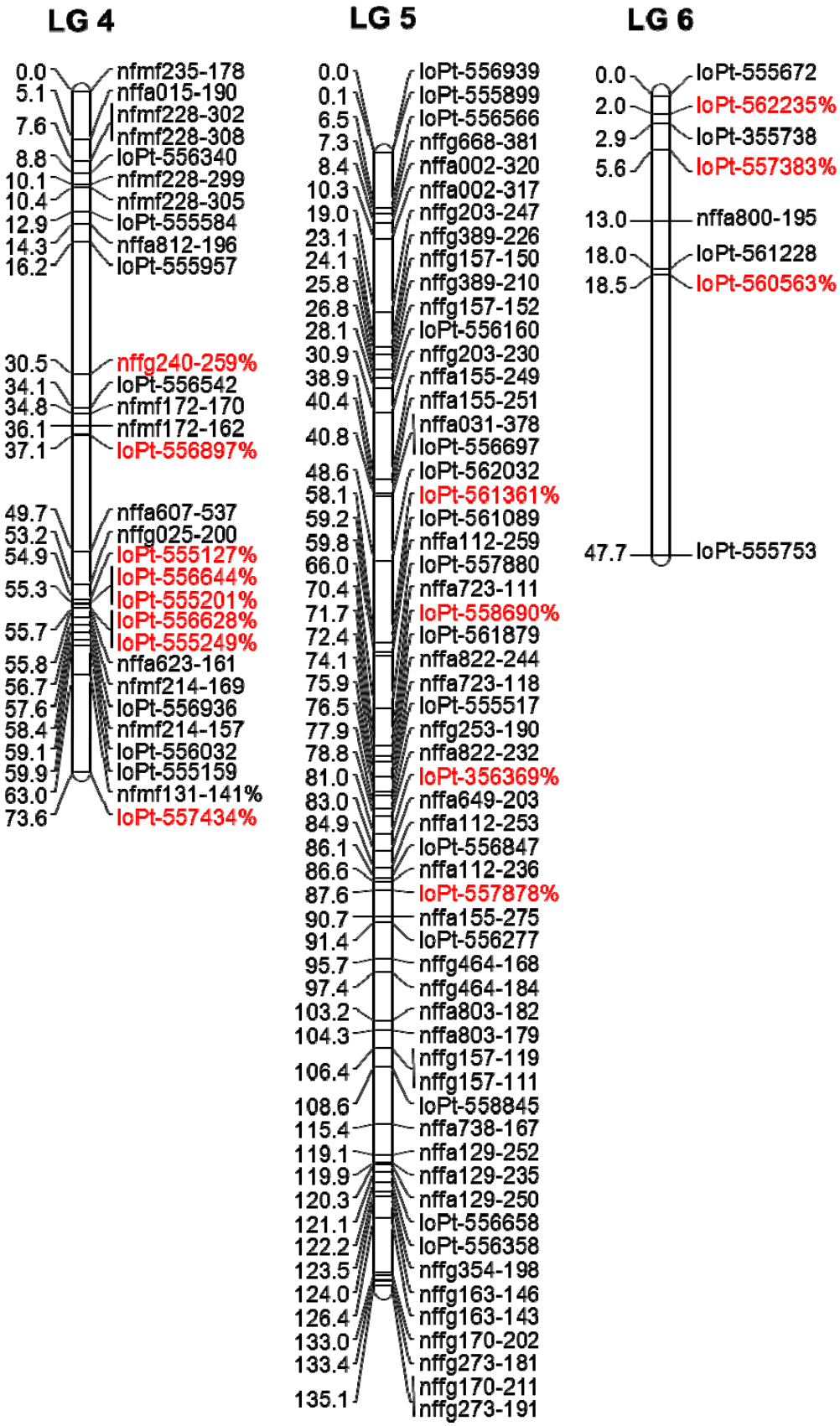


Figure 4.3. Linkage map of the 20 LGs created from the male parent R43-64. Numbers at left of each bar indicate the centiMorghan (cM) distance. Markers with % symbol segregated in a 3:1 ratio. Markers highlighted in red are common between the parental lines and were used to make a consensus map.


LG 1
0.0 nffg107-247
7.0 nffg107-251
15.2 nffa715-471
16.7 nffg219-264
nffg282-254
18.6 nffg413-256
nffg457-248
nffg219-260
nffg457-252
19.8 nffg413-260
nffg282-258
23.9 nffa383-358
25.0 nffa383-365
25.5 nffa715-478
25.8 loPt-558568
50.6 loPt-558676
54.4 nffa859-173
57.5 loPt-562342%
58.1 nffg111-230
58.8 nffg111-210
60.7 cnl039-253
62.1 nffa346-300
62.8 loPt-555101
63.2 loPt-556014
63.7 nffa815-120
64.0 nffa862-196
64.1 loPt-556698%
64.6 loPt-355938
65.5 nffa862-205
67.0 loPt-557711
69.9 loPt-560015
70.0 loPt-561279
70.9 nffg147-129
loPt-562376%
72.6 loPt-560941%
loPt-356077
loPt-556018%
73.2 loPt-559712%
loPt-555630%
74.0 loPt-558624%
74.8 nffa694-116
76.5 loPt-558264%
77.0 loPt-560491%
79.1 nffa739-113
82.1 nffa034-228
82.3 nffa043-233
84.6 loPt-558444%
86.5 loPt-556292%
88.9 loPt-560493%
89.8 loPt-560680%
90.7 nffg042-167
91.3 loPt-561062%
92.2 loPt-356316
92.7 loPt-556552%
93.2 loPt-562625%
95.4 loPt-558220%
96.9 loPt-559978%
98.8 nffa684-222
101.0 loPt-556840
103.3 loPt-562318%
107.3 nfmf055-185
112.7 loPt-559777
115.2 nfmf192-198
121.1 ctg023-484%
124.6 ctg023-483
145.8 loPt-555211%
LG 2
0.0 nfmf016-211
13.5 nfmf016-213
15.1 nfmf120-159
15.6 nffa030-209
16.2 nfmf189-174
16.3 nfmf178-156
17.8 nfmf178-165%
18.3 loPt-557783
18.5 loPt-557330
19.7 nffa289-153
21.6 nfmf120-167%
22.6 nffa030-217%
23.4 nfmf189-182%
23.9 loPt-555804
26.6 nfmf016-225
28.2 loPt-555839
29.5 ctg012-325
29.9 loPt-556516%
30.3 ctg012-326
33.9 ctg012-340
35.8 nfmf176-156
36.5 nffa788-305
nffg156-172
38.0 nffg197-192
39.2 nffa788-302
39.6 nffa788-308
54.8 nffg197-166
56.1 loPt-557515
63.2 nfmf194-170
63.9 loPt-562301
64.4 nffa034-232
65.6 nffg006-168
67.2 nffa034-247
68.5 nffa791-184
71.0 nffa730-197
71.9 nffg679-165
72.3 nffa602-160
72.9 nfmf229-157
73.1 nfmf186-188
73.6 nffa826-158
74.3 nffa730-191
74.5 nfmf229-169
74.8 nffa695-263
76.2 nfmf186-215
77.5 loPt-557595
78.5 nfmf075-166
82.4 nffa691-323
82.7 nffa692-204
83.1 loPt-561473
83.4 nffa692-194
83.7 nffa691-312
85.1 nffa233-200
85.4 loPt-561212
85.8 nffa234-200
87.1 nffg182-155
88.6 nffg182-139
88.9 loPt-557119
93.9 loPt-557916
94.9 nfmf103-159
96.3 nfmf103-156
97.4 loPt-355984
97.8 nffa809-138
101.2 nfmf220-173
114.5 loPt-555867
LG 3
0.0 nffg042-170
12.8 loPt-560565
14.7 nffa602-184
19.4 nffa826-176
20.5 nffa602-178
26.3 nfmf050-185
35.0 cnl151-172
40.6 ctg035-331
43.1 nffa648-229
51.1 loPt-555301%
51.2 nffa605-217
51.9 loPt-355814
52.2 nfmf200-173
loPt-555092
52.8 loPt-558071
60.4 nffg405-114
loPt-557982
65.9 loPt-557486
67.9 nffg331-168
68.7 loPt-557559
69.2 loPt-557522
70.2 nffg330-190
70.7 loPt-562648%
77.6 loPt-555749
78.6 nffg135-236
78.9 nffg493-218
83.0 loPt-556468
86.3 nffa877-145
94.6 loPt-561740
94.7 loPt-555797
97.2 nffa635-337
nffa787-461
97.7 loPt-557288
97.8 loPt-556241
100.0 nffa634-358
107.0 loPt-555703
115.6 loPt-557280%


LG 4
0.0 nfmf235-178
5.1 nffa015-190
7.6 nfmf228-302
nfmf228-308
8.8 loPt-556340
10.1 nfmf228-299
10.4 nfmf228-305
12.9 loPt-555584
14.3 nffa812-196
16.2 loPt-555957
30.5 nffg240-259%
34.1 loPt-556542
34.8 nfmf172-170
36.1 nfmf172-162
37.1 loPt-556897%
49.7 nffa607-537
53.2 nffg025-200
54.9 loPt-555127%
loPt-556644%
55.3 loPt-555201%
loPt-556628%
55.7 loPt-555249%
55.8 nffa623-161
56.7 nfmf214-169
57.6 loPt-556936
58.4 nfmf214-157
59.1 loPt-556032
59.9 loPt-555159
63.0 nfmf131-141%
73.6 loPt-557434%
LG 5
0.0 loPt-556939
0.1 loPt-555899
6.5 loPt-556566
7.3 nffg668-381
8.4 nffa002-320
10.3 nffa002-317
19.0 nffg203-247
23.1 nffg389-226
24.1 nffg157-150
25.8 nffg389-210
26.8 nffg157-152
28.1 loPt-556160
30.9 nffg203-230
38.9 nffa155-249
40.4 nffa155-251
40.8 nffa031-378
loPt-556697
48.6 loPt-562032
58.1 loPt-561361%
59.2 loPt-561089
59.8 nffa112-259
66.0 loPt-557880
70.4 nffa723-111
71.7 loPt-558690%
72.4 loPt-561879
74.1 nffa822-244
75.9 nffa723-118
76.5 loPt-555517
77.9 nffg253-190
78.8 nffa822-232
81.0 loPt-356369%
83.0 nffa649-203
84.9 nffa112-253
86.1 loPt-556847
86.6 nffa112-236
87.6 loPt-557878%
90.7 nffa155-275
91.4 loPt-556277
95.7 nffg464-168
97.4 nffg464-184
103.2 nffa803-182
104.3 nffa803-179
106.4 nffg157-119
nffg157-111
108.6 loPt-558845
115.4 nffa738-167
119.1 nffa129-252
119.9 nffa129-235
120.3 nffa129-250
121.1 loPt-556658
122.2 loPt-556358
123.5 nffg354-198
124.0 nffg163-146
126.4 nffg163-143
133.0 nffg170-202
133.4 nffg273-181
135.1 nffg170-211
nffg273-191
LG 6
0.0 loPt-555672
2.0 loPt-562235%
2.9 loPt-355738
5.6 loPt-557383%
13.0 nffa800-195
18.0 loPt-561228
18.5 loPt-560563%
47.7 loPt-555753

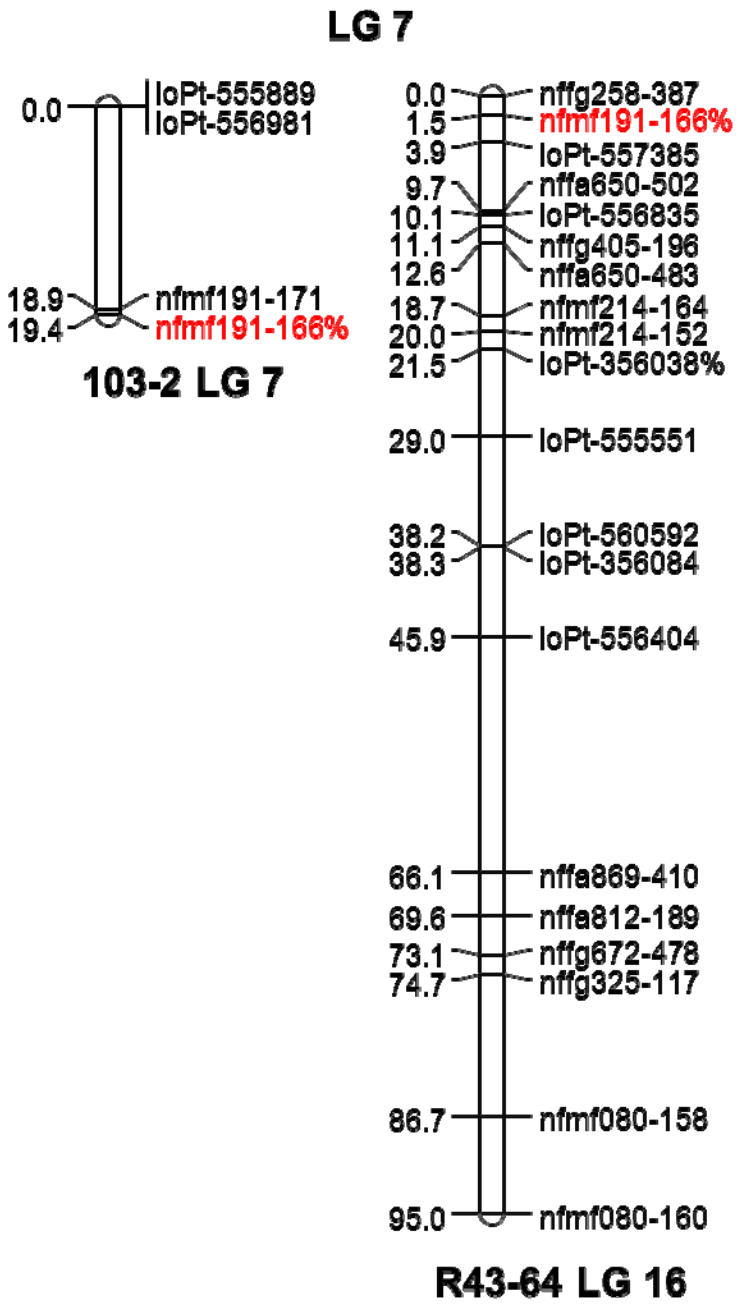


Figure 4.4. Consensus map of both parental lines. Markers with % symbol segregated in a 3:1 ratio. Markers highlighted in red are common between the parental lines and were used to make this consensus map. LG7 did not integrate due to only one marker from LG7 of parent 103-2 segregating in a 3:1 ratio.

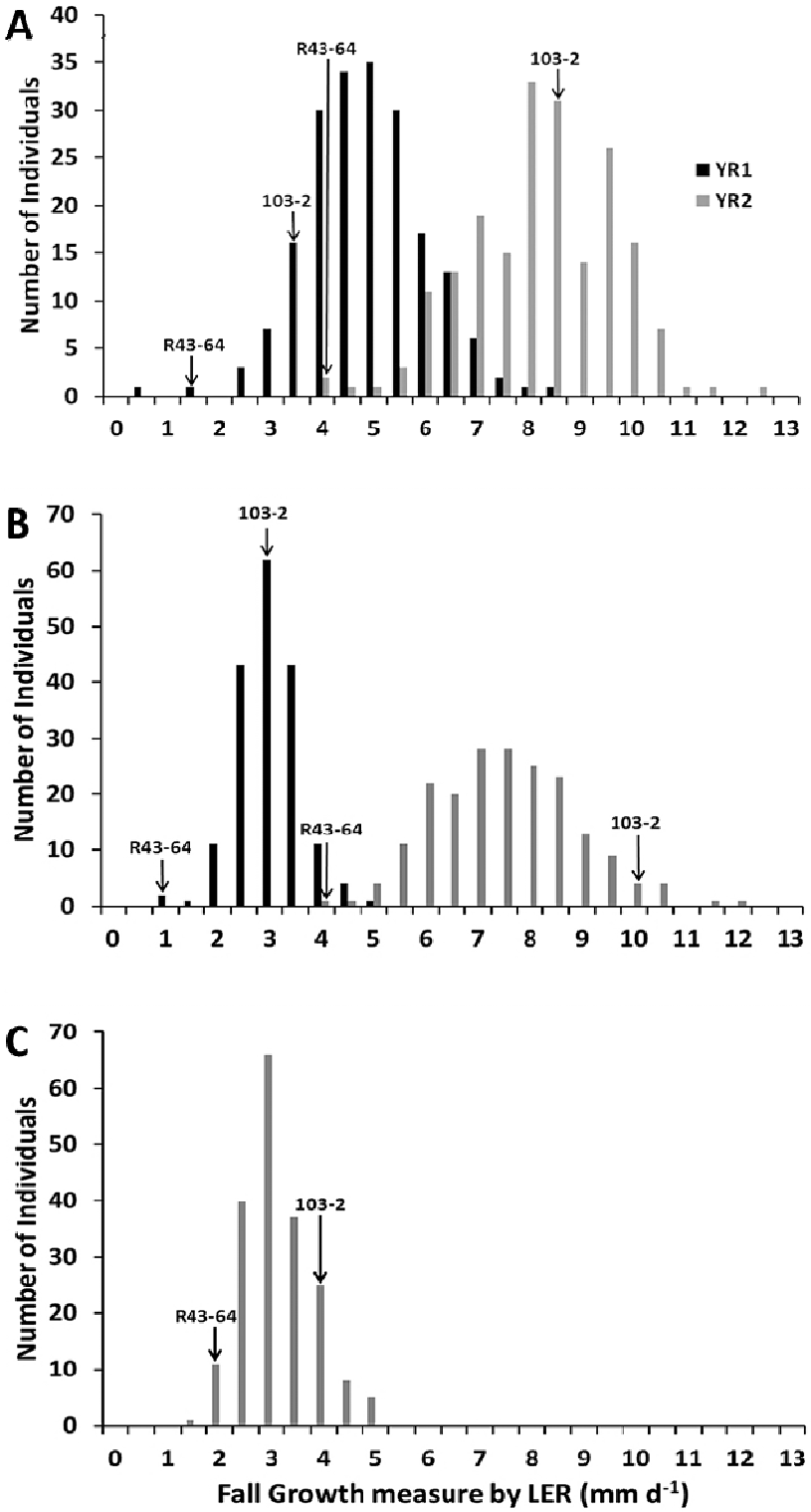


Figure 4.5. Progeny frequency distributions for the Mediterranean (103-2) × Continental (R43-64) population for fall growth at Arlington, Wisconsin (WI) (A), Columbia, MO (MO1) (B), and Mt. Vernon, MO (MO2) (C) measured in 2008-2009 (YR1) and 2009-2010 (YR2). The location of the means of the parents (103-2 and R43-64) are indicated by arrows.

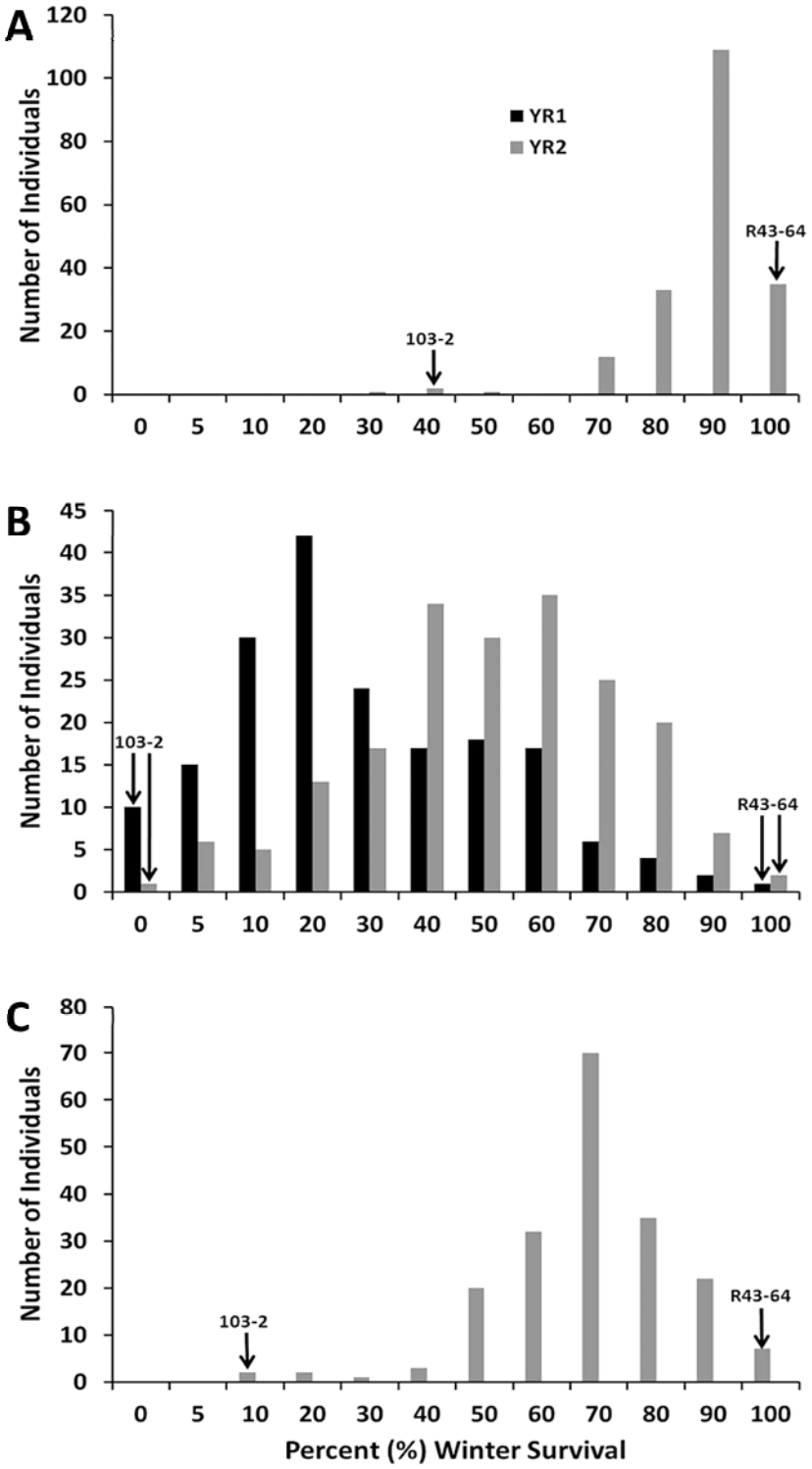


Figure 4.6. Progeny frequency distributions for the Mediterranean (103-2) × Continental (R43-64) population for winter survival at Arlington, Wisconsin (WI) (A), Columbia, MO (MO1) (B), and Mt. Vernon, MO (MO2) (C) measured in 2008-2009 (YR1) and 2009-2010 (YR2). The location of the means of the parents (103-2 and R43-64) are indicated by arrows.

APPENDIX:

HOMOLOGY OF TALL FESCUE EST SEQUENCES TO CBF, PROLINE (P5CS), AND A-TUBULIN GENES IN RELATED GRASS SPECIES

INTRODUCTION

In many plant species the increase in freezing tolerance when subjected to cold non-freezing temperatures are due the up-regulation of the CBF genes and their subsequent gene cascade. The CBF gene family encodes transcriptional activator proteins that contain the AP2/ERF (APETALA2/ethylene response factor) binding domain. These proteins bind to the CRT/DRE (C-repeat/dehydration-responsive element) promoter regions. This effectively begins their transcription, which ultimately aids in freezing tolerance.

These CBF genes are known to contain many homologous sequences. In *Arabidopsis,* where these genes were first described, there are three known sequences (Liu et al., 1998; Stockinger et al., 1997). This number is substantially higher in barley (*Hordeum vulgare*) with 20 identified CBF genes (Skinner, 2005), 15-25 genes in wheat (*Triticum aestvium*) (Badawi et al., 2007), and 7 genes in perennial ryegrass (*Lolium perenne*) (Tamura and Yamada, 2007). Currently, one CBF/DREB tall fescue sequence (FaDREB1) has been discovered (Tang et al., 2005). It has been shown in many plant species that exposure to chilling, cold, or freezing temperatures up-regulates CBF gene expression. Some CBF genes respond rapidly, within minutes or hours, while others do not exhibit a response until 12 to 24 hours after exposure. Additionally, the expression duration of the CBF genes varies from a couple of hours to longer than 20-45 days (Skinner et al., 2005; Tamura and Yamada, 2007). On the whole, CBF genes respond to multiple stresses such as cold, drought and salinity. It is thought that some CBF genes are

activated during multiple abiotic stresses. This multiple use has been observed in numerous plant species, and even tall fescue (Tang et al., 2005).

Free proline also accumulates during a cold stress event. As described in previous chapters, proline increased between 5 and 15-fold for Continental accessions of tall fescue when exposed to 4.5^{o}C as compared to 25.0^{o}C (Chapter 3). This increase in proline in response to cold stress has also been observed in wheat and radish (Chu et al. 1974; Dörffling et al. 2009). Like, the CBF genes, proline concentration increases in response to multiple abiotic stresses. In tall fescue, proline concentrations increased when plants were subjected to drought stress (Abernethy and McManus, 1998; Bokhari and Trent, 1985). Our objective was to identify CBF and proline (P5CS) genes in the Continental and Mediterranean accessions studied in chapter 3 and to use RT-PCR to quantify the expression of the CBF and proline genes in response to cold stress at 4.5^{o}C. For a RT-PCR control the α-tubulin gene was chosen. The α-tubulin sequences from other species were used to find tall fescue EST sequences. However, due to the complexity of the tall fescue genome this experiment could not be successfully completed.

METHODS AND MATERIALS

Many of the known CBF/DREB, proline (P5CS), and α-tubulin sequences from barley, wheat, rice (*Oryza sativa*), perennial ryegrass, maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugar cane (*Saccharum officinarum*), and hardy sugar cane (*Saccharum arundinaceum*) were used to blast against the tall fescue [*Lolium arundinaceum* (Schreb.) S.J. Darbysh.] EST (Expressed Sequence Tag) database. To acquire all available sequence data, the sequence grabber program was used at the Noble

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http://bioinfo.noble.org/gateway/index.php?option=com_content&task=view&id=43&Itemid=50. Once those CBF sequences were obtained and uploaded, using the FASTA format, they were blasted against the tall fescue EST library (FAIG). A total of eight CBF, one P5CS, and four α-tubulin sequences were identified.

The nucleotide sequence data was converted to amino acid sequence using the online resource Translator at http://www.fr33.net/translator.php. The amino acid sequence of the tall fescue ESTs was aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to determine protein sequence similarity and construct phylogeny trees. Conserved and non-conserved domains were located (Figure A.1.). Additionally, the tall fescue amino acid sequences with high homology to known CBF genes were aligned to construct phylogeny trees (Figures A.2-A.6) and placed in a tree from Tamura and Yamada (2007) (Figure A.7). This process was repeated for proline (Figure A.8) and α-tubulin (Figure A.9).

Primers were designed from the most similar known CBF sequences since many of the tall fescue ESTs were not full length. Primers were also designed from the ESTs sequences. Primer lengths ranged from 18 to 25 base pairs. The GC content was in the range of 65 to 75%. To help reduce amplification problems, betaine (1.3M) was used to aid the PCR reactions (Henke et al., 1997). Forward and reverse primers were used in all combinations to identify which pair gave the best amplification as well as the smallest number of products. PCR products were separated using agarose gels ranging from 1.0 to 3.0%. However, the amplification reactions produced many products which could not be clearly resolved on agarose gels. To try and overcome this issue, 6.0% polyacrylamide

gels were used, since they can separate individual bands with a two base pair difference, followed by silver staining. Several of the PCR products produced what seemed to be individual bands. These bands were collected from the gel using razor blades to cut the bands and pipet tips to collect the individual bands. Individual bands were placed in 10µl of ddH_2O for 24hs. This water solution was then used for further PCR using the same primers pairs. The refined PCR products were then run on a 2.0% agarose gel to detect if multiple bands were still present. The bands were removed from the agarose gel and cleaned. These were then sent for sequencing. The results indicated that there are multiple products still in the bands that were collected from the polyacrylamide gels.

FUTURE DIRECTION

For this experiment to succeed, the PCR products from the CBF genes should be cloned in a bacterial system where a single sequence would be integrated into a bacterial vector. From that vector, individual amplicons can be sequenced. A single transformation event can integrate nearly all amplicons into bacterial vectors. Each colony produced in the bacterial culture can be treated as individuals so that each of the amplicons can be sequenced.

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```
TC3426          ----------MDMGLEVSSSSTSSSSSSASVSASSEHSRVMAPAKRPAGRTKFRETRHP 49
DT704637        ------------------------------------------------ARGKFRETRHP 11
DT707777        ------MCPIKKEMSGDSGSPFSVDYYSPSTSSEPVQQKQLTPWTKRPAGRTKFRETRHP 54
DT696484        GTRLERTDGVGAYCGSDHSPSGTQSPVGGAGEQDSSSYMTVSSPPKRRTGRTKFKETRHP 60
DT695008        --------------------GTRVTSPASSSASPPLSPTSRRGLDKR---RREGGAGRHP 37
DT709888        ---HE-----------------------------------GEDG-------------RHP 9
DT709945        -MEHEHVLVVPQVSNDDSCTCSGSTTTSSSSLNASSPSSGDDGAVVAGGKKRPRRDLKHP 59
DT706530        ---------------GTRVSECDRSWKMVKNAQQQQQERLMNGGNAAAAAAARQRQHQQH 45
                                                                        ::

TC3426          VYRGVRRRGNAGRWVCEVRVPGKRGARLWLGTYATAEAAARANDAAMLTLGG-CSASCLN 108
DT704637        LYRGVRRRGTAGRWVCEVRVPGKRGARLWLGTYATAEAAARANDAAMIALGGRSAATCLN 71
DT707777        VYRGVRRXGNAGRWVCEVRVPGRRGSRLWVGTFDTAEIAARAHDATMLALAG-PGAARLN 113
DT696484        VYKGVRSR-NPGRWVCEVREPHGK-QRIWLGTFDTAEMAARAHDFAALALRG--RGACLN 116
DT695008        TYRGVRMR-AWGKWVSEIREPRKK-SRIWLGTFPTPEMAARAHDAAALVVKG-------- 87
DT709888        TYHGVRMR-SWGKWVSEIREPRKK-SRIWLGTFATAEMAARAHDVASLAIKGRG-AAHVN 66
DT709945        TYRGVRMR-TWGKWVSEIREPRKK-SRIWLGTFDTPEMAARAHDVAAIAIKGR--AAHLN 115
DT706530        QYKGVRMR-SWGSWVSEIRAPNQK-TRIWLGSYSTAEAAARAYDAALLCLRG--SAADLN 101
                 *:***     * **.*:* *  :  *:*:*:: *.* **** * : : : *

TC3426          FADSAWLLAVPCAVADLADVRRAA----VEAVEDFQRREAASVPLASQEPTSSDSSAVSS 164
DT704637        FADSAWLLAVPSQLSDLADIRAAG----IESVPDFQRRQTGTITEEV------------ 114
DT707777        FADSAELLAVPASYASLDEVR-------------------------------------- 134
DT696484        FSDSLRRLRAPPQVPSHDEIRRGGSGALFRPGPSVQRNAASEAAVVSPVDSWGEELVASC 176
DT695008        ----------P----------------------------------------------- 88
DT709888        FPELADLLPRPASAAPSARG-------GGRPSPDVPRRAHA------------------ 100
DT709945        FPDTAHELPRAASAAPKD------------VQAAAALAAAA------------------ 144
DT706530        FPVHLPFHVPAAAMSPKSIQRFAAAAASSPLQPTAPAWNAGAACQPCGGYGDASFGSSTE 161
                          .

TC3426          PDNAGSPETSEPPAHGEIDGRDMFRLDLFPEIDLGSYYLSLAEALLMDPPPMATANGSSW 224
DT704637        ------------------------------------------------------------
DT707777        ------------------------------------------------------------
DT696484        PYFPMVMGGLEFXMQGYLDMAQGM---------------------------------- 200
DT695008        ------------------------------------------------------------
DT709888        ------------------------------------------------------------
DT709945        ------------------------------------------------------------
DT706530        EXXAXXNX---------ADHNDDI---------------------------------- 176

TC3426          DN 226
DT704637        --
DT707777        --
DT696484        --
DT695008        --
DT709888        --
DT709945        --
DT706530        --
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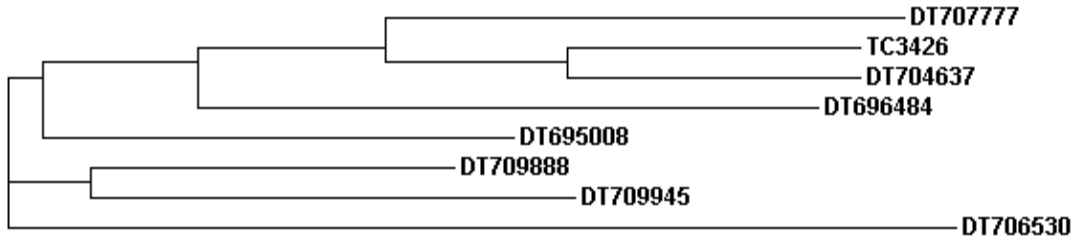

Figure A.1. Alignment of tall fescue EST sequences found to have high similarity to known CBF genes. The EST sequences were translated to amino acid sequences before being aligned. Also shown is the phylogeny of these sequences.

```
LpCBF3          MCQIKKEMSGESGSP--CNGE--YCSPSTSSE---QKQQTVWT----KRPAGRTKFRETR 49
DT707777        MCPIKKEMSGDSGSP--FSVD--YYSPSTSSEPVQQKQLTPWT----KRPAGRTKFRETR 52
HvCBF6          MCQIKKEMSGESGSP--CSGENYYYSPSTSPEHQQAKQQAAWTSAPAKRPAGRTKFRETR 58
TaCBF6          MCPIKREMSGESGSPSPCSGEN-FCSPSASPER-QQARQAGWTSAPAKRPAGRTKFRETR 58
                ** **:****:****   . :  : ***:*.*     : : **    *************

LpCBF3          HPVYRGVRRRGNAGRWVCEVRVPGRRGSRLWVGTFDTAEIAARAHDAAMLALA-AGDVCL 108
DT707777        HPVYRGVRRXGNAGRWVCEVRVPGRRGSRLWVGTFDTAEIAARAHDATMLALAGPGAARL 112
HvCBF6          HPVYRGVRRRGNAGRWVCEVRVPGRRGSRLWLGTFDTAEAAARANDAAMLALAAGGAGCL 118
TaCBF6          HPVYRGVRRRGNAGRWVCEVRVPGRRGSRLWLGTFDTAEAAARANDAVMLMLAAGGAACL 118
                ********* ********************:******* ****:**.** **  *   *

LpCBF3          NFADSAELLDMPASS-YRSLDEVRHAVTEAVEEFERRQALGEEDAL--SGTES---STLT 162
DT707777        NFADSAELLAVPAS--YASLDEVR----------------------------------- 134
HvCBF6          NFADSAELLAVPAASSYRSLDEVRHAVVEAVEDLLRREAHAEDDALSVSGTSSSAPSSIT 178
TaCBF6          NFADSAELLSVPVASSYRSLDEVRHAVVEAVEDLLRREALAEEDAL--SGTSSSAPSPLT 176
                ********* :*.:  * ******

LpCBF3          DDEESST-------PFELDVLSDMGWDLYYASLAQGMLMSSPFLAASAALGDYGEANLAD 215
DT707777        ------------------------------------------------------------
HvCBF6          DDDSSSSPA-DEGSPFELDVLSDMGWDLYYASLAQGMLMAPP-ASLAAALGDYGEAHLAD 236
TaCBF6          DDESSSSPLPEEDSPFEQDVLSEMGWDLYYASLAQAMLMAPP--AAAAALGDYGEAHLAD 234


LpCBF3          VPLWSYLS 223
DT707777        -------- 
HvCBF6          VPLWSYQS 244
TaCBF6          VPLWSYQS 242
```

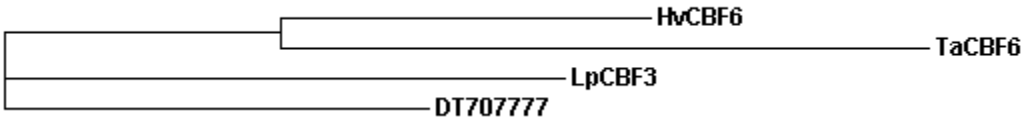

Figure A.2. Alignment of amino acid sequence from the putative tall fescue EST DT707777 compared to similar CBF amino acid sequence from barley (HvCBF6), wheat (TaCBF6) and perennial ryegrass (LpCBF3). Also shown is the phylogeny of these sequences.

```
HvCBF7          -----------MGATEDSSSGSETTTSSSVEALASPPSPTATTASSKKKRAR---NDGR 45
DT709888        ------------------------------------------------HEG---EDGR 7
DT709945        MEHEHVLVVPQVSNDDSCTCSGSTTTSSSSLNASSPSSGDDGAVVAGGKKRPR---RDLK 57
DT695008        --------------------GTRVTSPASSSAS-PPLSPTSRRGLDKRRREG---GAGR 35
OsDREB1F        -----------------MDTEDTSSASSSSVSPPSSPGGGHHHRLPPKRRAGRKKFRETR 43
                                                                        :

HvCBF7          HPTYRGVRMRSWG-KWVSEIREPRKKSRIWLGTFATAEMAARAHDVAALAIKGR-AAHLN 103
DT709888        HPTYHGVRMRSWG-KWVSEIREPRKKSRIWLGTFATAEMAARAHDVASLAIKGRGAAHVN 66
DT709945        HPTYRGVRMRTWG-KWVSEIREPRKKSRIWLGTFDTPEMAARAHDVAAIAIKGR-AAHLN 115
DT695008        HPTYRGVRMRAWG-KWVSEIREPRKKSRIWLGTFPTPEMAARAHDAAALVVKGP------ 88
OsDREB1F        HPVYRGVRARAGGSRWVCEVREPQAQARIWLGTYPTPEMAARAHDVAAIALRGERGAELN 103
                **.*:*** *: * :**.*:***: ::******: *.********.*::.::*

HvCBF7          FPDLAHELPRPATAAPKDVQAAAALAASAD--FPASATATANAGAKNPDGPEPNAASAST 161
DT709888        FPELADLLPRPASAAP---SARGGGRPSPD--VPRRAHA---------------------- 100
DT709945        FPDTAHELPRAASAAPKDVQAAAALAAAA---------------------------- 144
DT695008        ------------------------------------------------------------
OsDREB1F        FPDSPSTLPRARTASPEDIRLAAAQAAELYRRPPPPLALPEDPQEGTSGGGATATSGRPA 163


HvCBF7          PPDTAEDALFDLPDLLFDLRHGPPSCQVSCASTWDDDVAFAGPGAGVFRLEEPLQWEY 219
DT709888        ------------------------------------------------------------
DT709945        ------------------------------------------------------------
DT695008        ------------------------------------------------------------
OsDREB1F        AVFVDEDAIFDMPGLIDDMARGMMLTPPAIGRSLDDWAAIDDD-DDHYHMDYKLWMD- 219
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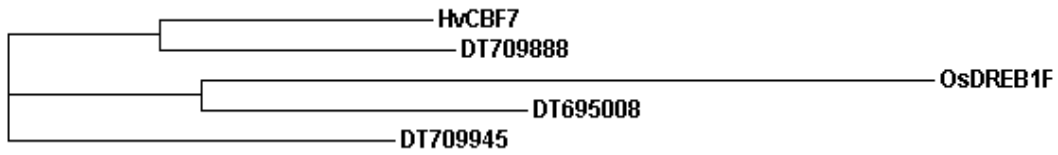

Figure A.3. Alignment of the amino acid sequences from putative tall fescue ESTs DT709888, DT695008, and DT709945 compared to similar CBF amino acid sequence from barley (HvCBF7) and rice (OsDREB1F). Also shown is the phylogeny of these sequences.

```
HvCBF11         ---MEW-----ACCGSGYSSSGTQSPAAGDGEEGS--YMTVSSAPPKRRAGRTKVRETRH 50
TaCBF11         ---MEW-----AYSGGGHSSSGTKSPAAGGREEGS--YMTVSSAPPKRRAGRTKVRETRH 50
OsDREB1E        ---MEW-----AYYGSGYSSSGTPSPVGGDGDEDS--YMTVSSAPPKRRAGRTKFKETRH 50
DT696484        GTRLERTDGVGAYCGSDHSPSGTQSPVGGAGEQDSSSYMTVSS-PPKRRTGRTKFKETRH 59
                   :*      *  *..:*.*** **..*  ::.*  ****** *****:****.:****

HvCBF11         PVYKGVRSRNPGRWVCEVREPQGKQRLWLGTFDTAEMAARAHDVAAMALRGRAACLNFAD 110
TaCBF11         PVYKGVRSRNPGRWVCEVREPHGKQRLWLGTFDTAEMAARAHDVAALALRGRAACLNFAD 110
OsDREB1E        PVYKGVRSRNPGRWVCEVREPHGKQRIWLGTFETAEMAARAHDVAAMALRGRAACLNFAD 110
DT696484        PVYKGVRSRNPGRWVCEVREPHGKQRIWLGTFDTAEMAARAHDFAALALRGRGACLNFSD 119
                ********************:****:*****:**********.**:*****.*****:*

HvCBF11         SPRRLPVPPQGAGHDEIRRAAVEAAELFRPAP-GQRN-AATVAAATAPPVALGNAELVAD 168
TaCBF11         SPRTLRVPPQGAGHEEIRRAAVEAAELFRPEP-GQRN-AATTEAPAASPADAGNAELVAN 168
OsDREB1E        SPRRLRVPPLGAGHEEIRRAAVEAAELFRPAP-GQHNAAAEAAAAVAAQATAASAELFAD 169
DT696484        SLRRLRAPPQVPSHDEIRRGG--SGALFRPGPSVQRN--AASEAAVVSPVDSWGEELVAS 175
                * * * .**  ..*:****..  :. **** *  *:*  *   *.... .   . **.*.

HvCBF11         SPYYP--MDGLESEMQGYLDMAHGMLIEPPPMAWPSTWIEEDYDCEISLWNY 218
TaCBF11         SPYHL--MDGLEFEMQGYLDMAHGMLIEPPPMAGPSTWIEEDYDCEVSLWNY 218
OsDREB1E        FPCYP--MDGLEFEMQGYLDMAQGMLIEPPPLAGQSTWAEEDYDCEVNLWSY 219
DT696484        CPYFPMVMGGLEFXMQGYLDMAQGM-------------------------- 200
                 * .   *.***  ********:**
```

Figure A.4. Alignment of the putative amino acid sequence from tall fescue EST DT696484, compared to similar CBF amino acid sequence from barley (HvCBF11 and HvCBF1), wheat (TaCBF11), and rice (OsDREB1E). Also shown is the phylogeny of these sequences.

```
HvCBF3          MDMGLEVSSSSPSSSPVSSSPEHAARRA---SPAKRPAGRTKFRETRHPVYRGVRRRGNT 57
HvCBF10A        MDMGLEVSSSPPSSS-----NENASGRS---STAKRPAGRTKFRETRHPVYRGVRRRGNA 52
TC3426          MDMGLEVSSSSTSSSSSSASVSASSEHSRVMAPAKRPAGRTKFRETRHPVYRGVRRRGNA 60
DT704637        ------------------------------------ARGKFRETRHPLYRGVRRRGTA 22
HvCBF13         --MGMDLCSSSPSSS-----VSSSPEHAS--GPAKRPVGRTKFRETRHPVYHDVRRRGNA 51
                                                .* ********:*:.*****.:

HvCBF3          ERWVCEVRVPGKRGARLWLGTYATAEVAARANDAAMLALGGRSAA-CLNFADSAWLLAVP 116
HvCBF10A        ERWVCEVRVPGKRGARLWLGTYATAEIAARANDAAMLALGGRSAA-RLNFSDSAWLLAVP 111
TC3426          GRWVCEVRVPGKRGARLWLGTYATAEAAARANDAAMLTLGGCSAS-CLNFADSAWLLAVP 119
DT704637        GRWVCEVRVPGKRGARLWLGTYATAEAAARANDAAMIALGGRSAATCLNFADSAWLLAVP 82
HvCBF13         GRWVCEVRVPSKRGARLWLGTYLTAGAAARANDAAMLALGGRSAR-RLNFADSAWLLAVP 110
                 *********.********** **  *********::*** **   ***:*********

HvCBF3          SALSDLADVRRAAVEAVADFQRREAADGSLAIAVPKEASSGAPSLSPSSGSDSAGSTGTS 176
HvCBF10A        SAHSDLADVRRAAVEAVSDLQRREAAGGSISATVDEEASCGAPAES-SSESDGAGSSETS 170
TC3426          CAVADLADVRRAAVEAVEDFQRREAA--SVPLASQEPTSSDSSAVS---SPDNAGSPETS 174
DT704637        SQLSDLADIRAAGIESVPDFQRRQTG------TITEEV---------------------- 114
HvCBF13         FALSDLADVRRTGLQAVANFQRREAASGLITRTVADSAD---------------SSETS 154
                   :****:* :.:::* ::***::.      :  . .

HvCBF3          EPSANGEFEGPVVMDSEMFRLDLFPEMDLGSYYMSLAEALLMDPPPTATIIHAYEDNG-- 234
HvCBF10A        KPSADGDLAVPVGMDIEMFRLDFFPEMEFGSYYASLAEALLMDPPPVANSTGAYWDNGEF 230
TC3426          EPPAHGEIDG-----RDMFRLDLFPEIDLGSYYLSLAEALLMDPPPMATANGSSWDN--- 226
DT704637        ------------------------------------------------------------
HvCBF13         EPSADGDFELPVAMDSDMFRLDFLPEMDLGSYYVSLAEALLMDPPSTATIIDAHRDNGDG 214


HvCBF3          -DGGADVRLWSYSVDM---------------------- 249
HvCBF10A        GEVATEFALWS--------------------------- 241
TC3426          ---------------------------------------
DT704637        ---------------------------------------
HvCBF13         AKVFLFWEKTLYSKDFEAVSNPSLNPEKGNIPSRLHVP 252
```

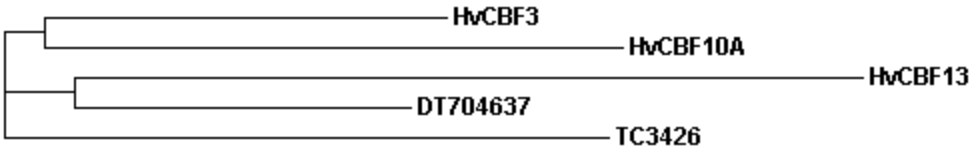

Figure A.5. Alignment of the putative amino acid sequences from tall fescue ESTs DT704637 and TC3426 compared to similar CBF amino acid sequence from barley (HvCBF3, HvCBF10A, and HvCBF13) Also shown is the phylogeny of these sequences.

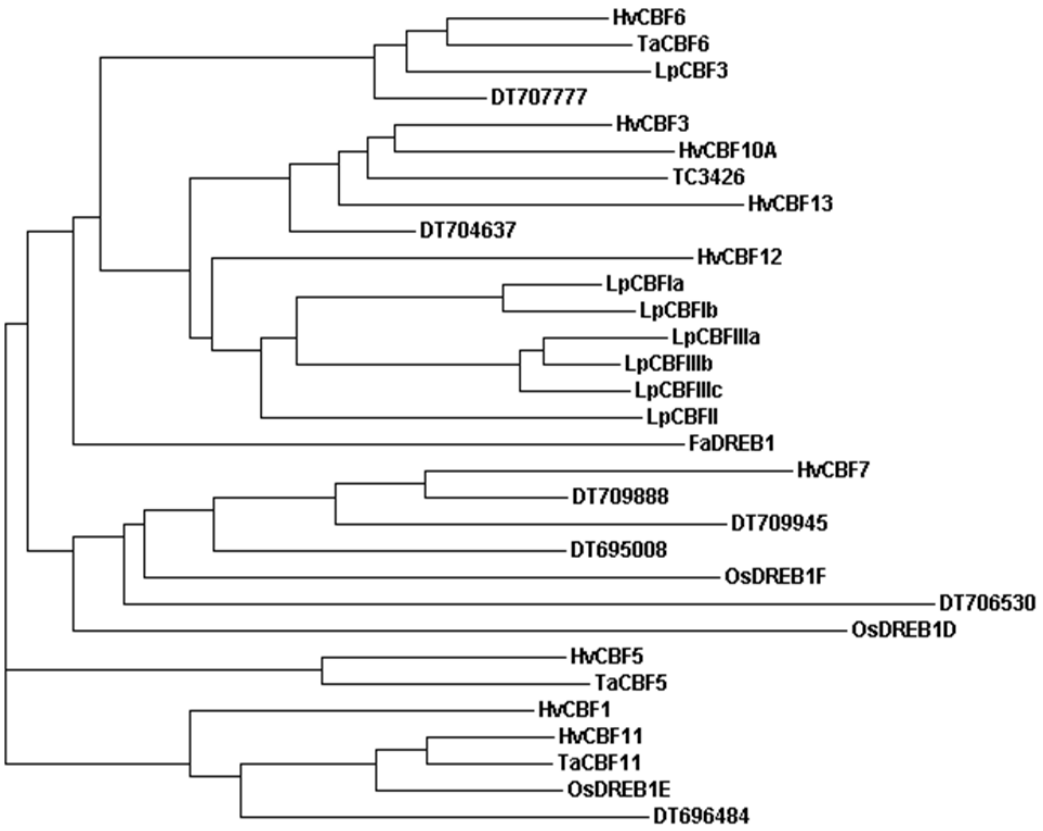

Figure A.6. Phylogeny of the putative amino acid sequences from all tall fescue ESTs compared to similar CBF amino acid sequence from barley, wheat, rice, and perennial ryegrass.

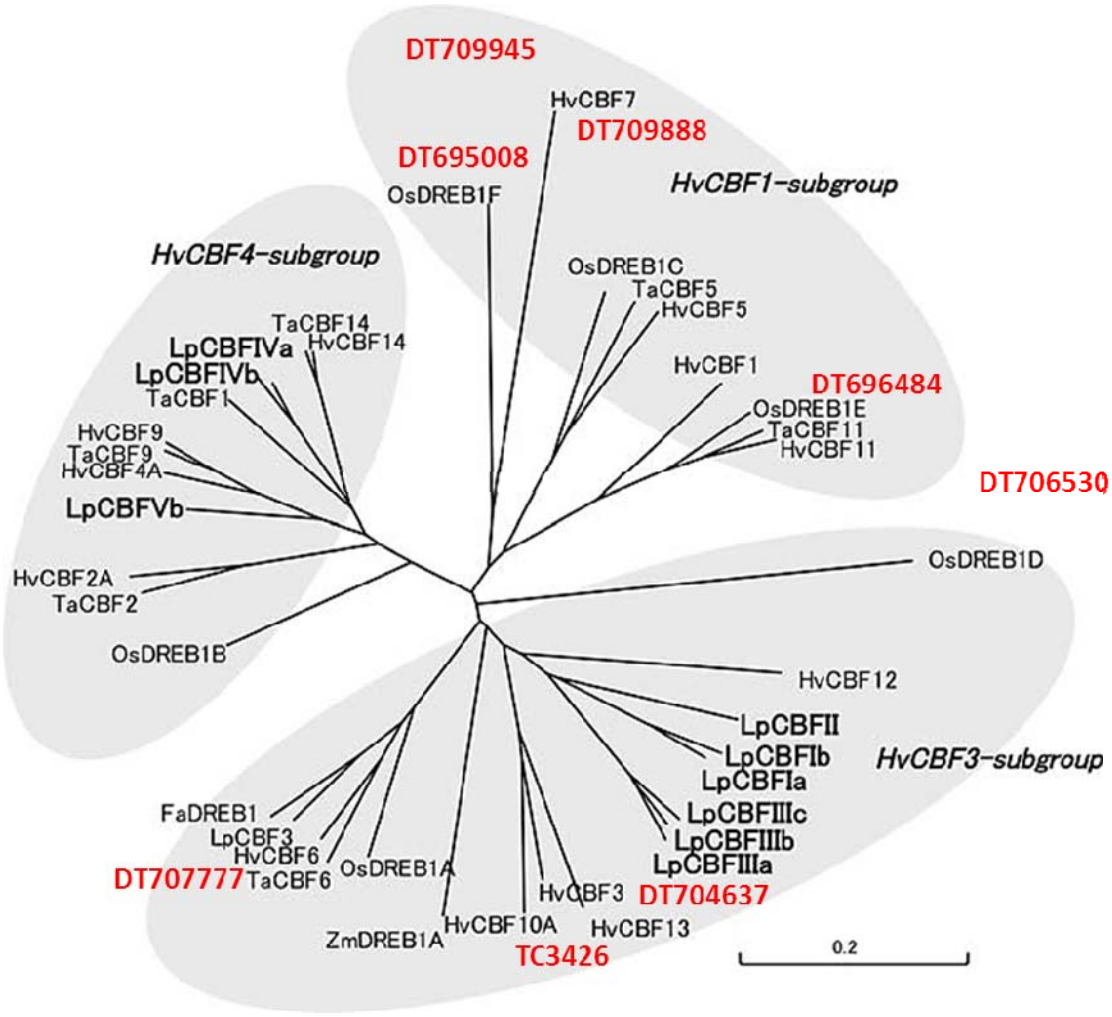

Figure A.7. Phylogenetic relationship of monocot CBF proteins. Scale indicates branch length (taken from Tamura and Yamada, 2007). Inserted (in red) are the tall fescue EST sequences that have highest similarity based on their putative protein sequence.

```
TaP5CS          ------------------------------------------------------------
HvP5Ca          ------------------MASADPNR-SFIKDVKRIIIKVGTAVITRNDGRLALGRIGS 40
OsP5CSa         ------------------MASVDPSRSAFVRDVKRVIIKVGTAVVSRQDGRLALGRVGA 41
SaP5CS          ------------------MATADRTR-TFMKDVKRVIIKVGTAVVTRHDGRLALGRLGA 40
SoP5CS          ------------------MATVDRTR-TFMKDVKRVIIKVGTAVVTTHDGRLALGRLGA 40
ZmP5CSa         ------------------------------------------------------------
TC144           ------------------MENSDSTR-CFVRDVKRIVIKVGTAVVTGQNGRLAMGRLGA 40
HvP5CSb         ---------------AAADLENSDSTR-CFVRDVKRIVVKVGTAVVTGQNGRLAMGRLGA 44
OsP5CSb         MGRGGIGGAGLVAAVAKADVENTDSTR-GFVKDVKRIIIKVGTAVVTGPNGRLAMGRLGA 59
ZmP5CSb         ------------------METADPAR-AFVKDVKRIIIKVGTAVVTGMNGRLAMGRLGS 40
SbP5CS          MGRGGIG------GAVGMAMENADSAR-AFVKDVKRIIIKVGTAVVTGQNGRLAMGGLGS 53


TaP5CS          ------------------------------RYRKLVNSSFADLQKPQMELDGKACAAVGQ 30
HvP5Ca          LCEQVKDLNAQGYEVIMVTSGAVGVGRQRLRYRKLVNSSFADLQKPQMELDGKACAAVGQ 100
OsP5CSa         LCEQVKELNSLGYEVILVTSGAVGVGRQRLRYRKLVNSSFADLQKPQMELDGKACAAVGQ 101
SaP5CS          LCEQVKELNALGYEVIIVTSGAVGVGKQRLKYRKLVNSSFADLQKPQMELDGKACAAVGQ 100
SoP5CS          LCEQVKELNALRYEVIIVTSGAVGVGKQRLKYRKLVNSSFADLQKPQMELDGKACAAVGQ 100
ZmP5CSa         ------------------------------------------------------------
TC144           LCEQVKELNFQGYEVILVTSGAVGVGRQRLKYRKLINSSFADLQNPQLDLDGKACAAVGQ 100
HvP5CSb         LCEQVKELNFQGYEVILVTSGAVGVGRQRLKYRKLINSSFADLQNPQLDLDGKACAAVGQ 104
OsP5CSb         LCEQVKQLNFEGYEVILVTSGAVGVGRQRLKYRKLVNSSFADLQNPQMDMDGKACAAVGQ 119
ZmP5CSb         LCEQVKQLNFQGYEVILVTSGAVGVGRQRLQYRKLIHSSFADLQNPQMNFDGKACAAVGQ 100
SbP5CS          LCEQVKQLNFQGYEVILVTSGAVGVGRQRLQYRKLIHSSFADLQNPQMNFDGKACAAVGQ 113

TaP5CS          SGLMALYDMLFTQLDVSSSQLLVTDSDFDNSNFRERLRETVESLLELRVIPIFNENDAIS 90
HvP5Ca          SGLMALYDMLFTQLDVSSSQLLVTDSVFDNSNFRERLRETVESLLELRVIPIFNENDAIS 160
OsP5CSa         SGLMALYDMLFNQLDVSSSQLLVTDSDFENPKFREQLTETVESLLDLKVIPIFNENDAIS 161
SaP5CS          SGLMALYDMLFTQLDVSSSQLLVTDSDFENPNFRERLRETVESLLDLKVVPIFNENDAIS 160
SoP5CS          SGLMALYDMLXTQLDVSSSQLLVTDNDFENPNFRERLRETVESLLDLKVVPIFNENDAIS 160
ZmP5CSa         ----------------------------------------------------PIFNENDAIS 10
TC144           SGLMAIYDTLFSQLDVTSSQLLVTDRDFRDPSFGHQLRETVVSLLDLKVIPVFNENDAIS 160
HvP5CSb         SGLMAIYDTLFSQLDVTSSQLLVTDRDFRDPSFGHQLRETVVSLLDLKVIPVFNENDAIS 164
OsP5CSb         SVLMAIYDTLFSQLDVTSSQLLVTDRDFMDPSFGNQLRETVNSLLDLKVIPVFNENDAIS 179
ZmP5CSb         SVLMAIYDTLFSQLDVTSSQLLVTDRDFKDPSFGDQLRETVFSLLDLKVVPLFNENDAIS 160
SbP5CS          SGLMAICDTLFSQLDVTSSQLLVTDRDFRDPSFGDQLRETVFALLNLKVIPLFNENDAIS 173
                                                          *:********

TaP5CS          TRKAPYEDSSGIFWDNDSLAGLLALELKADLLVLLSDVDGLYSGPPSEPSSKLIHTYIKE 150
HvP5Ca          TRKAPYEDSSGIFWDNDSLAGLLALELKADVLVLLSDVDGLYSGPPSEPSSKLIHTYIKE 220
OsP5CSa         TRKAPYEDSSGISWDNDSLAGLLALELKADLLILLSDVDGLYSGPPSEPSSKIIHTYIKE 221
SaP5CS          TRKAPYEDSSGIFWDNDSLAGLLAIELKADLLVLLSDVDGLYNGP-SEPQSKIIHTYIKE 219
SoP5CS          TRKAPYEDSSGIFWDNDSLAGLLAIELKADLLVLLSDVDGLYNGPPSEPQSKIIHTYIKE 220
ZmP5CSa         TRKAPYEDSSGIFWDNDSLAGNSARGLKADLLGLLSDVDGLYSGPPSEPGSKIIHTYIKD 70
TC144           TRKAPYEDSSGIFWDNDSLATLLAKELDADLLIMLSDVEGLFSGPPSDPQSKIIHTYINE 220
HvP5CSb         TRRAPYEDSSGIFWDNDSLATLLAKELDADLLIMLSDVEGLYSGPPSDPQSKIIHTYINE 224
OsP5CSb         TRRQPYEDSSGIFWDNDSLARLLAQELKADLLIMLSDVEGLYSGPPSDPQSKIIHTYVHE 239
ZmP5CSb         TRRQPYEDSSGIFWDNDSLAALLAAELNADLLIMLSDVEGLYSGPPSDPQSKIIHTYVNE 220
SbP5CS          TRRQSDEDSSGVFWDNDSLAALLAAELNADLLIMLSDVEGLYSGPPSDPQSKTIHTYVNE 233
                **: . *****: *******   *  *.**:* :****:**:.** *:* ** ****:::

TaP5CS          KHYH-EITFGDKSRVGRGG-MTAKVQAAVWAATGGVPVVITSGCASQSLVKVLRGEKIGT 208
HvP5Ca          KHYH-EITFGDKSRVGRGG-MTAKVQAAVWASTGGVPVVITSGCASQSLVKVLRGEKIGT 278
OsP5CSa         KHQQ-EITFGDKSRVGRGG-MTAKVKAAVLASNSGTPVVITSGFENRSILKVLHGEKIGT 279
SaP5CS          KHHN-EITFGDKSRVGRGG-MTAKVKAAFVASNSGTPVVITSRFASQSILRVLQGEKIGT 277
SoP5CS          KHHNGDHFFGGEVHVLVEEESQLKRRLLSWLQTVARLLLLQVDFASQSILRVLQGEKIGT 280
ZmP5CSa         KHYS-GITFGDKSRVGRGG-MTAKVKAAFVASNSGTPVVITSGFASQSIVRVLQGEKIGT 128
TC144           KHGK-LINFGEKSRVGRGG-MQAKVAAAVSAASKGVPAVIASGFVVDSIIKVMRGEKIGT 278
HvP5CSb         KHGK-LINFGEKSRVGRGG-MQAKVAAAVTAASKGVPAVIASGFVTDSIIKIMRGEKIGT 282
OsP5CSb         QHGK-LISFGEKSRVGRGG-MQAKVAAAFTASSKGIPVVIASGFAIDSIIKVMRGEKIGT 297
ZmP5CSb         KHGK-LISFGEKSSVGRGG-MQAKVTAAANAASKGVPVVIASGFATDSIITVLKGEKIGT 278
SbP5CS          KHGK-LISFGEKSNVGRGG-MQAKVAAAANAASKGVPVVIASGFATDSIIKVLKGEKIGT 291
                :*      ** :  *        *        . .   ::       *:: :::******
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TaP5CS          LFHKNASLWEPSKDTSVREMAVAARDCPRHLQNLSSEERKKILLDVADALEANEDLIRSE 268
HvP5Ca          LFHKNASSWEPSKDASVREMAVAARDCSRRLQNLSSEERKKILLDVADALEANEDLIRSE 338
OsP5CSa         LFHKNANLWESSKDVSTREMAVAARDCSRHLQNLSSEERKKILLDVADALEANEDLIRSE 339
SaP5CS          LFHKDASLWEPSKDVSAREMAVSARECSRRLQNLSSDERKKILLDVADALEENEDLIKAE 337
SoP5CS          LFHKDASLWEPSKDVSAREMAVSARECSRCLQNLSSDERKKILLDVADALEENEDLIKTE 340
ZmP5CSa         LFHKDASLWEPSKDVSAREMAVAARECSRRLQNLSSDERKKILLDIADALEQNEDLIRTE 188
TC144           LFHNEANIWDCSKEVTTREMAVAAKDCSRHLQNLSSEERKKILLDIADALEANEDLIISE 338
HvP5CSb         LFHNEANVWDCTKEVTTREMTVAAKDCSRHLQNLSSEERKKILLDIADALEANVGLIIAE 342
OsP5CSb         LFHREANQWGCSKEATAREMAVAARDCSRHLQKLSSEERKKILLDIADALEANEDLITSE 357
ZmP5CSb         LFHNEANLWACSKEATAREMSVAARDCSRRLQKLSSEERKQILLDIADALEANEDAIMSE 338
SbP5CS          LFHNEANLWECSKEATAREMAVAARDCSRRLQKLSPDERKKILLDIADALEANEGAIRSE 351
                ***.:*. *  :*:.:.***:*:*::*.* **:**.:***:****:***** *  . * :*

TaP5CS          NEADLAAAHEAGYESALVSRLTLKPGKIASLAKSVRTLANMEDPINEILKRTEVADGLVL 328
HvP5Ca          NEADVAAAHEAGYESALVARLTLKPGKIASLAKSVRTLANMEDPINEILKRTEVADGLVL 398
OsP5CSa         NEADVAAAQVAGYEKPLVARLTIKPGKIASLAKSIRTLANMEDPINQILKKTEVADDLVL 399
SaP5CS          NEADVAAAQDAGYEKSLIARLTLKPGKMASLAKSIRTLAHMEDPINQILKRTEVAEDLVL 397
SoP5CS          NEADVAAAQDAGYEKSLIARLTLKPGKIASLAKSIRTLAHMEDPINQILKRTEVAEDLVL 400
ZmP5CSa         NEADVSAAQDAGYQKSLVDRLTLKPEKIASLAKSIRTLANMEDPINQILKRTEVAEDLVL 248
TC144           NEADLAVAQDSGYEKSLVSRMTLKAGRITSLAGSIRAIADMEDPISHTVKKTEVAKDLVY 398
HvP5CSb         NEADLAAAQDAGYEKSLVARMTLKAGKITSLAESIRAIADMEDPISHTLKKTEVAKDLVF 402
OsP5CSb         NQADLDLAQDIGYDKSLVARMTIKPGKIKSLAGSIREIADMEDPISHTLKRTEVAKDLVF 417
ZmP5CSb         NDADVEAAQVAGYEKSLVARMTLKPGKITNLARSIRKTADMEDPISHTLKRTEVAKDLVF 398
SbP5CS          NEADVEAAQGAGYEKSLVARMTLKPGKITNLARSIRAIADMEDPISHTLKRTEVAKDLVF 411
                *:**:  *:  **:..*: *:*:*. :: .** *:*  *.*****.. :*:****..**

TaP5CS          EKTSCPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEAMRSNAILHKVITNAIPD 388
HvP5Ca          EKTSCPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEAMRSNAILHKVITNAIPD 458
OsP5CSa         EKTSCPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAIRSNTILHKVITDAIPR 459
SaP5CS          EKTSCPLGVLLIVFESRPDALVQIASLAIRSGNGLLLKGGKEAMRSNTVLHKVITGAIPS 457
SoP5CS          T--CCPLGVLLIFFESRPDALVQRASLAIRSGNPLLLKGGKQPMRSNTVLHKVITGAIPS 458
ZmP5CSa         EKTSCPLGVLLIVFESRPDALVQIASLAVRSGNGLLLKGGKEAMRSNTVLHKVITGAIPD 308
TC144           EKMYCPLGVLLIIFESRPDALVQIAALAIRSGNGLLLKGGKEAMRSNTILHKVITGVIPD 458
HvP5CSb         EKMYCPLGVLLIIFESRPDALVQIAALAIRSGNGLLLKGGKEAMRSNTILHKVITSVIPD 462
OsP5CSb         EKTYCPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEAMRSNTILHKVITGAIPD 477
ZmP5CSb         EKAYCPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEVMRSNAILHKVITGVIPD 458
SbP5CS          EKAYCPLGVLLIIFESRPDALVQIASLAIRSGNGLLLKGGKEAMRSNAILHKVITGVIPD 471
                    ********.********** *:**:**** *******: :***::******..**

TaP5CS          NVGEKLIGLITTRDEIADLLKHDDVIDLVIPRGSNKLVAQIKSSTKIPVLGHADGVCHVY 448
HvP5Ca          NVGEKLIGLITTRDEIADLLKLDDVIDLVIPRGSNKLVAQIKASTKIPVLGHADGVCHVY 518
OsP5CSa         NVGEKLIGLVTTRDEIADLLKLDDVIDLVIPRGSNKLVSQIEASTKIPVLGHADGICHVY 519
SaP5CS          NVGEKLIGLVTSRDKIADLLKLDDVIDLAIPRGSNKLVSQIKASTKIPVLGHADGICHVY 517
SoP5CS          NVGEKLIGLVTSRDEIADLLKLDDVIDLVIPRGSNKLVSQIKTSTKIPVLGHADGICHVY 518
ZmP5CSa         NVGQKLIGLVTSRDEIADLLKLDDVIDLVIPRGSNKLVSQIKASTKIPVLGHADGICHVY 368
TC144           DVGKKLIGLVKSKDEIADLLKLDDVIDLVIPRGSNKLVSQIKAATKIPVLGHADGICHVY 518
HvP5CSb         GVGKKLIGLVKSKEEIADLLKLDDVIDLVIPRGSNRLVSQIKATTKIPVLGHADGICHVY 522
OsP5CSb         VVGKKLIGLVKNKDEIADLLKLDDVIDLVIPRGSNKLVSQIKAATKIPVLGHADGICHVY 537
ZmP5CSb         TVGKKLIGLVTSKEEIADLLALDDVIDLVIPRGSKSLVSQIKATTKIPVLGHADGICHVY 518
SbP5CS          IVGKKLIGHVTSKDEIADLLALDDVIDLVIPRGSKNLVSQIKATTKIPVLGHAGGICHVY 531
                 **:**** :.:::*****  ******.*****: **:**:::*********.*:****

TaP5CS          IDKSADMDMAKRIVMDAKIDYPAACNAMETLLVHKDLMKIPELNDILVALKAAGVNLYCG 508
HvP5Ca          IDKSADMDMAKRIVVDAKIDYPAACNAMETLLVHKDLMKTPELDDILVALKTAGVNLYCG 578
OsP5CSa         IDKSADMDMAKHIVMDAKIDYPAACNAMETLLVHKDLMKSPGLDDILVALKTEGVNIYGG 579
SaP5CS          IDKSADMNMAKRIVMDAKIDYPAACNAMETLLVHKDLIKAPGLEDLLLSLKTEGVTLYGG 577
SoP5CS          IDKSADMNMAKRIVMDAKIDYPAACNAMETLLVHKDLIKAPGLEDLLLSLKTEGVTLYGG 578
ZmP5CSa         IDKSADMNMAKRIVMDAKTDYPAACNAMETLLVHKDLIKAPGLDDILLSLKTEGVAIYGG 428
TC144           IDKSADMDMAKRIVLDAKVDYPAACNAMETLLVHKDLNKTEGLDDLLMELANEGVVIYGG 578
HvP5CSb         IDKSADMDMAKRIVLDAKVDYPAACNAMETLLVHKDLNKTEGLDDLLMELAKEGVVIYGG 582
OsP5CSb         IDKSADMDMAKRIVLDAKVDYPAACNAMETLLVHKDLNRTEGLDDLLVELEKEGVVIYGG 597
ZmP5CSb         IDKSADMDMAKRIVLDAKIDYPAACNAMETLLVHKDLNKSEGLDDLLVELEKEGVVIYGG 578
SbP5CS          IDKSADMDMAKRIVLGAKVDYPAACNAMETLLVHKDLNKSEGLDDLLVELEKEGVVIYGG 591
                *******:***:**:.** ****************** :   *:*:*: *   ** :* *
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TaP5CS          PVAHKVLGYPKADSLHLEYSSMACTVEIVDDVQSAIDHIHRYGSAHTDCVVTTDDKVAET 568
HvP5Ca          PVARKILGYPKADSLHLEYSSMACTVEIVDDVQSAIDHIHRYGSAHTDCVVTTDDTVAET 638
OsP5CSa         PIAHKALGFPKAVSFHHEYSSMACTVEFVDDVQSAIDHIHRYGSAHTDCIVTTDDKVAET 639
SaP5CS          PVAQELLCIPKADSFHHEYSSMACTIEFVDDVQAAIDHIHRYGSGHTDCIVTTDDKVAET 637
SoP5CS          PVAQELLCIPKADSFHHEYSSMACTIEFVDDVQAAIDHIHRYGSGHTDCIVTTDDKVAET 638
ZmP5CSa         PVAHEVLCIPKADSFHHEYSSMACTIEFVDDVQSAINHIHRYGSAHTDCIITTDDKVAET 488
TC144           PVAHDTLKVPKVDSFHHEYSSMACTLEFVDDVQSAIDHINRYGSAHTDCIITTDKKSADT 638
HvP5CSb         PVAHDTLKVPKVDSFHHEYSSMACTLEFVDDIQSAIDHINRYGSAHTDCIITTDKKSADT 642
OsP5CSb         PVAHDTLKLPKVDSFHHEYNSMACTLEFVDDVQSAIDHINRYGSAHTDCIITTDGKAAET 657
ZmP5CSb         PVAHDKLKVPKVDSFRHEYSSMACTVEFVDDVQSAIDHINRYGSAHTDCIITTDRSAAEA 638
SbP5CS          PVAHDKLKVPKVDSFRHEYSSMACTLEFVDDVQSAIDHINRYGSAHTDCIITTDESAAEA 651
                *:*:. *  **. *:: **.*****:*:***:*:**:**:****.****::*** . *::

TaP5CS          FLRQVDSAAVLYNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWLLRGKGQ 628
HvP5Ca          FLRQVDSAAVLYNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWLLRGKGQ 698
OsP5CSa         FLRRVDSAAVFHNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWILRGRGQ 699
SaP5CS          FLRQVDSAAVFYNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWIMRGSGQ 697
SoP5CS          FLRQVDSAAVFYNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWIMRRSGQ 698
ZmP5CSa         FLRQVDSAAVFHNASTRFSDGARFGLGAEVGISTGRIHARGPVGVEGLLTTRWIMRGSGQ 548
TC144           FLQQVDSA---------------------------------------------------- 646
HvP5CSb         FLQQVDSAAVFHNASTRFCDGTRFGLGAEVGISTGRIHARGPVGVDGLLTTRCILRGSGQ 702
OsP5CSb         FLQQVDSAAVFHNASTRFCDGARFGLGAEVGISTGRIHARGPVGVDGLLTTRCILRGSGQ 717
ZmP5CSb         FLQQVDSAAVFHNASTRFCDGTRFGLGAEVGISTERIHARGPVGVDGLLTTRCILRGSGQ 698
SbP5CS          FLQQVDSAAVFHNASTRFCDGTRFGLGAEVGISTGRIHARGPVGVDGLLTTRCILRGSGQ 711
                **::****

TaP5CS          VVNGDKDVEYTHKSLPLQ 646
HvP5Ca          IVNGDKDVVYTHKSLPLQ 716
OsP5CSa         VVNGDKDVVYTHKSLPLQ 717
SaP5CS          VVNGDKDIAYTHKNLPLQ 715
SoP5CS          VVNGDKDIAYTHKNLPLQ 716
ZmP5CSa         VVNGDKNVAYTHKNLPLQ 566
TC144           ------------------
HvP5CSb         VVNGDKGVVYTHKDLPLQ 720
OsP5CSb         VVNGDKGVVYTHRELPLQ 735
ZmP5CSb         VVNGDKGVVYTHKDLPLQ 716
SbP5CS          VVNGDKGVVYTHKDLPLQ 729
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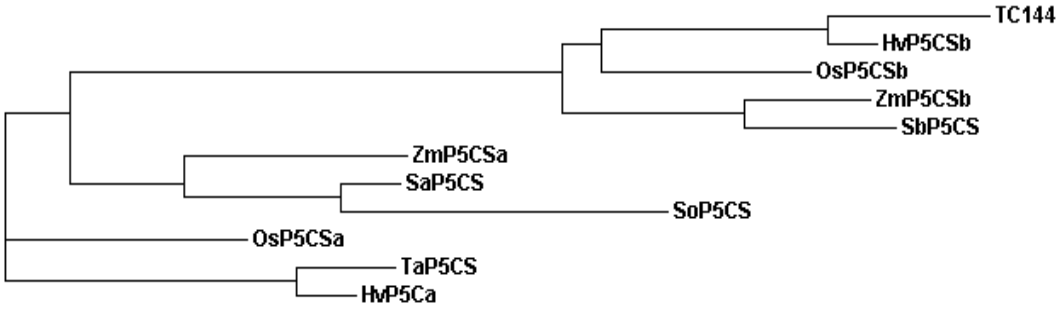

Figure A.8. Alignment of the putative amino acid sequence from tall fescue EST TC144 compared to similar proline (P5CS) amino acid sequence from barley (HvP5CSa and b), wheat (TaP5CS) rice (OsP5CSa and b) Corn (ZmP5CSa and b) sorghum (SbP5CS) sugar cane (So P5CS) and hardy sugar cane (SaP5CS). Also shown is the phylogeny of these sequences.

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Taalpha1A       MREIISIHIGQAGIQVGNACWELYCLEHGIQQDGTMPSDTTVGVAHDAFNTFFSETGAGK 60
Taalpha1B       MREIISIHIGQAGIQVGNACWELYCLEHGIQQDGTMPSDTTVGVAHDAFNTFFSETGAGK 60
Taalpha1D       MREIISIHIGQAGIQVGNACWELYCLEHGIQQDGTMPSDTTVGVAHDAFNTFFSETGAGK 60
TC159           MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQTSGDKTIGGGDDAFNTFFSETGAGK 60
TC9395          MRECISIHIGQAGIQVGSACWELYCLEHGIQPDGQTSGDKTIGGGDDAFNTFFSETGAGK 60
Taalpha4B       MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQTNGDKTIGGGDDAFNTFFSETGAGK 60
Taalpha4D       MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQTNGDKTIGGGDDAFNTFFSETGAGK 60
Taalpha4A       MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQTNGDKTIGGGDDAFNTFFSETGAGK 60
Taalpha3A       MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPGDKTVGGGDDAFNTFFSETGAGK 60
Taalpha3B       MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPGDKTVGGGDDAFNTFFSETGAGK 60
TC4022          MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPGDKTVGGGDDAFNTFFSETGAGK 60
TC6235          MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPGDKTVGGGDDAFNTFFSETGAGK 60
OsalphatubA1    MRECISIHIGQAGIQVGNACWELYCLEHGIQADGQMPSDRTVGGGDDAFNTFFSETGAGK 60
Taalpha5B       MREIISIHIGQAGIQVGNSCWELYCLEHGIQPDGLMPSDTSVGVAKDAFNTFFSETGSGK 60
Taalpha5D       MREIISIHIGQAGIQVGNSCWELYCLEHGIQPDGLMPSDTSVGVAKDAFNTFFSETGSGK 60
                *** ************.:************ **   .* ::* ..***********:**

Taalpha1A       HVPRAIFVDLEPTVIDEVRTGAYRQLFHPEQLISGKEDAANNFARGHYTVGKEIVDLCLD 120
Taalpha1B       HVPRAIFVDLEPTVIDEVRTGAYRQLFHPEQLISGKEDAANNFARGHYTVGKEIVDLCLD 120
Taalpha1D       HVPRAIFVDLEPTVIDEVRTGAYRQLFHPEQLISGKEDAANNFARGHYTVGKEIVDLCLD 120
TC159           YVPRAVFVDLEPTVIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
TC9395          YVPRAVFVDLEPTGIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha4B       YVPRAVFVDLEPTVIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha4D       YVPRAVFVDLEPTVIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha4A       YVPRAVFVDLEPTVIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha3A       HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha3B       HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
TC4022          HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
TC6235          HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD 120
OsalphatubA1    HVPRAVFVDLEPTVIDEVRTGCYRQLFHPEQLINGKEDAANNFARGHYTIGKEIVDLCLD 120
Taalpha5B       HVPRALFVDLEPTVIDEVRTGAYRQLFHPEQLISHNEDAANNFARGHYTVGREVVDLCLD 120
Taalpha5D       HVPRALFVDLEPTVIDEVRTGAYRQLFHPEQLISHNEDAANNFARGHYTVGREVVDLCLD 120
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Taalpha1A       RVRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTIYPSPQVSTA 180
Taalpha1B       RVRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTIYPSPQVSTA 180
Taalpha1D       RVRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTIYPSPQVSTA 180
TC159           RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
TC9395          RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha4B       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha4D       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha4A       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha3A       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha3B       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
TC4022          RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
TC6235          RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
OsalphatubA1    RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS 180
Taalpha5B       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGRKSKLGFTIYPSPQISTA 180
Taalpha5D       RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGRKSKLGFTIYPSPQISTA 180
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Taalpha1A       VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLISQIISSLTT 240
Taalpha1B       VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLISQIISSLTT 240
Taalpha1D       VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLISQIISSLTT 240
TC159           VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT 240
TC9395          VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT 240
Taalpha4B       VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT 240
Taalpha4D       VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT 240
Taalpha4A       VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT 240
Taalpha3A       VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA 240
Taalpha3B       VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA 240
TC4022          VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA 240
TC6235          VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA 240
OsalphatubA1    VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA 240
Taalpha5B       VVEPYNSVLSTHSLIEHTDVVVLLDNEAIYDICKRSLDIERPTYTNLNRLISQVISSLTT 240
Taalpha5D       VVEPYNSVLSTHSLIEHTDVVVLLDNEAIYDICKRSLDIERPTYTNLNRLISQVISSLTT 240
                **************:***** :***********:****************:**:*****:

Taalpha1A       SLRFDGAINVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVPEITNAVFEPSS 300
Taalpha1B       SLRFDGAINVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVPEITNAVFEPSS 300
Taalpha1D       SLRFDGAINVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVPEITNAVFEPSS 300
TC159           SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS 300
TC9395          SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS 300
Taalpha4B       SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS 300
Taalpha4D       SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS 300
Taalpha4A       SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS 300
Taalpha3A       SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS 300
Taalpha3B       SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS 300
TC4022          SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS 300
TC6235          SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS 300
OsalphatubA1    SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS 300
Taalpha5B       SLRFDGAINVDITEFQTNLVPYPRIHFMLSSYAPIISAEKAFHEQHSVPEITNSVFEPSS 300
Taalpha5D       SLRFDGAINVDITEFQTNLVPYPRIHFMLSSYAPIISAEKAFHEQHSVPEITNSVFEPSS 300
                *******:***:.*********************:******:*** **.****:.*****

Taalpha1A       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTVQFVDWCPTGFKCGINYQPP 360
Taalpha1B       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTVQFVDWCPTGFKCGIKYQPP 360
Taalpha1D       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTVQFVDWCPTGFKCGINYQPP 360
TC159           MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
TC9395          MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha4B       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha4D       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha4A       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha3A       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha3B       MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
TC4022          MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
TC6235          MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
OsalphatubA1    MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP 360
Taalpha5B       VMAKCDPRHGKYMACCLMYRGDVVPKDVNSAVHSIKTKRTVQFVDWCPTGFKCGINYQPP 360
Taalpha5D       VMAKCDPRHGKYMACCLMYRGDVVPKDVNSAVHSIKTKRTVQFVDWCPTGFKCGINYQPP 360
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Taalpha1A       SVVPGGDLAKVQRAVCMISNNTAVAEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha1B       SVVPGGDLAKVQRAVCMISNNTAVAEVFLRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha1D       SVVPGGDLAKVQRAVCMISNNTAVAEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
TC159           TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
TC9395          TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha4B       TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha4D       TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha4A       TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha3A       SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha3B       SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
TC4022          SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
TC6235          SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
OsalphatubA1    SVVPSGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYSKRAFVHWYVGEGMEEGEFSE 420
Taalpha5B       TVVPGGDLAKVRRAVCMISNNTAVAEVFSRIDRKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
Taalpha5D       TVVPGGDLAKVRRAVCMISNNTAVAEVFSRIDRKFDLMYAKRAFVHWYVGEGMEEGEFSE 420
                :***.******:********.*:*.*** ***:******:********************

Taalpha1A       AREDLAALEKDYEEVGAEGADDE-GDEGDDY 450
Taalpha1B       AREDLAALEKDYEEVGAEGADDE-GDEGDDY 450
Taalpha1D       AREDLAALEKDYEEVGAEGADDE-GDEGDDY 450
TC159           AREDLAALEKDYEEVGAEGGDDEEGEDEDDY 451
TC9395          AREDLAALEKDYEEVGAEGGDHEEGEDEDDY 451
Taalpha4B       AREDLAALEKDYEEVGAEGGDDEDGEEDDDY 451
Taalpha4D       AREDLAALEKDYEEVGAEGGDDDDGEEDDDY 451
Taalpha4A       AREDLAALEKDYEEVGAEGGDDEDGEEDDDY 451
Taalpha3A       AREDLAALEKDYEEVGAEFDEGEDGDEGDEY 451
Taalpha3B       AREDLAALEKDYEEVGAEFDEGEDGDEGDEY 451
TC4022          AREDLAALEKDYEEVGAEFDEGEDGDEGDEY 451
TC6235          AREDLAALEKDYEEVGAEFDEGEDGDEGDKY 451
OsalphatubA1    AREDLAALEKDYEEVGAESDENEDGDDGDEY 451
Taalpha5B       AREDLAALEKDYEEVGAEGEDDE--DEGDEY 449
Taalpha5D       AREDLAALEKDYEEVGAEGEDDE--DEGDEY 449
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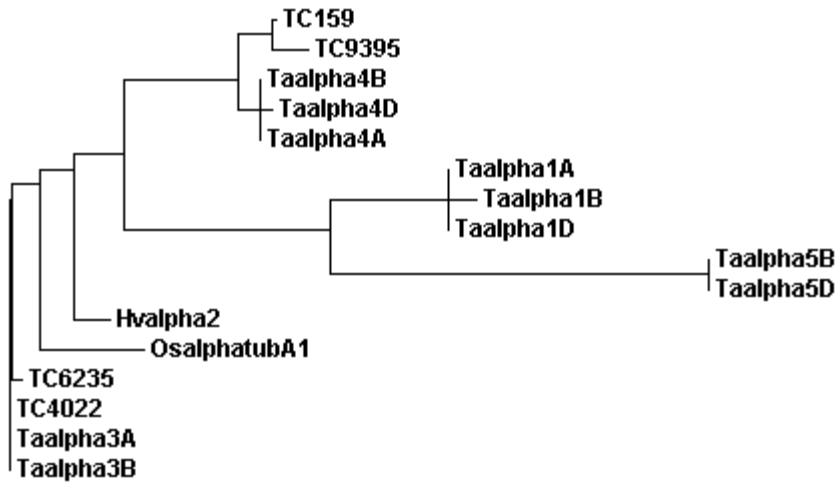

Figure A.9. Alignment of the putative amino acid sequences from tall fescue ESTs TC159, TC9395, TC6235, and TC4022 compared to similar α-tubulin amino acid sequence from barley (Hvalpha2), wheat (Taalpha1A, B, C; 3A and B; 4A, B, and D; 5B and D) and rice (OsalphatubA1). The EST TC4022 is highlighted in red since that EST had the greatest homology to primer sequence that was used for perennial ryegrass expression analysis of CBF. Also shown is the phylogeny of these sequences.

Conclusions and Final Remarks

This work has provided insights into the responses of endophyte and Mediterranean and Continental tall fescue germplasm production of metabolites when subjected to cold stress. Additionally, the creation of the first Mediterranean × Continental link map as well as identifying underlying genomic regions responsible for fall growth and winter survival was discussed. In chapter 2 the presence or absence of endophyte was examined in five diverse genotypes of tall fescue on the production of proline, fructan, mono- and disaccharides, and leaf extension rate (LER). Additionally freezing survivability of these genotypes at -3, -6, -9, and -12^{o}C was assessed in the presence or absence of endophyte. It was discovered, that unlike the enhancement found during other abiotic and biotic stresses, endophyte infection had little to no influence on LER, mono- and disaccharides, and freezing survivability. However, proline was different between E+ and E- at $P < 0.1$ and fructan concentrations at $P < 0.008$. It was apparent that plant genotype had a greater influence than endophyte infection on these measures at cold (4.5^{o}C) temperatures. However, results suggest that for proline and fructan responses there may be a specific plant genotype by endophyte interaction.

In chapter 3, seven genotypes were further examined for LER, proline, abscisic acid (ABA), fructan, mono- and disaccharides at 25.0 and 4.5^{o}C. Three Mediterranean (PI 200339, PI 610956, and Flecha) and four Continental accessions (PI 172423, PI 283297, PI 314684, and Kentucky-31) were investigated. At 4.5^{o}C the Continental accessions had greater concentrations of proline, ABA, and mono- and disaccharides; however, the Mediterranean accessions had greater LER and fructan concentrations. Proline and ABA concentrations were correlated to LER among the accessions examined.

Interestingly, one Continental genotype, PI 172423, had high concentrations of both proline and ABA while maintaining an LER comparable to that of the Mediterranean accessions. Therefore, it appears that these traits are separable and can be integrated into a single variety.

To accelerate breeding, it is useful to have a genetic map to identify portions of the genome responsible for traits of interest. In chapter 4, linkage maps were created from a cross between a Mediterranean (103-2) and Continental (R43-64) parents. This work represents the first linkage map of Mediterranean tall fescue as well as the use of DArTFest markers for the construction of these maps. Twenty-three linkage groups (LGs) were created for 103-2 and 20 LGs for R43-64. The parental maps were distinctly different in their sizes and marker densities. The parental map of 103-2 was only 1373 cM with 7.19 cM marker^{-1} while R43-64 had a total length of 1939 cM and a density of 3.44 cM marker^{-1}. Only seven LGs were common between the parental genotypes; it appears that the Mediterranean and Continental tall fescue types share the P genome which is comprised of seven chromosomes. The traits for fall growth (FG), measured by LER, and winter survival (WS) were collected over two years and three locations. The parental genotypes had QTL for FG and WS near the same genomic regions in both parental lines.

Lastly, expressed sequence tags (ESTs) were found in tall fescue that had high homology to know CBF, proline, and α-tubulin genes in various monocot species. However, expression analysis of these putative genes at 4.5^{o}C could not be completed due to multiple products being amplified during PCR.

Based on this work it appears that these two types of tall fescue are distinctive species. Furthermore, the development of the linkage map serves a tool for other breeders to accelerate breeding for specific traits of interest. In light of the crossing barriers between Mediterranean and Continental tall fescue and the inability to introgress the superior fall growth of Mediterranean types the following breeding strategy is suggested. Within the Mediterranean germplasm superior fall growth is present however winter hardiness is lacking; however, the Continental germplasm possess the opposite phenotypes. Therefore, selection for winter hardiness needs to be the primary objective of Mediterranean tall fescue. To this end, hundreds or even thousands of Mediterranean lines could be screened in cold chambers, as was described in chapter 2. This could identify superior genotypes in a matter of weeks. Additionally, if a rhizomatous phenotype can be found in Mediterranean germplasm, this may also aid or increase the ability of that germplasm pool to survive prolonged freezing conditions. The selection for Continental genotypes possessing heightened fall growth appears to be more complicated and labor intensive. Due to genotypes having different tillering capacity, which can affect the yield measurement, having plants on an equal playing field will be vital. Additionally, this measure could take longer due to the necessity of needing the correct lighting and temperature conditions to make an accurate assessment. This may mean being able to screen for this trait only during the fall months which could limit the number of individual genotypes that can be evaluated. In either case, progress seems possible and currently more achievable in increasing fall growth within the Continental germplasm pool. This is supported by the identification of a genotype in accession PI 172423 discussed in chapter 3 containing both winter survival and superior fall growth.

Vita

Ryan Michael Dierking was born to the parents of Michael Ray and Victoria Lynn Dierking on March 11, 1983. At the moment in time I had already made my parents proud and their love for me would only continue to bloom as I ventured on my life's, and at times, wayward path. Life, as it often happens, would deliver me to my first passion, and education, farming! However, at times it seems, and is still present, that my passion would be a blessing and a curse. More about that later. I am the eldest of two children. My sister, Amanda, was born a few year later in 1986. I first entered small rural school in 1988 at Beaufort Elementary, just west of Union, MO. I even remember the kindergarten teacher, Mrs. Burk. There I met my one lifelong best friend, Jason Lee Green. However, it was deemed that my friendship should be put on hold for roughly 10 years as my parents thought the best interest would be to move about 8 miles northwest outside the small river town of New Haven, MO. There, I completed my remaining primary and secondary education. At the time I thought that this was the worst idea that my parents have ever dreamt up. However, it seems fate thought it was a pretty good idea too. There I tried to fit in playing sports; since some athleticism was granted in my favor. These would include basketball, cross country, and track and field. I was a decent runner where my abilities led me to state six times. Additionally, as it turned out, I wasn't all that dumb and was able to graduate third in my high school class of 36. Well maybe there just wasn't any competition. Either way, due to this ranking some people thought that I should receive some free money, i.e. scholarships, for which I am eternally grateful. And so I was off to begin the next portion of my life at the University of Missouri. College life was great! In all reality college was fun, but I did try to focus on my studies of which

wait now where was I, Oh yeah I did manage a 3.7 GPA. Additionally, that is the time when I met my future wife, Emily Catherine Dierking née Dunn. It was on Friday March, 15 2003 sometime between 1:30 and 3:30 in the afternoon at Lake Genevia,WI for the 45th Maize Genetic Conference. It was that memorable. After some time and some convincing she decided that she would like to meet (i.e. go out on a date) for the first time in July. She actually came to watch me pull in the local tractor pull. After that, really, the rest is history. She became my wife on September, 24 2005. And then in June of 2006 we got the best dog money can buy for $150.00. We decided to name him, an Airedale Terrier, Fisher after the company Fischer Scientific. He is quite literally one of my pals that was able to help me unwind after some challenging days at work. Yes, I know I got a little ahead of myself, but I thought it would be a litter easier for all you to read if I tried to keep with one continuous line of thought. Ok back to 2005. I graduated that May with a B.S. in Agronomy and we, my wife and I, Fisher was not yet a part of our family, had decided to attend graduate school. I started my graduate school career, because in all essence that is really what it is, in January of 2006. I decided that it would be in my best interest to have Dr. Robert Kallenbach as my advisor. He mentored me throughout my M.S. degree and continued to do so though my studies as a Ph.D. student along with Dr. David Sleper. Rob and I had our disagreements at times mainly over my writing style that at times where he thought I would add somewhere around 15 subjects in every sentence and the reader was left wondering which particular topic matter I was discussing along with having a very long winded and complex sentence structure, and where I would be somewhat kind of most likely indecisive or that it would make some novelist scratch their heads wondering either where is the sentence going or, maybe more importantly, when it

is ever going to end; which my response would be I think all of what I had written was necessary to communicate my point to the reader; however, I seldom won except for this one instance. This entire journey has led to some valuable experiences that will no doubt have some influence on my remaining life. Speaking of which, I mentioned at the beginning of the immensely long paragraph that my first passion was farming. I truly love the land along with all it has to offer. I hope and dream of one day that I can return to my passion and fulfill a desire that eats at me every day. Currently, I dabble in the husbandry of registered Red Angus cattle on my father's family farm near Beaufort, MO. I hope that I can continue this in some form as I enter a new chapter on life's grand stage. I learned about hard work during my youth working with this piece of land with my father, and I think that lesson has paid off in the pages of this work. Additionally, I would like to mention my mother and her passion to see her children succeed. I definitely received a great deal of passion from my mother as well as some great recipes as she is one of the best cooks in the surrounding area where I grew up. I may be biased, but I think many a folk in that area would agree. And I would like to put in a word in for the rest of my family that has aided me throughout the years. My grandparents Edward and Darlene Buenemann for our Saturday morning fishing trips and our lazy afternoon out on the porch looking for airplanes and turtles; my deceased grandparents Raymond (Grandpa Dick) and Helen Dierking for our times on the farm and the smell of fresh bread; My Aunts Brenda and Beth for our outings (sixflags and the country fairs) as well as helping me pay for a few speeding tickets when money was short in college. My aunts Edlene and Pam and my cousins for our timeless memories. Thank you all for the impressions you laid upon me. And yes, I know that this is just one HUGE paragraph of

jumbled thoughts, but this is the only section throughout this entire body of work were I could finally let loose and jump around from one thought to the next. I hope you readers out there in space and time, or more accurately spacetime, will find this work informative and that it added to the collective knowledge of *Homo sapien* and perhaps was slightly entertaining too.