ALTERATIONS IN PROTEIN SYNTHESIS ASSOCIATED WITH COLD ACCLIMATION IN BERMUDA-GRASS (*CYNODON* SPP.)

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PREFACE

This study was conducted to provide new knowledge pertinent to improving turf bermudagrass for tolerance to freezing stress. Bermudagrass plants exhibit increased tolerance to freezing stress after cold acclimation (CA). Proteins synthesized in association with CA may mediate many of the physiological changes that contribute to increases in freezing tolerance. Specific objectives of this research were to characterize (a) changes in freezing tolerance exhibited by turf bermudagrasses 'Midiron' and 'Tifgreen' after CA in growth chambers, and (b) alterations in protein synthesis by crowns associated with CA of these varieties utilizing polyacrylamide gel electrophoresis (PAGE) of *in vivo* radiolabeled crown proteins. SDS (sodium dodecyl sulfate)-PAGE was used for one-dimensional analyses. For two-dimensional analyses, proteins were first separated by charge using IEF (isoelectric focusing)-PAGE, before being separated by size using SDS-PAGE.

I sincerely thank my doctoral committee--Drs. Charles M. Taliaferro (Chair), Jeffrey A. Anderson, David R. Porter, and Michael P. Anderson--for guidance and support in the completion of this research. I also thank Mrs. Rose M. Edwards and Mr. Glenn T. Drown for willing assistance in this research, and Dr. Dennis Martin, Mr. David Gerken, and Mr. Craig Evans for sharing expertise

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In conclusion, I include Psalm 30 in thanksgiving to God:

I will praise you, Lord, you have rescued me
and have not let my enemies rejoice over me.

O Lord, I cried to you for help
and you, my God, have healed me.
O Lord, you have raised my soul from the dead,
restored me to life from those who sink into the grave.

Sing psalms to the Lord, you who love him,
give thanks to his holy name.
His anger lasts a moment; his favor through life.
At night there are tears, but joy comes with the dawn.

I said to myself in my good fortune:
"Nothing will ever disturb me."
Your favor had set me on a mountain fastness,
then you hid your face and I was put to confusion.

To you, Lord, I cried,
to my God I made appeal:
"What profit would my death be, my going to the grave?
Can dust give you praise or proclaim your truth?"

The Lord listened and had pity.
The Lord came to my help.
For me you have changed my mourning into dancing.
you removed my sack cloth and clothed me with joy.
So my soul sings psalms to you unceasingly.
O Lord my God, I will thank you for ever.

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CHAPTER I

INTRODUCTION

Plant Gene Expression in Response to Environmental Stresses

Environmental stresses challenging plants include those caused by cold (Graham and Patterson, 1982; Guy, 1990a), heat (Vierling, 1991), anaerobic conditions (often associated with flooding), drought, salinity, heavy metals, and *uv* light (Sachs and Ho, 1986). Wounding stresses (Hennig et al., 1989) caused by abiotic factors also are among environmental stresses which challenge plants. Drought and cold are the two most important stress factors which limit the distribution of plants (Weiser, 1970). In particular, crop damage caused by freezing stress is of major economic significance. On the basis of percentages of indemnities attributed to specific hazards, as recorded by the Federal Crop Insurance Agency in 1986, freezing stress accounts for greater losses of fruits and vegetables than any other environmental or biological hazard (USDA, 1986). To highlight the importance of tolerance to environmental stresses in crop plants, Weiser (1982) notes that average yields of most crops are 3 to 7-fold less than

potential yields. He estimates that 10% of the shortfall between potential and average yield is due to diseases and insects, and 90% is due to environmental factors and weed competition.

A better understanding of alterations in gene expression underlying plant acclimation to environmental stresses can facilitate crop improvement for tolerance to these stresses. Increased colaboration between plant physiologists and plant breeders, however, will be required. The conclusion of Blum (1988) in a review of plant breeding for freezing resistance is relevant here:

> ". . . while knowledge on the physiology of freezing stress and resistance has developed in recent years, very little of that information was applied to selection work. There is a serious gap between our knowledge on freezing resistance and the application of that knowledge to breeding, in the form of advanced methodology. The ongoing breeding programs for winter survival in the annuals still rely heavily on field evaluations, in spite of their shortcomings -- largely because breeders do not see or are not convinced of the alternatives. The problem is accentuated by the fact that selection work must become more accurate and finely tuned as the demand for higher levels of resistance increases. A need for more research in developing physiological selection criteria for freezing resistance is really an understatement."

Selection of freezing tolerant cereals based on screening plants for freezing tolerance marker (FTM) proteins (Houde et al., 1992) appears promising. In addition, genes identified as important in cold acclimation or freezing tolerance may be utilized for germplasm enhancement via genetic transformation strategies. For example, ice recrystallization was inhibited in extracts of leaf tissues of tomato (*Lycopersicon esculentum*) transformed with antifreeze protein genes from winter flounder (*Pseudopleuronectes americanus*) (Hightower et al., 1991)

Cold Hardiness and Value of Bermudagrass Turf

Species of the genus *Cynodon* have been described morphologically (Harlan et al., 1970a), cytogenetically (Harlan et al., 1970b), and geographically (Harlan et al., 1970c). Several species are used for turf and forage in the United States, including *C. dactylon* (L.) Pers. (common bermudagrass), *C. nlemfuensis* Vanderyst (stargrass), *C. transvaalensis* Burtt-Davy (transvaalensis or floridagrass), *C. magennisii* Hurcombe (magennis), and *C. bradleyi* Stent (bradley) (Hanson, 1972). *C. dactylon* x *C. transvaalensis* bermudagrass hybrids are commonly used for turf. Popular varieties among these hybrids are the 'Tif-' series varieties (e.g., 'Tiffine', 'Tifgreen', 'Tiflawn', 'Tifway', etc.) developed in Glenn W. Burton's breeding program in Tifton, Georgia, and the more winter-hardy 'Mid-' series varieties (e.g., 'Midiron', 'Midway', etc.) developed in cooperation with Ray A. Keen's program in Hays, Kansas (Hanson, 1972; Taliaferro, personal communication).

In an effort to generate winter-hardy mutants of the forage bermudagrass Coastcross 1 (*C. dactylon* x *C. nlemfuensis*) by unmasking genes for rhizome development, Burton et al. (1980) irradiated green stems with ^{60}Co gamma rays. One mutant selected for rhizome development had forage production characteristics similar to parental Coastcross 1 and had marginally better winter-hardiness. The small gain in winter-hardiness indicates, not unexpectedly, that there are several genes controlling rhizome development and winter-hardiness in bermudagrass.

Other researchers have studied physiological changes associated with chilling stress and cold acclimation (CA) in turf bermudagrass. Rogers et al. (1977) found that chloroplasts took on a globular shape during late summer, and by November, grana lamellae of chloroplasts were usually damaged. These ultrastructural changes were associated with reduced photosynthesis in bermudagrass, when compared to zoysia (*Zoysia* spp.). White and Schmidt (1989) studied the effects of chilling on Midiron and Tifgreen turf bermudagrass maintained under 10/7 °C cycles photosynthetic photon flux density of 450 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ during 12-h photoperiods. After 4 days of chilling, Midiron chlorophyll content was relatively unchanged, while chlorophyll content of Tifgreen was significantly reduced. Midiron had higher CO_2 exchange rates (CER) than Tifgreen before, during, and after chilling treatment. In addition, nonreducing sugar content in stolons and rhizomes was higher in Midiron (versus Tifgreen) after the chilling regime. These and other findings led White and Schmidt to conclude that Midiron should be classified as chill tolerant, and Tifgreen, chill sensitive.

Midiron should also be classified as more winter-hardy (Fry, 1990) and freezing tolerant than Tifgreen. Anderson et al. (1988) utilized electrolyte leakage tests and regrowth assays to estimate the freezing tolerance of field-grown Midiron and Tifgreen bermudagrasses. Killing temperatures during December or January were -11 °C and -7 °C for Midiron and Tifgreen,

respectively; killing temperatures during June increased to approximately -5 °C and -3 °C for Midiron and Tifgreen, respectively.

Cold hardiness of *Cynodon* spp. plants defines the northern limits of bermudagrass distribution in the United States (Juska and Hanson, 1964; Chalmers and Schmidt, 1979). The transition zone for bermudagrass crosses through Maryland, Kentucky, Oklahoma, and Texas (Fig. 1). States further south comprise the zone of adaption for bermudagrass. Within these zones, bermudagrass is a major turfgrass species of home and commercial-building lawns, schools, airports, parks, highway roadsides, playgrounds, and golf courses.

Considering turfgrass species of various golf-course areas for example, we find bermudagrass on almost all greens, tees, fairways, and roughs in Florida, as well as on most tees, fairways, and roughs of golf courses in the surrounding Southeast region, as well as in the Mid-Continent region, as defined by the U.S. Golf Association (Center for Golf Course Management, 1992) (Table I). However, because a cold-tolerant bermudagrass possessing characteristics desirable for superior putting green turf (e.g., uniformity, green color retention, high shoot density, fine leaf texture, wear tolerance, and tolerance of frequent and low mowing) is not yet available (Fry, 1990), many golf course superintendents of the Southeast and Mid-Continent regions maintain greens of *Poa* and/or bentgrass (*Agrostis palustris*, *A. stolonifera*) (Table II), syringing, if necessary, these greens of cool-season turfgrass species on hot summer days in order to lessen potential damage from heat stress.

Figure 1. Distribution of Bermudagrass in the United States (Juska and Hanson, 1964)

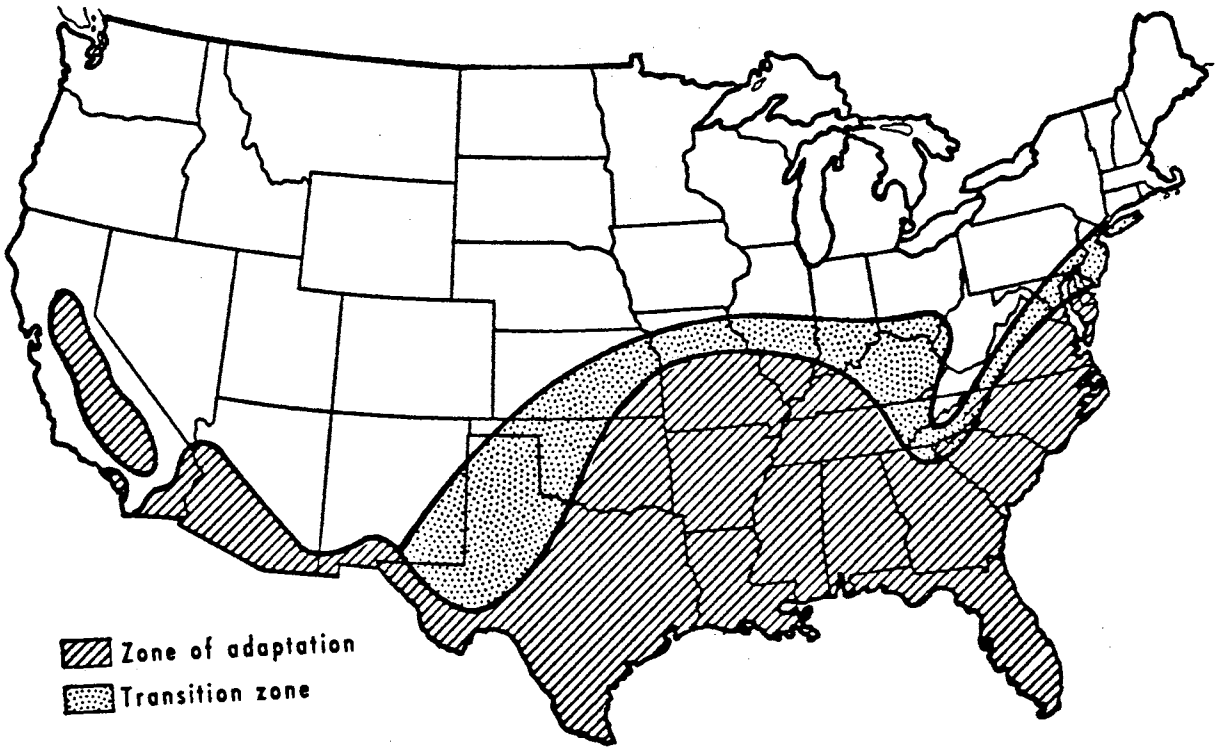
Zone of adaptation
Transition zone

Table I. Percentages of Golf Courses by USGA Region Using Bermudagrass on Greens, Tees, Fairways, and Roughs. (Center for Golf Course Management, 1992)

USGA Region*	Greens	Tees	Fairways	Roughs
NE	0	0	0	2
MA	1	14	19	14
SE	35	90	90	85
GL	0	1	1	3
MC	24	54	59	50
W	13	47	52	48
FL	97	100	100	95

Table II. Percentages of Golf Courses by USGA Region Using Bentgrass, *Poa*, and Bermudagrass on Greens (Center for Golf Course Management,1992).

USGA Region*	Bentgrass	*Poa*	Bermudagrass
NE	99	68	0
MA	99	59	1
SE	67	7	35
GL	99	49	0
MC	78	20	24
W	82	56	13
FL	6	0	97

* USGA Regions: NE = Northeast; MA = Mid-Atlantic; SE = Southeast; GL = Great Lakes; MC = Mid-Continent; W = West; FL = Florida.

Martin (1990) estimated the total 1987 turfgrass acreage maintained in Oklahoma to be approximately 690,000 acres (280,000 hectares). Of this acreage, Taliaferro (1992, personal communication) estimated eighty to ninety percent to be bermudagrass (*Cynodon* spp.). Martin (1990) also estimated the 1987 replacement value of turf in Oklahoma to exceed $1.7 billion. He arrived at this value by calculating the product of turf establishment cost per acre and the total number of turf acres maintained. Since establishment cost of bermudagrass turf is comparable to that of turf composed of other grass species, and possibly more expensive if establishment is by sodding (Taliaferro, personal communication), eighty percent of $1.7 billion, i.e., approximately $1.3 billion, estimates the 1987 1987 replacement value of bermudagrass turf in the state of Oklahoma.

Replacement values do not fully measure the benefits of bermudagrass turf. Turfgrasses contribute to the well-being of human communities and environments in several ways. Beard and Green (1993) divided benefits of turfs into three categories: functional, recreational, and aesthetic (Table III). Bermudagrass turf contributes to the well-being of human communities and environments in each of these categories.

Freeze damage to bermudagrass turf in transition zone regions, such as in regions of northern and western Oklahoma, is not uncommon and, during particularly severe winters, may extend into regions of the zone of adaption for bermudagrass. For example, injury caused by freezing stress, in combination with injury from desiccation and traffic stresses, contributed to considerable turf loss

Table III. Benefits Derived from Turfs (Beard and Green, 1993)

Functional Benefits	**Recreational Benefits**	**Aesthetic Benefits**
Soil erosion control	Low-cost surfaces	Beauty
Dust prevention	Physical health	Quality of life
Groundwater recharge	Mental health	Mental health
Flood control	Safety	Social harmony
Carbon storage	Spectator entertainment	Community pride
Organic chemical degradation		Increased property values
Heat dissipation		Complements trees and shrubs in landscapes
Noise abatement		
Glare reduction		
Air pollution control		
Nuisance animal reduction		
Allergic pollen control		
Fire hazard reduction		
Wildlife habitat		

in Oklahoma during the winter of 1989 (D. Martin, personal communication; Kenna, 1989).

Maintaining bermudagrass turf at different cutting heights during 1988 influenced the level of turf susceptibility to damage from freezing stresses during the winter of 1989. For example, on bermudagrass (probably variety U3) fairways of the Meadowbrook Country Club of Tulsa, Oklahoma, that had been maintained at one half-inch cutting height during 1988, loss of turf was extensive, while damage was less severe on those fairways which had been maintained at a three-quarter-inch cutting height (G. Hallet, personal communication). Golf course members, however, may prefer that superintendents maintain fairways at a half-inch, rather than a three-quarter-inch, cutting height, and greens at a one-eighth-inch, rather than a three-sixteenth-inch, cutting height, in order to facilitate fairway shots and putts, respectively. Maintaining bermudagrass turf at lower cutting heights, though, increases the susceptibility of bermudagrass plants to injury from freezing stress.

In addition to maintaining bermudagrass at cutting heights of three-quarter inch or higher, avoiding succulent top growth will enhance the hardening process of bermudagrass for winter weather (Green, 1984). Development of succulent top growth of bermudagrass in the fall may be minimized by avoiding excessive applications of nitrogen fertilizer, especially in the late fall, and by avoiding excessive irrigation. Reducing soil compaction by core cultivation during the growing season, but not in late fall, and limiting traffic in early spring until

bermudagrass turf has thawed to a two or three-inch depth, are other management practices which contribute to reducing winter injury in bermudagrass turf (Green, 1984). The development of superior-quality bermudagrass varieties with greater winter hardiness, and specifically, tolerance to freezing temperatures, would complement management practices that minimize potential conditions which favor winter injury to bermudagrass turf.

Cold Acclimation and Protein Synthesis

Among biochemical mechanisms underlying plant adaption and acclimation to environmental stresses (Alscher and Cumming, 1990), alterations in protein synthesis are common. For adaption to cold stresses specifically, proteins synthesized in response to low temperatures may mediate cold hardening, or CA, in plants (Guy, 1990a; Thomashow, 1990). Proteins synthesized by genes persistently upregulated during CA are referred to as cold-regulated (COR) proteins. A dramatic manifestation of CA shown by many plant species is increased tolerance to freezing temperatures, after extended exposure to low, nonfreezing temperatures (Thomashow, 1990). In addition to modifying plant metabolism for survival at low temperatures, COR proteins may contribute directly towards increasing freezing-stress tolerance in plant species after CA (Guy, 1990b).

Davis and Gilbert (1970) studied alterations in protein composition associated with CA in Tifgreen and 'Tifdwarf' turf bermudagrass. Disc

electrophoresis was the electrophoretic technique used by Davis and Gilbert, restricting their characterization to alterations in protein composition revealed by one-dimensional analyses. Based on these analyses, however, Davis and Gilbert did find alterations in soluble protein composition of rhizomes in association with cold hardening.

Objectives

To provide new knowledge pertinent to improving turf bermudagrass (*Cynodon dactylon* x *C. transvaalensis*) for tolerance to freezing stress, the primary objective of this study was to characterize alterations in protein synthesis by turf bermudagrass crowns that are associated with CA. Crowns are a major regenerative tissue of bermudagrass from which new shoots emerge after plants break winter dormancy. An initial, though secondary, objective of this research was to further characterize the potential of turf bermudagrass varieties for tolerance to freezing stress after CA in growth chambers (rather than under field conditions).

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CHAPTER II

Cold Acclimation and Alterations in Protein Synthesis by Bermudagrass Crowns

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Environmental Stress Physiology

Cold Acclimation and Alterations in Protein Synthesis by Bermudagrass Crowns

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Abstract. Freezing tolerances of 'Midiron' and 'Tifgreen' turf bermudagrasses (*Cynodon dactylon* x *C. transvaalensis*) were determined without, and after cold acclimation (CA). Alterations in protein synthesis by crowns associated with CA were investigated. Freezing treatments were applied to plants after four weeks in growth chambers under acclimating [8/2C (day/night) cycles with a 10 hour photoperiod] or non-acclimating (28/24C) conditions. Proteins synthesized by isolated crowns were *in vivo* radiolabeled for 16 hours with ^{35}S-Methionine and ^{35}S-Cysteine. After CA, LT_{50} (lethal temperature for 50% of plants) values for 'Midiron' plants decreased from -6.5 to -11.3C, and for 'Tifgreen' plants, decreased from -3.6 to -8.5C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography revealed alterations in protein synthesis. Cold-acclimated crowns of both cultivars exhibited increased synthesis of several cold-regulated (COR) proteins. Prominantly revealed were COR proteins ranging in size from ≈ 14 to 37-kDa. 'Midiron' crowns synthesized

intermediate molecular weight (MW) (≈ 32 to 37 kDa) and low MW (≈ 20 to 26 kDa) COR proteins in greater amounts than 'Tifgreen' crowns.

Introduction

Warm-season, perennial grasses of the genus *Cynodon* (*C. dactylon* L. Pers., *C. transvaalensis* Burtt-Davy, and *C. dactylon x C. transvaalensis* hybrids) are widely used for turf in tropical and subtropical regions throughout the world. Their use also extends into regions of transition between warm- and cool-season species. While substantial differences exist in the winterhardiness of turf bermudagrass cultivars (Fry, 1990), all are subject to freezing injury. Even in relatively mild climatic regions such as the southern United States, severe injury or death of bermudagrass turf may result from freezing stress during unusually severe winters. Tolerance of turf bermudagrass cultivars to freezing, consequently, is a major concern of turfgrass managers in regions subject to these climatic conditions. A better understanding of the genetics and physiology of tolerance to freezing stresses would facilitate the development of new cultivars with improved cold tolerance.

Cold acclimation (CA), or cold hardening, is the process by which organisms adjust their metabolism for survival at low temperatures. A dramatic manifestation of CA is the increased tolerance to freezing demonstrated by many plants after extended exposure to low, nonfreezing temperatures (Thomashow, 1990). Alterations in protein synthesis are associated with CA in a range of plant

species (Guy, 1990a; Thomashow, 1990). Though the functions and identities of most proteins synthesized in association with CA remain undetermined, some of these cold-regulated (COR) proteins are likely adaptive and function to enhance plant tolerance to freezing (Guy, 1990b).

Bermudagrass plants cold acclimate. 'Tifgreen' and 'Tifdwarf' bermudagrasses (*C. dactylon x C. transvaalensis*) sampled from field plots at Raleigh, North Carolina during January and February, tolerated lower temperatures than when sampled during typically warmer months (Davis and Gilbert, 1970). 'Tifgreen' sampled during January or February tolerated freezing to -8.3C, while 'Tifgreen' plants sampled during September tolerated freezing to only -2.2C before a 50% reduction in growth (as determined by dry weight measurements taken 4 weeks after freezing treatments) was observed. 'Tifdwarf' plants grown in a controlled environment chamber for 15 and 30 days at 3.3C under 10/14-h : light/dark photoperiods tolerated freezing to -3.9 and -5.0C, respectively, before a 50% reduction in growth was observed.

Anderson et al. (1988) presented additional information on cold hardening in turf bermudagrass. Using both regrowth assays and electrolyte leakage tests, they found that field-grown plants of 'Midiron' and 'Tifgreen' bermudagrasses sampled during December or January tolerate lower freezing temperatures than those sampled during June. Based on electrolyte leakage from crown tissues, LT_{50} (lethal temperature for 50% of plants) values during December or January for 'Midiron' and 'Tifgreen' were -11C and -7C, respectively. LT_{50} values for

plants sampled during June increased to ≈ -5C and -3C for 'Midiron' and 'Tifgreen', respectively. Regrowth assays were also used to estimate LT_{50} values for both cultivars from February through June. There was close agreement between the electrolyte leakage and regrowth procedures in the estimation of LT_{50} values for plants sampled in March, April, May, or June.

Davis and Gilbert (1970) utilized disc electrophoresis through polyacrylamide to examine alterations in the composition of soluble proteins isolated from cold-hardened 'Tifdwarf' rhizomes taken either from plants grown in field plots in November, or cold hardened in a controlled environment chamber for 15 or 30 days. Several alterations in soluble protein compositions were revealed in samples from both field- and growth-chamber-hardened rhizomes when compared to those from nonhardened rhizomes.

Our objective was to characterize alterations in protein synthesis associated with CA of 'Midiron' and 'Tifgreen' turf bermudagrasses as revealed by *in vivo* radiolabeling and SDS-PAGE.

Materials and Methods

Plant material and treatments. 'Midiron' and 'Tifgreen' plants maintained in pots under greenhouse conditions (temperatures maintained between ≈ 22 and 37C) for one year or less were used as source plants for transplantation to cone-tainers (38 mm diameter x 204 mm height) (Ray Leach 'Cone-tainer' Nursery, Canby, OR). Both cultivars are vegetatively propagated F_1 hybrid plants

derived from interspecific crosses of tetraploid (2n = 4X = 36 chromosomes) *C. dactylon* x diploid (2n = 2X = 18 chromosomes) *C. transvaalensis*. One phytomer (crown-stem-root unit) from 'Midiron' or 'Tifgreen' turf was planted per cone-tainer in a soil mix composed of fine sand, peat moss, and vermiculite in a volume ratio of 2 : 3 : 2, respectively. After 6 to 8 weeks under greenhouse conditions, cone-tainers were transferred to controlled environment chambers (Model No. PGW36, Conviron, Ashville, NC). For CA, 'Midiron' and 'Tifgreen' plants were maintained under 8/2C cycles for 28 days with a photosynthetic photon flux of about 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ during 10-h photoperiods (Anderson et al, in press). Plants were watered in growth chambers as needed with a dilute fertilizer solution (240 mg 20:20:20 formula per liter distilled water) (No-Stain Formula Peter's Professional Plant Food, Grace-Sierra Horticultural Products Company, Milpitas, CA). Control plants were maintained similarly, except temperatures for 10/14-h : light/dark periods were ≈ 28/24C.

Determining LT_{50} values. LT_{50} values for plants of four treatment combinations ('Midiron' control, 'Midiron' after CA, 'Tifgreen' control, and 'Tifgreen' after CA) were determined using a regrowth test procedure (Anderson et al. (in press). Crushed ice was layered on soil surfaces to minimize supercooling of soil water, and plants within cone-tainers were cooled at a rate of 1C per hour in a low temperature chamber (Rheem Scientific, Ashville, NC). Ranges of freezing temperatures applied to plants of the four treatment combinations were as follows: 'Midiron' control (-4 to -10C), 'Midiron' after CA

(-7 to -13C), 'Tifgreen' control (-3.6 to -9C), and 'Tifgreen' after CA (-5 to -11C). Except for the non-integer initial freezing temperature to which 'Tifgreen' control plants were cooled (-3.6C), plants were cooled to integer temperature values within each temperature range, inclusively. At each integer temperature (± 0.1C), four cone-tainers per treatment combination were removed from the low temperature chamber and allowed to thaw at 4C overnight. After thawing, all cone-tainers were returned to control conditions (28/24C : 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light/dark : 10/14-h periods) in a controlled environment chamber. Plant survival was determined visually as regrowth on a weekly schedule for 8 weeks following the application of freezing temperatures. Only plants having one or more vigorously growing shoots after 8 weeks were considered to be viable. After calculating the percent survival of plants removed at each temperature, LT_{50} values were estimated by linear interpolation.

Collecting and *in vivo* radiolabeling crown tissue. Crown tissue cores from cold acclimated plants were excised in a cold room (4 to 5C). Crown tissues from control plants were excised at room temperature (22 to 26C). Only tissue cores from non-necrotic crowns lacking chlorophyll, but supporting root primordia and green shoots, were excised. Attached roots and shoots, as well as browning tissues surrounding crowns, were removed. Excised crown tissues were washed in distilled water, swabbed with paper tissues, and placed in pre-weighed 1.5 ml microcentrifuge tubes, before being *in vivo* radiolabeled using a procedure of Porter et al. (1989) modified for labeling crown tissues.

Incubation buffer [0.35 ml of 20 mM Tris (pH 7.5), 5 mg/ml chloramphenicol] was added to ≈ 180 mg crown tissue in 1.5 ml microcentrifuge tubes. Chloramphenicol was added to inhibit protein synthesis by microbes associated with isolated crown tissues. Tran^{35}S-label Reagent (ICN Biomedicals, Irvine, CA), an aqueous solution of ^{35}S-labeled amino acids comprising ≈ 70% L-Methionine [^{35}S], 15% L-Cysteine [^{35}S], and various non-labeled amino acids, was added in the amount of 0.236 mCi to mixtures containing crown tissue from control plants. Mixtures were infiltrated under a mild vacuum for 3 X 10 min. Incubation buffer pre-cooled to 2 to 6C was added to cold-acclimated crown samples, and these samples were vacuum infiltrated while on ice. Crown tissue from control plants was incubated at 22 to 26C, while crown tissue from plants after CA was incubated at 2 to 6C. Incubation buffer was removed after 16 h. Crown tissue was rinsed in distilled water and stored at -70C for up to several months. The Tran^{35}S-label Reagent was added at a 2.5 X rate to acclimated crowns to compensate partially for a reduction in radioactive amino acid incorporation into proteins relative to controls. For sources of non-radiolabeled protein, additional crown tissue samples from each treatment combination were treated as described above, except that no Tran^{35}S-label Reagent was added to the incubation buffer. All crowns were stored at -70C following treatments.

Protein extraction. Total protein samples were extracted, using a procedure modified from Damerval et al. (1986), from crowns that had been stored at -70C. Crown tissue (≈ 180 mg) was ground to a fine powder in liquid

nitrogen in a mortar and pestle. Powdered tissue was suspended in 12 ml of chilled (-20C) 10% trichloroacetic acid (TCA) in acetone with 0.07% *B*-mercaptoethanol (*B*-ME) and tranferred to microcentrifuge tubes. After 45 min at -20C, tubes were centrifuged at 12,000x *g* for 15 min. Supernatants were removed by pipet and discarded. Each pellet was thoroughly resuspended in 1.5 ml chilled (-20C) acetone with 0.07 % *B*-ME. After 1 h at -20C, tubes were again centrifuged at 12,000x *g* for 15 min. Supernatants were removed by pipet and discarded. Pellets were vacuum dried for 3 X 10 min. The mass of each dried acetone-insoluble pellet remaining in each tube was determined by subtracting the pre-determined tube mass. Pellets were stored at -70C.

Protein solubilization, quantification, and scintillation counting. To solubilize proteins, 10-μl solubilization buffer (62.5 mM trizma base, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol in purified, distilled H_2O) (Laemmli, 1970) was added per milligram acetone-insoluble pellet in 1.5 ml microcentrifuge tubes. Solubilization was for 1 h at 22 to 26C with agitation by hand every 15 min. After centrifugation at 12,000x *g* for 15 min, supernatants were transferred to new 1.5 ml microcentrifuge tubes and centrifuged at 12,000x *g* for 15 min to be further clarified. Aliquots used for determinations of protein concentration were taken from resulting supernatants. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Chemical Division, 1991). Solubilization buffer containing 10% *B*-ME and 0.0025% bromphenol blue dye (BPB) was added 1:1 (v/w) to remaining supernatants to reconstitute a sample buffer with

5% *B*-ME and 0.00125% BPB for SDS-PAGE. After removing aliquots of radio-labeled samples needed for scintillation counting, solubilized protein samples were stored at -70C for subsequent SDS-PAGE.

A procedure modified from the filter-paper disk method of Mans and Novelli (1961) was followed to determine levels of ^{35}S-Methionine and ^{35}S-Cysteine incorporated by *in vivo* radiolabeling in solubilized proteins. Aliquots (5-μl) of solubilized protein samples were applied to squares (1 to 2 cm^2) cut from Whatmann 3MM paper sheets. After incubating squares in 10% TCA on ice for 30 min, then in 5% TCA on ice for 15 min, followed by 30 min at 90C in 5% TCA, the squares were washed for 2 min in 95% ethanol. After drying, each square was placed in 2 ml of Ultima Gold LSC-Cocktail (Packard Chemical, Groningen, The Netherlands) fluor solution in plastic scintillation counting vials. A Packard (Meriden, CT) Model No. B1900 Liquid Scintillation Analyzer was used for scintillation counting. For estimating total sample radioactivity (i.e., radioactivity from both free ^{35}S-amino acids and those incorporated into isolated proteins), 2-μl aliquots of solubilized protein samples were added directly to 2 ml of Ultima Gold LSC-Cocktail fluor solutions in plastic scintillation counting vials.

After protein quantification and scintillation counting, the fraction of solubilized ^{35}S-Methionine and ^{35}S-Cysteine incorporated into isolated proteins was calculated for each sample by dividing radioactivity (Kdpm/μl) of proteins remaining adhered to the 3MM squares, by total sample radioactivity (Kdpm/μl).

From protein concentration (μg/μl) and specific activity (Kdpm/μg) values for each solubilized protein sample, appropriate volumes of ^{35}S-labeled protein samples, non-radiolabeled protein samples, and solubilization buffer were mixed to prepare samples for each treatment combination containing 32-μg protein with equal specific activities solubilized in 20 μl volumes of solubilization buffer containing 5% *B*-ME and 0.00125% BPB.

Gel electrophoresis, silver staining, fluorography. For SDS-PAGE, protein samples (7.5-μg) in solubilization buffer with 5% *B*-ME and 0.00125% BPB were electrophoresed per gel lane through 8 to 10 mm stacking gels [4% (w/v) acrylamide, 4.01% T: 2.74% C)] containing 0.1% (w/v) SDS. Separation gels [14% (w/v) acrylamide, 14% T: 2.56% C)] containing 0.15% SDS were poured in 16 cm x 20 cm x 1 mm PROTEAN II xi (Bio-Rad, Richmond, CA) vertical electrophoresis cells. Radioactive ^{14}C-methylated marker proteins (Amersham, United Kingdom) of 200.0, 97.4, 69.0, 46.0, 30.0, and 14.3 kDa were electrophoresed for molecular weight estimations of sample proteins. Electrophoresis buffer as described by Laemmli (1970) was used.

A current of 16 mA per gel at maximum voltage from an ISCO Model # 470 power supply was used for electrophoresis through stacking and separation gels. Electrophoresis was stopped after the BPB dye front had migrated to within 1 cm of the bottom edge of gels. Gels were silver stained (Blum et al., 1987), photographed, and stored for several days at 4C in 50% methanol. To destain for fluorography, gels were placed for 6 to 7 h in 1X Kodak GBX Fixer and

Replenisher and then washed three times in distilled water for 30 min (Kulsar and Prestwich, 1988). For impregnation of the fluor 2,5-diphenyloxazole (PPO), gels were placed in glacial acetic acid for 10 min to remove excess water, soaked in 20% (w/v) PPO in acetic acid for 90 min, then soaked in distilled water for 30 min (Skinner and Griswold, 1983). The gels were dried (Porter and Gatschet, 1992) and exposed to preflashed Kodak X-Omat AR film at -70C. Gels were exposed for ≈ 9 days (decay densities in sample lanes were ≈ 7.29 Kdpm cm^{-2}).

Image analysis. Scanned images of silver stained proteins separated by SDS-PAGE, as well as of corresponding fluorographic patterns, were analyzed using a PDI (Huntington Station, NY) densitometer (Krauss et al., 1989). Estimations of protein size (kDa), based on electrophoretic mobilities, and values of band optical density x height (OD x mm) were made using PDI's "Quantity One" software application. Two fluorographs from each of two gels were scanned. Peak OD values ranged from 0 for transparent regions to 1.47 for saturated images of ^{14}C-labeled marker protein bands. Band OD x height values were estimated near centers of lanes. When comparing band OD x mm values in lanes of protein samples after CA to control lanes, and increase of at least 100% was used to indicate COR protein synthesis. These values for COR protein bands of ≈ 14 to 15 kDa, 20 to 26 kDa, and 32 to 37 kDa were compared for lanes of 'Midiron' and 'Tifgreen' samples after CA using paired comparison *t* tests and analysis of variance (SAS Institute, 1988).

Results and Discussion

Freezing tolerances. After CA for 28 d, LT_{50} values for both 'Midiron' and 'Tifgreen' cultivars were lowered by ≈ 5C. Using re-growth assays, LT_{50} values decreased from -6.5 to -11.3C, and from -3.6 to -8.5C, for 'Midiron' and 'Tifgreen' plants, respectively. Under these test conditions, 'Midiron' was slightly more freezing tolerant (2.8C lower LT_{50}) than 'Tifgreen' after CA. These LT_{50} values are in close agreement with those reported by Anderson et al. (1988) using field-acclimated crown tissues and electrolyte leakage tests. The greater freezing tolerance of 'Midiron' compared to 'Tifgreen' is also supported by other field studies (Fry, 1990), and by experience from long-term, widespread commercial use of both varieties. As expected, LT_{50} values of cold-acclimated 'Midiron' and 'Tifgreen' are higher than LT_{50} values reported for cold-acclimated cool-season turfgrasses. Rajashekar et al. (1983) reported LT_{50} values based on electrolyte leakage for cultivars of chewings fescue (*Festuca rubra*) to range from -27.0C for 'Wintergreen' to -17.5C for 'Atlanta'. LT_{50} values for hardened cultivars of perennial rye (*Lolium perenne*), Kentucky bluegrass (*Poa pratensis*), and red fescue (*Festuca rubra*) were bracketed by these LT_{50} values of 'Wintergreen' and 'Atlanta' chewings fescues.

SDS-PAGE. Densitometry of silver-stained gels revealed that proteins of ≈ 34 kDa decreased by 10% or more in samples from 'Midiron' and 'Tifgreen' crowns after CA (Fig. 2). Apparently, proteins of ≈ 34 kDa are degraded more rapidly at low temperatures and/or are synthesized to a lesser extent by crowns

in response to CA than in crowns maintained under control conditions. Few other marked changes in crown protein composition after CA were revealed by silver staining. Using Coomassie blue staining, Perras and Sarhan (1989) found decreases in 34 kDa proteins of wheat (*Triticum aestivum*) seedlings after CA for 10 and 40 days.

Fluorography of radiolabeled proteins revealed marked alterations in protein synthesis by crowns after CA. Both 'Midiron' and 'Tifgreen' crowns synthesized intermediate molecular weight (MW) COR proteins of ≈ 32 to 37 kDa, and low MW COR proteins of two size ranges: ≈ 20 to 26 kDa, and ≈ 14 to 15 kDa (Fig. 3). Peak OD values of all sample protein bands of 37 kDa or less in size were not saturated. 'Tifgreen' crowns synthesized COR proteins of ≈ 14 to 15 kDa in greater amounts than 'Midiron' crowns (Table IV). 'Midiron' crowns, however, synthesized COR proteins of ≈ 20 to 26 and 32 to 37 kDa in greater amounts than 'Tifgreen' crowns (Table IV). The largest difference in COR protein synthesis between cold-acclimated 'Midiron' and 'Tifgreen' crowns occurred in the 20 to 26 kDa range.

Several *in vivo* radiolabeled COR proteins isolated from cold-acclimated spinach (*Spinacia oleracea*) seedlings (Guy and Haskell, 1988) are similar in size to the low and intermediate MW COR proteins of 'Midiron' and 'Tifgreen' crowns. COR proteins of 15 and 24 kDa recovered after *in vitro* translation of *cor* RNA sequences from *Arabidopsis thaliana* (Lin et al., 1990) plants are also within size ranges for low MW COR proteins of 'Midiron' and 'Tifgreen'

bermudagrasses. *Arabidopsis* 15 kDa COR proteins may have cryoprotective functions (Lin and Thomashow, 1992). Other COR proteins [e.g., 39-kDa COR proteins wheat and 47 kDa COR proteins from *Arabidopsis thaliana* (Guo et al., 1992)] have sequence similarities to late embryogenesis abundant (LEA) proteins and may help mediate tolerance to desiccation stresses which accompany freezing stresses. COR proteins synthesized by bermudagrass crowns may also be non-enzymatic and function in desiccation tolerance or cryoprotection, or they may be enzymes important in altering crown metabolism for survival at low temperatures.

If proteins characterized on the basis of electrophoretic mobility are immunologically related to known COR proteins, these proteins may be useful as molecular markers of freezing tolerance. Houde et al. (1992) found that levels of proteins ranging from 14 to 200 kDa that were immunologically related to a 50 kDa COR protein of wheat correlated well to levels of freezing tolerance in cold-acclimated Graminaceous plants. Assaying bermudagrass plants for levels of proteins cross-reactive with COR proteins of bermudagrass or other species may be useful in selecting plants having superior tolerance to freezing.

Results of this study indicate a positive association between the synthesis of low MW COR proteins (≈ 20 to 26 kDa) and freezing tolerance. Higher levels of low MW COR proteins (≈ 20 to 26 kDa) were detected after CA in the more freezing-tolerant 'Midiron' when compared to 'Tifgreen'. Additional study will be needed to further characterize this association and understand the functions of these COR proteins in freezing tolerance.

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Table IV. Band Optical Density x Height (OD x mm) Averages (± SE) of Regions of COR Protein Bands on Fluorographs.

	Band optical density x height (OD x mm)		
	Molecular weight (kDa)		
Genotype	14 to 15	20 to 26	32 to 37
Midiron	1.37 ± 0.06	12.52 ± 0.18	11.83 ± 0.45
Tifgreen	1.80 ± 0.08	5.53 ± 0.39	10.14 ± 0.70
Significance			
Genotype	**	***	*

*, **, *** Significant at $P < 0.05, 0.01, 0.001$, respectively, using either paired comparisons *t* tests, or analysis of variance with fluorographs as replications.

Fig. 2. Silver-stained Crown Proteins after SDS-PAGE. Left lane: silver-stained ^{14}C-labeled marker proteins of 200.0, 97.4, 69.0, 46.0, 30.0, and 14.3 kDa; Mo: 'Midiron' control; Ma: 'Midiron' after CA; To: 'Tifgreen' control; Ta: 'Tifgreen' after CA. Protein bands of ≈ 34 kDa from crown protein samples of control 'Midiron' and 'Tifgreen' plants are marked by open arrows. Intermediate molecular weight (≈ 32 to 37 kDa) and low molecular weight (≈ 20 to 26 kDa; and ≈ 14 to 15 kDa) regions corresponding to those marked in Fig. 3 are indicated by lines to the right of lanes of protein samples from acclimated crowns.

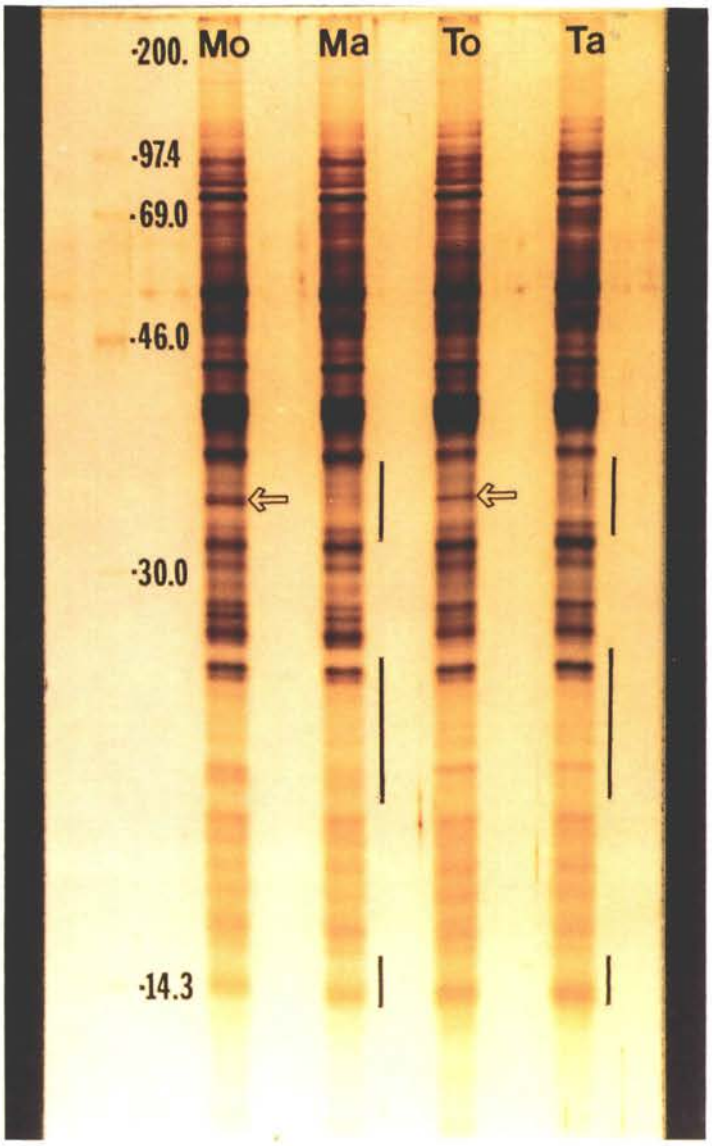
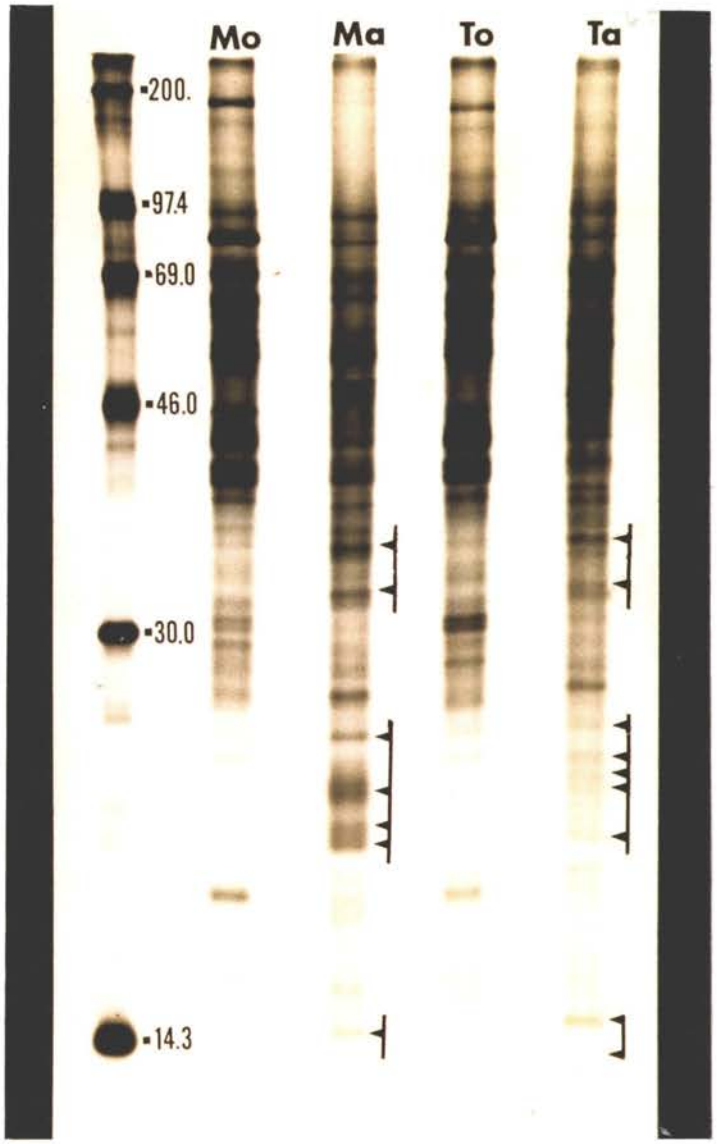

-200.
Mo
Ma
To
Ta
-97.4
-69.0
-46.0
-30.0
-14.3

Fig. 3. Fluorograph of ^{35}S-labeled Crown Proteins after SDS-PAGE. Left lane: ^{14}C-labeled marker proteins of 200.0, 97.4, 69.0, 46.0, 30.0, and 14.3 kDa; Mo: 'Midiron' control; Ma: 'Midiron' after CA; To: 'Tifgreen' control; Ta: 'Tifgreen' after CA. Intermediate molecular weight (≈ 32 to 37 kDa) and low molecular weight (≈ 20 to 26 kDa; and ≈ 14 to 15 kDa) regions corresponding to those of Fig. 2 are indicated by lines to the right of lanes of protein samples from acclimated crowns. Filled arrowheads mark COR protein bands.


Mo
Ma
To
Ta
200.
97.4
69.0
46.0
30.0
14.3

CHAPTER III

TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF COLD-REGULATED PROTEINS FROM TURF BERMUDAGRASS CROWNS

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ABSTRACT

Injury from freezing stress is a major factor limiting the distribution of bermudagrass (*Cynodon* spp.). In many plant species, cold-regulated (COR) proteins--proteins synthesized by genes persistently upregulated during cold acclimation (CA)--may mediate tolerance to freezing stress. This study was conducted to characterize COR protein synthesis by crowns of 'Midiron' and 'Tifgreen' turf bermudagrasses (*Cynodon dactylon* x *C. transvaalensis*) utilizing isoelectric focusing (IEF) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), i.e., two-dimensional-PAGE. Midiron bermudagrass tolerates freezing to lower temperatures than Tifgreen, both without, and after CA. Plants were grown for 4 wk in growth chambers under acclimating [8/2 °C (day/night) cycles with a 10 h photoperiod] or nonacclimating (28/24 °C) conditions. Proteins synthesized by isolated crowns were *in vivo* radiolabeled for 16 h with ^{35}S-Methionine and ^{35}S-Cysteine. 2D-PAGE and fluorography revealed that after CA, crowns of both cultivars synthesized groups of basic and acidic COR proteins of several size ranges. Midiron crowns synthesized low molecular weight (ca. 20 to 28 kD) basic (isoelectric points from ca. 9.0 to 7.3) COR proteins in greater numbers and amounts than Tifgreen crowns in association with CA.

Keywords: bermudagrass, cold-regulated (COR) proteins, *Cynodon dactylon* x *C. transvaalensis*, protein gel electrophoresis

Abbreviations: 2D- = two-dimensional; ABA = abscisic acid; AFP = antifreeze protein; APS = ammonium persulfate; *B*-ME = *B*-mercaptoethanol; BPB = bromphenol blue; CA = cold acclimation; COR = cold-regulated; HIB = high intermediate MW basic; IA = intermediate MW acidic; IB = intermediate MW basic; IEF = isoelectric focusing; LA = low MW acidic; LB = low MW basic; LEA = late embryogenesis abundant; LT_{50} = lethal temperature for 50% of plants; MW = molecular weight; PAGE = polyacrylamide gel electrophoresis; pI = isoelectric point; PPO = 2,5-diphenyloxazole; SDS = sodium dodecyl sulfate; SS = sucrose synthase; TCA = trichloroacetic acid; TEMED = N,N,N',N'-tetramethylethylenediamine; UKS = urea, K_2CO_3, SDS.

INTRODUCTION

Bermudagrasses (*Cynodon* spp.) are warm-season, perennial, sod-farming plants typically propagated vegetatively. Northern limits of the zone of adaption for bermudagrasses are largely dependent on tolerances of individual plants to cold (Juska and Hanson, 1964). Two *Cynodon* species, *C. dactylon* L. Pers. and *C. transvaalensis* Burtt-Davy, as well as hybrids of these two species, are widely used as turfgrasses in tropical and subtropical regions throughout the world. While substantial differences in winterhardiness of turf bermudagrass cultivars have been observed (Fry, 1990), all are subject to freezing injury.

The process in which organisms, including plants, adjust their metabolism for survival at low temperatures is called cold acclimation (CA), or cold hardening. After extended exposure to low, nonfreezing temperatures (and/or reduced photoperiods), many plant species cold acclimate and exhibit tolerance to lower freezing temperatures (Guy, 1990b). Alterations in protein synthesis are associated with CA in a range of plant species (Guy, 1990a). Proteins synthesized by genes persistently upregulated during CA are referred to as cold-regulated (COR) proteins (Thomashow, 1990).

Some plant COR proteins have cryoprotective activity *in vitro* (Lin and Thomashow, 1992; Rosas et al., 1986). A 6.6 kD COR protein from *Arabidopsis thaliana*, COR6.6, is hydrophilic, synthesized in response to ABA and water stress, and is alanine-rich (Gilmour et al., 1992). COR6.6 is similar to KIN1, another small alanine-rich COR protein of *Arabidopsis thaliana* (Kurkela and

Franck, 1990). Some antifreeze proteins (AFPs) from flounder are also alanine-rich. Gilmour et al. (1992), and Kurkela and Franck (1990), suggest that proteins like COR6.6, and KIN1, respectively, may have antifreeze functions in *Arabidopsis*.

Other non-enzymatic COR proteins may function in conferring tolerance to osmotic stress that accompanies freezing stress. For example, two boiling-stable COR proteins, one from wheat (*Triticum aestivum*), COR39, and one from *Arabidopsis thaliana*, COR47, have lysine-rich repeats similar to those found in late embryogenesis abundant (LEA) proteins (Guo et al., 1992). Despite these sequence similarities, however, as a result of the diversity of factors involved in freezing stress, most COR proteins likely evolved at separate times and are not highly conserved among plant species (Guy, 1990a). A lack of conservation among small fish AFPs also appears to rule out a common evolutionary origin for these proteins (Kurkela and Franck, 1990).

Enzymatic COR proteins likely include isozymes of cold-labile enzymes, enzymes for cryoprotectant synthesis, and enzymes for membrane lipid modifications (Guy, 1990a). Though the identities and functions of most COR proteins remain unknown, sucrose synthase (SS) has been identified as a COR protein. Crespi et al. (1991) found that both SS gene expression and SS activity are increased in wheat during CA. Rice (*Oryza sativa*) is considered more cold-susceptible than wheat, and consistent with this character, Hahn and Walbot (1989) found that a SS transcript of rice is suppressed by cold.

Turf bermudagrass plants cold acclimate. Davis and Gilbert (1970) found that Tifgreen and 'Tifdwarf' bermudagrass (*C. dactylon x C. transvaalensis*) plants, sampled from field plots at Raleigh, North Carolina, during January and February, tolerated lower temperatures than plants sampled during typically warmer months. Tifgreen plants sampled during January or February tolerated temperatures of -8.3 °C, while Tifgreen plants sampled during September tolerated temperatures to only -2.2 °C before a 50% reduction in growth (as determined by dry weight measurements taken 4 wk after freezing treatments) was observed.

Similarly, Anderson et al. (1988) reported that field-grown plants of Midiron and Tifgreen bermudagrasses sampled during December or January tolerated lower freezing temperatures than those sampled during June. Killing temperatures based on electrolyte leakage for Midiron plants during December or January were -11 °C, and for Tifgreen plants during the same periods were -7 °C. Killing temperatures for plants sampled during June increased to ca. -5 °C and -3 °C for Midiron and Tifgreen, respectively. Regrowth assays were also used in later months to estimate lethal temperatures for 50% of plants (LT_{50} values). Killing temperatures based on electrolyte leakage from crowns and LT_{50} values estimated from regrowth of plants were in close agreement. Greater freezing tolerance of Midiron, compared with Tifgreen, was also supported by determinations of LT_{50} values following CA in controlled-environment chambers (Anderson et al., 1993; Gatschet et al, submitted).

Protein composition of bermudagrass rhizomes is altered during cold hardening. Davis and Gilbert (1970) utilized disc electrophoresis through polyacrylamide to examine alterations in the composition of soluble proteins isolated from cold-hardened Tifdwarf rhizomes taken either from plants grown in field plots in November, or cold hardened in a controlled environment chamber for 15 or 30 d. Alterations in the composition of soluble proteins in samples from hardened rhizomes, when compared to those from nonhardened rhizomes, were revealed by altered band patterns after electrophoresis.

This study was conducted to characterize COR protein synthesis by crowns of Midiron and Tifgreen turf bermudagrasses after CA by utilizing isoelectric focusing (IEF) and sodium dodecyl sulfate (SDS)-PAGE (i.e., two-dimensional (2D)-PAGE). Midiron is tolerant to lower freezing temperatures than Tifgreen, both without, and in association with CA (Anderson et al., 1988; Fry, 1990; Anderson et al., 1993; Gatschet et al., submitted). If proteins are synthesized to a greater extent by cold-acclimated Midiron crowns, than by cold-acclimated Tifgreen crowns, these proteins could contribute to the greater freezing tolerance of Midiron as compared to Tifgreen plants, and/or they may be useful as markers for freezing tolerance in bermudagrass.

MATERIALS AND METHODS

Plant materials

Both Midiron and Tifgreen are vegetatively-propagated F_1 hybrid plants derived from interspecific crosses of tetraploid (2n = 4X = 36 chromosomes) *C. dactylon* x diploid (2n = 2X = 18 chromosomes) *C. transvaalensis*. Genetic uniformity among cloned plants of these vegetatively propagated cultivars would be expected to contribute to uniformity in protein synthetic responses between cloned plants after CA.

Test plants of four treatment combinations (Midiron control, Midiron after CA, Tifgreen control, and Tifgreen after CA) were acclimated in controlled environment chambers for 4 wk under 8/2 °C (day/night) cycles with a 10 h photoperiod as described by Anderson et al. (1993). A dilute fertilizer (20:20:20 No-Stain Formula Peter's Professional Plant Food, Grace-Sierra Horticultural Products Company, Milpitas, CA) solution (240 mg L^{-1}) was used to water plants in chambers. Control plants were maintained in controlled environment chambers under the same light periods and intensities as cold-acclimating plants, but under non-acclimating (28/24 °C) temperature conditions.

Collecting and in vivo radiolabeling crown tissue

Crown tissue cores from cold-acclimated and control plants were excised and *in vivo* radiolabeled at 2 to 6 °C, and 22 to 26 °C, respectively. Only tissue cores from non-necrotic crowns lacking chlorophyll, but supporting root primordia and green shoots, were excised by removing attached roots and shoots,

as well as surrounding brown tissues. Excised crowns were washed in distilled water, then *in vivo* radiolabeled using the procedure of Porter et al. (1989) modified for labeling crown tissues. Incubation buffer [0.35 ml 20 mM Tris (pH 7.5), 5 mg/ml chloramphenicol (an inhibitor of microbial protein synthesis)] was added to ca. 180 mg crown tissue. Tran^{35}S-label Reagent (ICN Biomedicals, Irvine, CA), an aqueous solution of ^{35}S-labeled amino acids comprising ca. 70% L-Methionine [^{35}S], 15% L-Cysteine [^{35}S], and various non-labeled amino acids, was added in the amount of 0.236 mCi to mixtures containing crown tissue from control plants, and at a 2.5 X rate to acclimated crowns to partially compensate for a reduction in radioactive amino acid incorporation into proteins of acclimated crowns, relative to controls. For sources of non-radiolabeled protein, Tran^{35}S-label Reagent was omitted from incubation mixtures of additional crown tissue samples from each treatment combination. Mixtures were infiltrated under a mild vacuum for 3 X 10 min. Incubation buffer was removed after 16 h and crown tissue was washed once in incubation buffer without Tran^{35}S-label Reagent before being stored at -70 °C.

Protein extraction, solubilization, quantification, and scintillation counting

Samples of trichloroacetic acid-precipitable, acetone-insoluble material were extracted (Damerval et al., 1986) from 180 mg crown tissue samples that had been stored at -70 °C. To solubilize proteins for SDS-PAGE or IEF, 10-μl 'SDS'-solubilization buffer [62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol in purified, distilled H_2O], or 10-μl 'UST' solubilization buffer [9.5 M urea, 2.5%

sodium dodecyl sulfate (SDS), 2.5% Triton X-100 in purified, distilled H_2O] were added per mg acetone-insoluble pellet, respectively. SDS-solubilization buffer was as described by Laemmli (1970) except without *B*-mercaptoethanol (*B*-ME) and bromphenol blue (BPB). Solubilization of proteins in either buffer was for 1 h at 22 to 26 °C with agitation every 15 min. After centrifugation at 12,000x *g* for 15 min, supernatants were re-centrifuged at 12,000x *g* for 15 min for further clarification. Concentrations and radioactivities of proteins in aliquots taken from resulting supernatants were determined using the DC Protein Assay (Bio-Rad Chemical Division, 1991) and by scintillation counting proteins remaining adhered to filter-paper disks (Mans and Novelli, 1961), respectively. SDS-solubilization buffer containing 10% *B*-ME and 0.0025% bromphenol blue (BPB), or 'Ampholyte' buffer [9.5 M urea, 9.5% (v/v) Titron X-100, 4% (v/v) Servalyt 3-10 carrier ampholyte mixture (Crescent Chemical, Hauppauge, NY), 10 mM K_2CO_3, 64.8 mM dithiothreitol (DTT), in purified, distilled H_2O], was added 1:1 (v/w) to remaining supernatants to reconstitute SDS-solubilization buffer with 5% *B*-ME and 0.00125% BPB for SDS-PAGE, or urea, K_2CO_3, SDS (UKS) sample buffer (Damerval et al., 1986) with 2% Servalyt 3-10 carrier ampholyte mixture for IEF, respectively. For SDS-PAGE, from protein concentration (μg/μl) and specific activity (Kdpm/μg) values for each solubilized protein sample, appropriate volumes of ^{35}S-labeled protein samples, non-radiolabeled protein samples, and SDS-solubilization buffer containing 5% *B*-ME and 0.00125% BPB were mixed to prepare samples for each treatment combination

containing 32-μg protein with equal specific activities in ca. 20-μl volumes. For IEF, appropriate volumes of ^{35}S-labeled protein samples, non-radiolabeled protein samples, and UKS buffer were mixed to prepare samples for each treatment combination containing 32-μg protein with equal specific activities in ca. 20-μl volumes. Solubilized protein samples were stored at -70 °C.

To prepare samples initially solubilized in SDS-solubilization buffer for IEF in Mini-PROTEAN II (Bio-Rad, Richmond, CA) tubes, 32-μg protein samples were acetone-precipitated, vacuum dried, and re-solubilization in ca. 20-μl UKS buffer. After IEF, tubes containing focused proteins were subject to SDS-PAGE in large format PROTEAN II xi units. All protein samples that underwent IEF and then SDS-PAGE in Mini-PROTEAN II units had been solubilized in UST buffer directly, i.e., only 2D-PAGE patterns on large-format PROTEAN II xi gels were of protein samples that had been acetone-precipitated from SDS-solubilization buffer and then re-solubilized in UKS buffer.

Gel electrophoresis, silver staining, fluorography

For IEF-PAGE, 4% (w/v) acrylamide (4% T, 5.5% C) gels [(0.8% (v/v) Servalyte 3-10, 1.6% (v/v) Servalyte 5-7, and 1.6% (v/v) Bio-lyte 7-9 carrier ampholyte mixtures; 9.5 M urea; 4% (w/v) 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS)] were polymerized in casting tubes of Mini-PROTEAN II electrophoresis units with ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED). Protein samples (32-μg) in ca. 20-μl UKS buffer were loaded through 0.1 M sodium hydroxide

catholyte buffer onto IEF tubes immersed in 6 mM phosphoric acid anolyte buffer. Samples were overlayed with 20-μl UKS-overlay buffer (2:1 / H_2O:UKS buffer / v:v) and focused for 10 min at 500 volts, then for 3.5 h at 750 volts and maximum current using a ISCO (Lincoln, NE) Model # 470 power supply. Tube gels were extruded (Porter et al., 1992) onto Parafilm strips, soaked in equilibration buffer [0.292 M sucrose, 2.3% (w/v) SDS, 62.5 mM Tris · HCl, pH 6.8] (O'Farrell, 1975) for 45 min, and then stored on Parafilm at -70 °C.

For second dimension SDS-PAGE in Mini-PROTEAN II units, 8 cm x 7.5 cm x 1.0 mm separation gels [12% (w/v) acrylamide (12% T: 2.5% C), 0.15% (w/v) SDS, 29.2 mM sucrose, 0.5 M Tris · HCl, pH 8.8] were polymerized in vertical electrophoresis cells with APS and TEMED. Thawed IEF tube gels were layered over separation gels and sealed in place with 0.5% agarose containing bromphenol blue (BPB) dye. Second dimension electrophoresis was for 45 min at 100 volts per gel and maximum current using a ISCO Model # 470 power supply. Electrophoresis buffer as described by Laemmli (1970) was used. Samples were electrophoresed 40 to 45 min. For second dimension SDS-PAGE through large-format (16 cm x 20 cm x 1 mm) PROTEAN II xi units of protein samples focused in tube gels or in SDS-solubilization buffer, separation gels [14% (w/v) acrylamide, 14% T: 2.56% C)] containing 0.15% SDS were polymerized in vertical electrophoresis cells. To associate protein bands revealed after SDS-PAGE of samples through large-format PROTEAN II xi units with 2D-PAGE spot patterns revealed by 2D-PAGE in Mini-PROTEAN II units,

crown protein samples (3.75-μg) in SDS-solubilization buffer were electrophoresed adjacent to Mini-PROTEAN II IEF tube gels with focused sample proteins through large-format 4% polyacrylamide stacking and 14% polyacrylamide separation gels. For estimations of protein molecular weight (MW), radioactive^{14}C-methylated marker proteins (Amersham, United Kingdom) of 200.0, 97.4, 69.0, 46.0, 30.0, and 14.3 kDa, were electrophoresed in lanes side by side with crown protein samples in SDS-solubilization buffer (Gatschet et al., submitted). Electrophoresis was at 16 mA per gel at maximum voltage using a ISCO Model # 470 power supply for ca. 9 h until BPB dye was about 1 cm from gel bottoms.

Gels were silver stained (Blum et al., 1987), photographed, and stored for several days at 4 °C in 50% methanol. Gels were destained (Kulsar and Prestwich, 1988), impregnated with the fluor 2,5-diphenyloxazole (PPO) (Skinner and Griswold, 1983), dried for fluorography (Porter and Gatschet, 1992), and exposed to preflashed Kodak X-Omat AR film at -70 °C. Exposure of film to Mini-PROTEAN II gels was for 3 to 4 wk (decay densities for gels were ca. 2.1 x 10^7 decays cm^{-2}). Film exposed to dried PPO-impregnated large-format PROTEIN II xi gels was developed after lower decay densities than ca. 2.1 x 10^7 decays cm^{-2} in order to generate fluorographs for estimation of the size (kD) of COR proteins.

Image analysis

Scanned images of silver-stained proteins, as well as of corresponding fluorographic patterns, were analyzed using "Quantity One" and "PDQuest" software applications on a PDI (Huntington Station, NY) densitometer (Krauss et al., 1989). Estimations of protein isoelectric points (pIs) were made assuming a linear pH gradient from pH 10.0 to pH 3.0 in the IEF dimension.

RESULTS AND DISCUSSION

Acidic proteins M1 (34/5.5) (i.e., size of ca. 34 kD, and pI of ca. 5.5) and M2 (34/5.3) in Mo gels, and T1 (34/5.8) and T2 (34/5.3) in To gels, were revealed to be diminished markedly in samples from crowns of Midiron and Tifgreen, respectively, after CA based on silver-staining gels (Figs. 4 and 5). Few other obvious changes in crown protein composition after CA were revealed by silver staining. Using Coomassie blue staining and SDS-PAGE, Perras and Sarhan (1989) found decreases in 34 kD proteins of wheat (*Triticum aestivum*) seedlings after CA for 10 and 40 days. Perras and Sarhan (1989) also reported that, based on 2D-PAGE of *in vivo* radiolabled proteins of wheat, synthesis by leaves and crowns of 34 kD proteins having pIs between ca. 5.5 and 6.0 decreased after CA. The diminished 34 kD proteins of bermudagrass crowns have similar electrophoretic mobilities to the down-regulated 34 kD proteins from wheat leaves and crowns. However, M1 and M2, and T1 and T2 proteins, of Midiron and Tifgreen bermudagrass crowns, respectively, were not *in vivo* radiolabeled in either control or acclimated crowns (Fig. 4). To identify proteins of spots M1, M2, T1, and T2 may require antibody cross-reactivity tests or microsequencing of these proteins after preparative 2D-PAGE.

Fluorography of radiolabeled proteins revealed alterations in protein synthesis by crowns in association with CA. Both Midiron and Tifgreen crowns synthesized several COR proteins in association with CA. Five of the more prominent COR protein groups were: high intermediate MW basic [HIB:45 to

55/9.5 to 8.5 (i.e., sizes from ca. 45 to 55 kD, and pIs from ca. 9.5 to 8.5)]; intermediate MW basic (IB:32 to 37/8.9 to 7.8); intermediate MW acidic (IA:32 to 37/5.3 to 4.1); low MW basic (LB:20 to 28/9.0 to 7.3); and low MW acidic (LA:13 to 15/3.3 to 3.1) (Fig. 4). On large-format PROTEAN II xi silver-stained gels used for estimations of MW, HIB proteins of both Midiron and Tifgreen crowns were resolved into two spots, HIB1 (ca. 53 kD), and HIB2 (ca. 51 kD) (Fig. 5; fluorographs of large-format PROTEAN II xi gel not shown).

On Mini-PROTEAN II gel fluorographs, most labeled protein spots which were greater than 37 kD in size from acclimated crowns generally were reduced in optical density in comparison protein spots greater than 37 kD in size from control crowns, as incorporation of ^{35}S was more prevelant in COR proteins having MWs of 37 kD or less (Fig. 4). HIB COR protein spots, though, were examples of spots of proteins greater than 37 kD in size from acclimated crowns which were not reduced in comparison with spots of control proteins greater than 37 kD in size.

Fluorography indicated that acclimated Midiron crowns synthesized LB COR proteins in greater amounts and numbers than acclimated Tifgreen crowns (Fig. 4). Midiron crowns apparently also synthesized IA COR proteins in greater amounts than Tifgreen crowns after CA (Fig. 4). In addition, Tifgreen crowns synthesized LA COR proteins in slightly greater amounts than Midiron crowns, and Tifgreen crowns apparently synthesized IB COR proteins in greater numbers than Midiron crowns (Fig. 4).

Guy and Haskell (1988) found, also utilizing *in vivo* radiolabeling and 2D-PAGE, that leaves of spinach (*Spinacia oleracea*) seedlings which had been cold-acclimated at 5 °C for 4 d synthesized COR proteins in the size ranges of the low and intermediate MW COR proteins of bermudagrass, as well as of higher MWs (e.g., 160, 117, 85, and 79 kD). Most spinach COR proteins were acidic, having pIs of less than 6.5. Similarly, *in vitro* translation of mRNAs from cold-acclimated spinach leaves produced COR proteins of 160, 85, 79, 55, 45, 28, 27, 26, and 23 kD (Guy and Haskell, 1988). COR proteins of 160 kD had pIs of slightly less than 4.5, while 23 kD COR proteins appeared to have pIs between 6.5 and 10.0, and were likely basic (pIs $\geq$ 7). COR proteins between 160 and 23 kD in size had pIs between 6.5 and 4.6.

The basic pIs of many COR proteins synthesized by Midiron and Tifgreen crowns in association with CA are unusual. In order to further characterize the basic nature of these proteins, research utilizing non-equilibrium pH gradient electrophoresis (NEPHGE) (O' Farrell et al., 1977; Danyluk and Sarhan; 1990) and/or marker proteins of known pI (Dunbar, 1987) may be helpful. Detecting synthesis of COR proteins having apparent pIs greater than 7 was made possible by using basic carrier ampholytes for IEF. In reported 2D-PAGE analyses of COR proteins of other plant species, carrier ampholyte mixtures as basic as those of this study appear not to have been utilized for IEF.

Cattevelli and Bartels (1990) deduced from sequence analysis of cDNA plasmids of cold-induced mRNAs from barley (*Hordeum vulgare*) leaves that the

longest open reading frames of two cDNA clones contain arginine-rich basic domains. They did not report pIs of proteins nor identify possible proteins encoded by these open reading frames. COR proteins having basic charges may have nucleic acid binding functions (Hajela, personal communication). It is possible that some of the basic COR proteins synthesized by acclimated Midiron and Tifgreen crowns have nucleic acid binding functions and regulate alterations in gene expression associated with CA. Some LB COR proteins of acclimated Midiron and Tifgreen crowns were not detectable by silver staining, but only on fluorography (Fig. 4), indicating that some LB COR proteins are present only at low concentrations. Proteins regulating gene expression may function at low concentrations.

Characterizing COR proteins of bermudagrass and other plant species improves understanding of the physiological changes underlying CA and freezing tolerance in plants. Though little of this information has been applied to selecting more freezing tolerant plants (Blum, 1988), assaying plants for levels of proteins cross-reactive with COR proteins of bermudagrass or other species may be useful in selecting plants having superior tolerance to freezing. Houde et al. (1992) found that levels of proteins that were immunologically related to a 50 kDa COR protein of wheat correlated well to levels of freezing tolerance in cold-acclimated Graminaceous plants.

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Figure 4. Crown Proteins after 2D-PAGE in Mini-PROTEAN II Units. Mo: Midiron control; Ma: Midiron after CA; To: Tifgreen control; Ta: Tifgreen after CA. Left panels are silver stained gels. Right panels are corresponding fluorographs of destained gels. In Mo gels, protein spots M1 (ca. 34 kD/pI of 5.5, i.e., 34/5.5) and M2 (34/5.3), and in To gels, protein spots T1 (34/5.8) and T2 (34/5.3), are labeled with open arrows in 2D-PAGE gels of protein samples from control crowns. Five more prominent COR protein groups are labeled on both silver stained gels and fluorographs of protein samples from acclimated crowns: HIB (high intermediate MW basic; ca. 45 to 55 kD and pIs from 9.5 to 8.5, i.e., 45 to 55/9.5 to 8.5); IB (intermediate MW basic; 32 to 37/8.9 to 7.8); IA (intermediate MW acidic; 32 to 37/5.3 to 4.1); LB (low MW basic; 20 to 28/9.0 to 7.3 for Ma, and 20 to 28/9.0 to 7.7 for Ta); and LA (low MW acidic; 13 to 15/3.3 to 3.1).

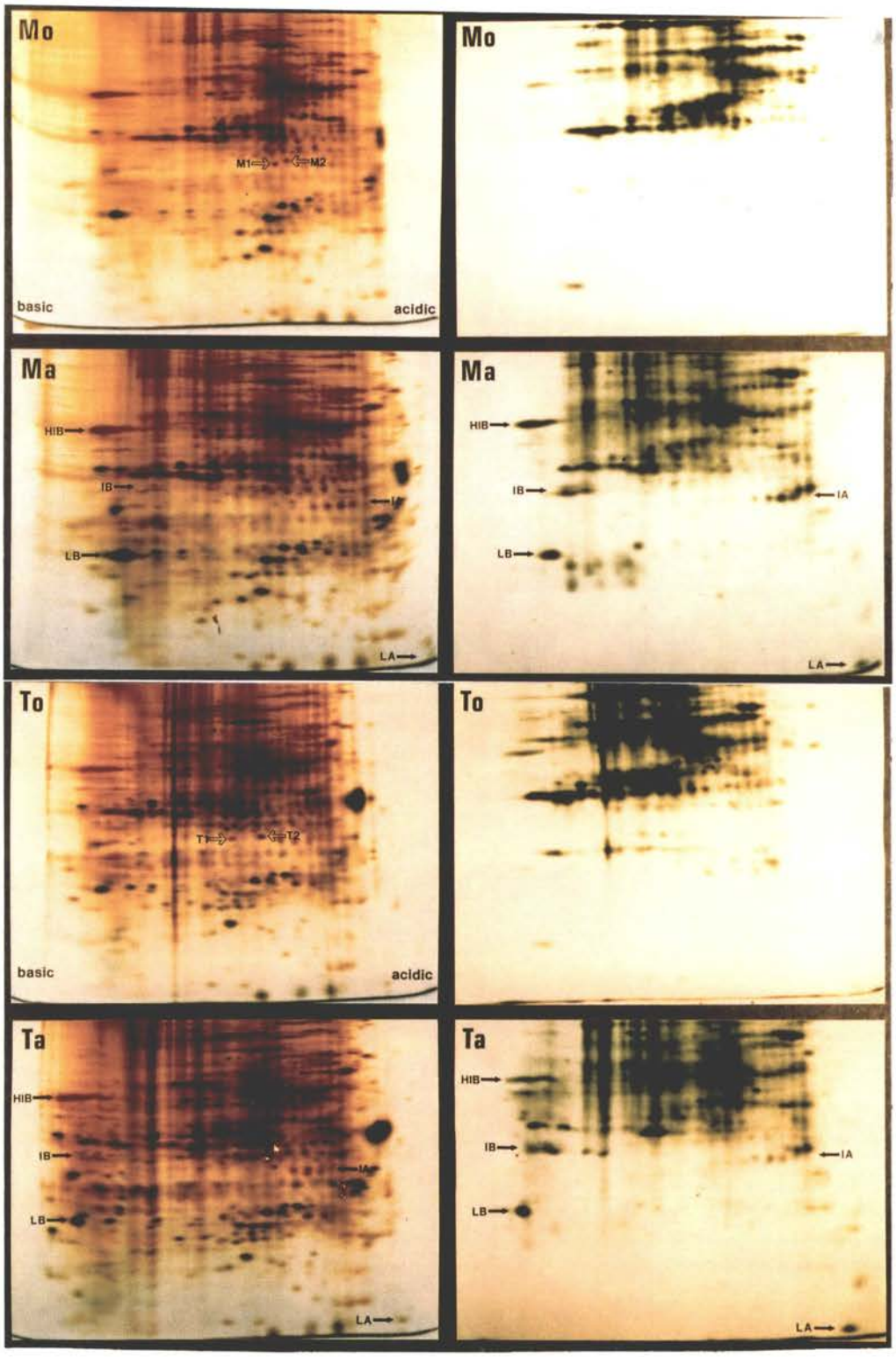
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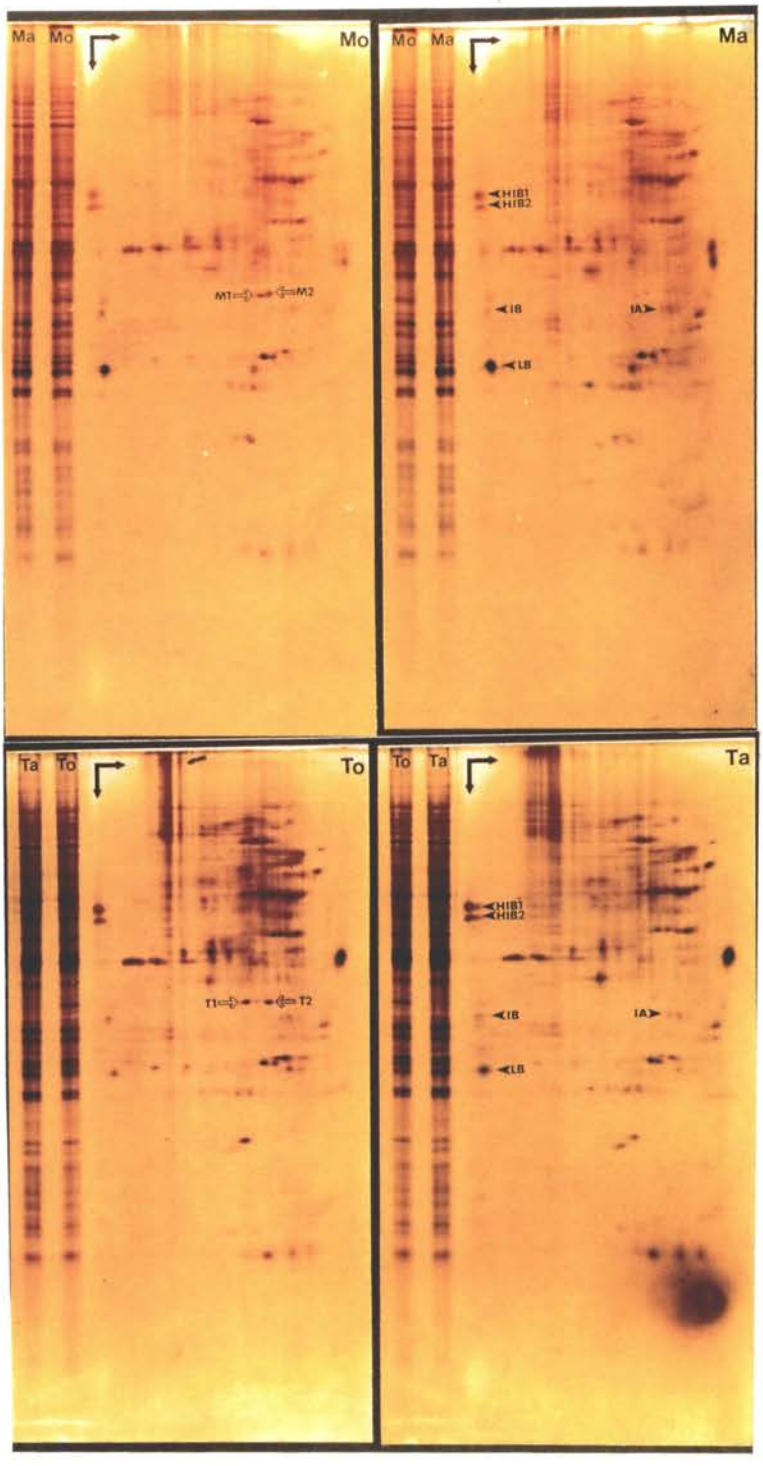
Figure 5. Silver-stained Crown Proteins after 2D-PAGE in Large-format PROTEAN II xi Units. Mo: Midiron control; Ma: Midiron after CA; To: Tifgreen control; Ta: Tifgreen after CA. Protein spots M1 (ca. 34 kD/pI of 5.5, i.e., 34/5.5) and M2 (34/5.3) in Mo gels, and T1 (34/5.8) and T2 (34/5.3) in To gels, are marked by open arrows. Representative protein spots of HIB1 (high intermediate MW basic of ca. 53 kD) and HIB2 (high intermediate MW basic of ca. 51 kD), IB (intermediate MW basic of ca. 33 kD), IA (intermediate MW acidic of ca. 33 kD), and LB (low MW basic of ca. 27 kD) are marked. The dark region on the lower right of the Ta gel is a staining artifact.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APPENDIX A

DETERMINATION OF EXPOSURE TIMES FOR AUTORADIOGRAPHY AND FLUOROGRAPHY OF GELS CONTAINING PROTEINS LABELED WITH RADIOACTIVE ISOTOPES

Introduction

Radioactive isotopes are used to label biomolecules for detection in autoradiography or fluorography (Hahn, 1983). In particular, isotopes ^{3}H, ^{14}C, and ^{35}S (and, in special cases, ^{59}Fe and ^{131}I) are used as tracers of protein metabolism (Sheeler and Bianchi, 1980). These five isotopes all decay by emitting β-particles, though total average radiation energy (keV) per disintegration of a neutron varies from 5.70 keV for ^{3}H, 49.5 keV for ^{14}C, 48.6 keV for ^{35}S, 118 keV for ^{59}Fe, to 182 keV for ^{131}I (Browne and Firestone, 1986). The average radiation energy per neutron disintegration for ^{3}H, relative to other radioactive isotopes, is low. This being the case, fluorography may be preferred to autoradiography for timely detection of proteins labeled with ^{3}H, while either direct exposure autoradiography with intensifying screens, or fluorography, may be used for detection of proteins labeled with ^{14}C, ^{35}S, or other radioactive isotopes that emit higher radiation energies per neutron disintegration than ^{3}H.

In addition, since the average energy of γ-radiation per neutron disintegration exceeds the average energy of β-radiation per disintegration for ^{59}Fe and ^{131}I (Browne and Firestone, 1986), the contribution of γ-radiation to latent image formation on X-ray film would facilitate direct exposure autoradiography for ^{59}Fe- and ^{131}I-labeled proteins. For ^{3}H, ^{14}C, and ^{35}S, on the other hand, average energy of γ-radiation per neutron disintegration is 5000-fold or more less than the average energy of β-radiation per disintegration (Browne and Firestone, 1986). Therefore, latent image formation on X-ray film may largely result from β-radiation for radioactive isotopes ^{3}H, ^{14}C, and ^{35}S.

Whether exposure of X-ray film for the detection of labeled proteins is by autoradiography or fluorography, exponential functions describing the decay of radioactive isotopes may be used to calculate levels of radioactivity for samples of labeled proteins at discrete times after an initial determination of radioactivity incorporated into proteins has been made using scintillation counting. Decay of isotopes is described by the exponential function (Salas and Hille, 1974):

$y = y_o \cdot e^{k \cdot t}$ where 'k' is a constant specific for the isotope and the units of time used to measure 't',

't' is elapsed time since scintillation counting,

'*e*' is the natural logarithm base,

'y' is the radioactivity of the labeled sample at time 't', and

'y_o' is the radioactivity of the labeled sample at 't' = 0.

Initially, when 't' = 0, $y = y_o \cdot e^{k \cdot 0} = y_o \cdot e^{0} = y_o$.

For isotope ^{35}S, a numeric value for constant 'k' can be calculated for 't' measured in units of days (d) as follows [the half-life of ^{35}S is approximately 87.51 d (Browne and Firestone, 1986)]:

$$\text{at } y = {}^1/_2 \cdot y_o,\ y = y_o \cdot e^{k \cdot 87.51},$$

$$\text{so } {}^1/_2 = e^{k \cdot 87.51},$$

$$\ln({}^1/_2) = k \cdot 87.51,$$

$$k = \ln({}^1/_2) \div 87.51 = -\ln(2) \div 87.51 \quad (\text{or } k \doteq -0.007921).$$

Similarly, values of 'k' specific for both an isotope and the units of time used to measure a half-life value of that isotope may be calculated (half-life values from Browne and Firestone, 1986):

Isotope	**Half-life**	**'k'**
^3H	12.33 yr	- 0.05621
^{14}C	5730 yr	-1.210×10^{-4}
^{35}S	87.51 d	- 0.007921
^{59}Fe	44.496 d	- 0.015578
^{131}I	8.040 d	- 0.08621

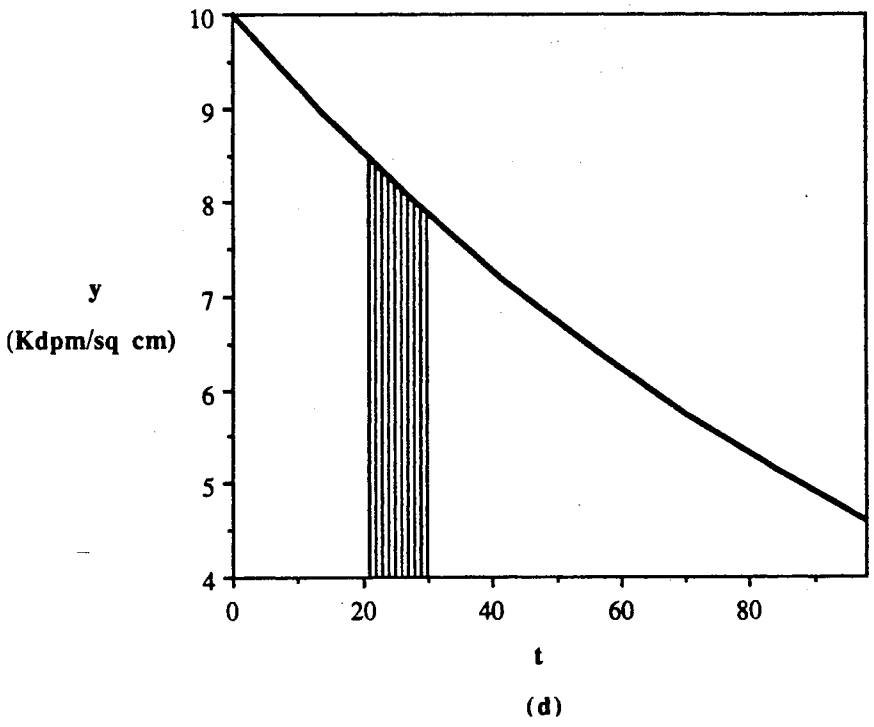
If a polyacrylamide gel in which separated proteins labeled with ^{35}S-methionine and/or ^{35}S-cysteine has a radioactivity density of 10 Kcpm cm^{-2} (i.e., 10 thousand counts per minute per cm^2) at the time of exposure to X-ray film, it would have a radioactivity density of 5 Kcpm cm^{-2} after 87.51 d. Radioactivity density at, for example, 't' = 14 d, of this gel having y_o = 10 Kcpm cm^{-2}, could be calculated using the exponential function for isotope decay and the value of 'k' calculated for ^{35}S half-life expressed in d units of time:

$$y = 10 \cdot e^{k \cdot t} = 10 \cdot e^{(-\ln 2/87.51) \cdot 14} \doteq 8.950 \text{ Kcpm cm}^{-2}.$$

Since y_o is expressed in Kcpm cm^{-2} units, 'y' is expressed in these units, too. As in the above equation, the constant, 'k', needs to be calculated using half-life values expressed in the same units of time as the variable 't'.

Values of radioactivity density may be calculated at intervals of 14 d over a 98d period as listed in the following table. Furthermore, these radioactivity density values at 14 d intervals also may be represented graphically, too (as presented after the following table; the shaded region between 't' = 21 and 30 d depicts an area of integration).

't' (d)	'y' (Kcpm cm^{-2})
14	8.950
28	8.011
42	7.170
56	6.417
70	5.744
84	5.141
98	4.601



If a gel containing ^{35}S-labeled proteins and having y_o = 10 Kcpm cm^{-2} were used to expose X-ray film exactly 21 d after scintillation counting, and the X-ray film were developed exactly 9 d later, the cumulative count total per cm^2 (C cm^{-2}) of decayed ^{35}S isotopes of the gel during this 9 d period would be the definite integral of the exponential decay function $y = y_o e^{(-\ln 2/87.51)\cdot t}$ between 't' = 21 d (time of exposure) and 't' = 30 d (time of development). This integral value corresponds to the shaded area in the preceding graph.

Using integral calculus, and remembering that the indefinite integral of $e^{k\cdot t}$, i.e., $\int e^{k\cdot t}\,dt = (1/k)\cdot e^{k\cdot t}$, a numeric value for C cm^{-2} between exposure at time 't' = 21 d and development at time 't' = 30 d may be calculated as follows:

$$\int_{21}^{30} y_o \cdot e^{k\cdot t}\,dt = y_o \cdot (1/k)\cdot \left\{ e^{k\cdot t}\Big|_{21}^{30} \right\}$$

$$= 10\cdot (87.51/-\ln 2)\cdot \left\{ e^{k\cdot t}\Big|_{21}^{30} \right\}$$

$$\doteq -1262.5 \cdot \left\{ e^{\{(-\ln 2)/87.51\}\cdot 30} - e^{\{(-\ln 2)/87.51\}\cdot 21} \right\}$$

$$\doteq 73.555 \text{ Kcpm } cm^{-2}\cdot \text{ d}$$

$$\doteq 73.555 \text{ Kcpm } cm^{-2}\cdot \text{ d}\cdot \{1440 \text{ min} / \text{d}\}$$

$$\doteq 1.0592 \times 10^8 \text{ counts } cm^{-2}$$

Similarly, if X-ray film that were exposed by a gel exactly 21 d after scintillation counting of ^{35}S-labeled proteins contained in the gel--the proteins of this gel having a radioactivity density of 10 Kcpm cm $^{-2}$ at the time of scintillation counting--and the X-ray film was to be developed after having been exposed to a potential C cm^{-2} of 1.0592 x 10^8 counts cm^{-2}, this X-ray film would need to be developed exactly 9 d after exposure, or exactly 30 d after scintillation counting.

As this example indicates, exposure times required for targetted C cm^{-2} values may also be calculated. If X-ray film exposed to ^{35}S-labeled proteins (of a gel having y_o = 10 Kcpm cm^{-2}, and exposed exactly 21 d after scintillation counting) was to be developed after 1.0 x 10^9 counts cm^{-2}, the duration of exposure required could be calculated as follows:

Note: 1.0 x 10^9 counts cm^{-2}]$\cdot \left\{ {}^{d}/_{1440\ min} \right\}$ = 694.44 Kcpm cm^{-2} $\cdot$ d, so

$$\int_{21}^{td} y_o \cdot e^{k \cdot t} dt = y_o \cdot \left({}^{1}/_{k}\right) \cdot \left\{ e^{k \cdot t} \Big|_{21}^{td} \right\} = 694.44 \text{ Kcpm cm}^{-2} \cdot \text{d}$$

$$y_o \cdot \left({}^{1}/_{k}\right) \cdot \left\{ e^{k \cdot td} - e^{k \cdot 21} \right\} = 694.44$$

$$e^{k \cdot td} - e^{k \cdot 21} = {}^{(k \cdot 694.44)}/_{y_o}$$

$$e^{k \cdot td} = {}^{(k \cdot 694.44)}/_{y_o} + e^{k \cdot 21}$$

$$k \cdot t_d = \ln \left[{}^{(k \cdot 694.44)}/_{y_o} + e^{k \cdot 21} \right]$$

$$t_d = \ln \left[{}^{(k \cdot 694.44)}/_{y_o} + e^{k \cdot 21} \right] \div k$$

$$t_d = \ln\left[\left(-\ln 2 / 87.51\right)\cdot 694.44/_{10} + e^{(-\ln 2 / 87.51)\cdot 21}\right] \div \left\{-\ln 2/_{87.51}\right\}$$

$$= \left\{87.51/_{-\ln 2}\right\}\cdot \ln\left[\left(-\ln 2 / 87.51\right)\cdot 69.444 + e^{(-\ln 2 / 87.51)\cdot 21}\right]$$

$$= 153.39 \text{ d}$$

Hence, exposure duration = $t_d - t_i = 153.39 - 21.000 = 132.39$ d

$\doteq$ 132 d, 9 h, 22 min.

Summary

In summary, three equations were derived. First, for a given isotope and specific units of time used to measure half-life ('h'), a numeric value for the constant 'k' is the solution to:

$$k = \ln(1/2) \div h \quad , \text{ or } \quad k = -\ln 2 \div h. \qquad (1)$$

Second, knowing times for X-ray film exposure initiation (t_i) and for film development (t_d) (in units of time since scintillation counting of a sample), as well as 'y_o' (Kcpm cm^{-2} at scintillation counting), a cumulative count total per cm^{-2} (C cm^{-2}) of exposed X-ray film is:

$$C\ cm^{-2} = \int_{ti}^{td} y_o \cdot e^{k \cdot t} = y_o \cdot \left({}^{1}/_{k} \right) \cdot \left\{ e^{k \cdot t} \Big|_{ti}^{td} \right\}$$

$$\text{or, } C\ cm^{-2} = y_o \cdot \left({}^{1}/_{k} \right) \cdot \left\{ e^{k \cdot td} - e^{k \cdot ti} \right\} \qquad (2)$$

Third, for any gel containing isotope-labeled proteins (e.g.,^{35}S-labeled) for which y_o and t_i are known, and a targetted C cm^{-2} (= 'C' in Kcpm·d cm^{-2} below) is set, the time of development (t_d) since scintillation counting is the solution to:

$$t_d = \ln \left[{}^{k \cdot C}/_{y_o} + e^{k \cdot ti} \right] \div k \quad \left(k = {}^{-\ln 2}/_{87.2} \text{ for } {}^{35}S \right). \qquad (3)$$

Exposure duration would be $t_d - t_i$.

These equations may be useful for calculation of exposure times for autoradiography and fluorography of gels containing proteins labeled with radioactive isotopes. Though integral calculus was used to derive these equations, integration is not needed in the equations to determine exposure times (equation 3), nor cumulative counts (equation 2), nor 'k' values (equation 1). A pocket calculator with exponential function powers is sufficient. Finally, these equations may be useful for the development of spreadsheets. After entering values of independent variables and constants (e.g., values for 'k', 'y_o', 't_d', and 't_i' in equation 2), a spreadsheet program could calculate values for dependent variables (e.g., a value for C cm^{-2} in equation 2).

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APPENDIX B

REGROWTH DATA AND ESTIMATIONS OF LT_{50} VALUES FOR MIDIRON AND TIFGREEN BERMUDAGRASSES

Introduction

LT_{50} values for plants of four treatment combinations ('Midiron' control: Mo, Midiron after CA: Ma, 'Tifgreen' control: To, and Tifgreen after CA: Ta) were estimated using a regrowth test procedure of Anderson et al. (1993) as outlined in Gatschet et al. (submitted). Original data for regrowth evaluations, as well as linear interpolations used to estimate LT_{50} values, are presented here.

Materials and Methods

Eight weeks after application of freezing treatments, cone-tainer-grown bermudagrass plants (maintained in a controlled environment chamber set for control conditions) were scored for regrowth. Plants were then transferred to a greenhouse. There they also were scored for regrowth 16 weeks after freezing. Plants were scored according to five categories based on condition of the plant and extent of regrowth--O = apparently dead, 1 = very weak, 2 = weak, 3 = good, 4 = excellent. More specific descriptions of each category follow:

Category	Description
0	No apparent evidence for whole plant viability, though hidden subepidermal cells and tissues below soil surface not examined.
1	Green coloration on some shoots.
2	One (or a few) short ($\leq$ 4 cm) shoot(s) green throughout.
3	One long ($\geq$ 8 cm), and/or several medium-length (ca. 4 to 8 cm), shoots green throughout.
4	Two or more long ($\geq$ 8 cm) green shoots.

Approximate shoot length was an important measure of regrowth, for before application of freezing treatments, all plants were trimmed to < 4 cm. Plants rated as being dead (0), very weak (1), or weak (2) after eight or 16 week periods of regrowth were not considered to be viable plants for turf re-establishment after freezing stress. The proportion of the four plants of each treatment combination (Mo, Ma, To, or Ta) equilibrated at each freezing temperature having good (3) or excellent (4) regrowth eight or 16 weeks after freezing stress was determined (e.g., 0/4 = 0; 1/4 = 0.25, 2/4 = 0.5, 3/4 = 0.75, 4/4 = 1.0) . LT_{50} values (temperatures, estimated by linear interpolation, at which 50% of the plants would be expected to recover to have good or excellent regrowth) were estimated using regrowth data taken after eight weeks, and after 16 weeks.

Results

Table V. Regrowth Data for Control Midiron (Mo) Plants. Regrowth scores (O=dead, 1=very weak, 2=weak, 3=good, 4=excellent) were made 8 and 16 weeks after equilibration of plants to ca. 1 °C decrements of freezing stress.

Temp	8 wk	16 wk	Temp	8 wk	16 wk
control	4	4	-7.1	0	0
control	4	4	-7.0	0	0
control	4	4	-7.0	0	0
control	4	4	-7.0	0	0
-4.0	4	4	-8.0	0	0
-4.0	4	4	-8.0	0	0
-4.0	4	4	-8.0	0	0
-4.0	4	4	-8.1	0	0
-5.0	0	0	-9.0	0	0
-5.0	4	4	-9.1	0	0
-5.0	4	4	-9.0	0	0
-5.0	4	3	-9.1	0	0
-6.0	3	3	-10.0	0	0
-6.0	3	0	-10.0	0	0
-6.0	4	3	-9.9	0	0
-6.0	4	4	-10.0	0	0

Table VI. Regrowth Data for Cold-acclimated Midiron (Ma) Plants. Regrowth scores (O=dead, 1=very weak, 2=weak, 3=good, 4=excellent) were made 8 and 16 weeks after plants were equilibrated to ca. 1 °C decrements of freezing stress.

Temp	8 wk	16 wk	Temp	8 wk	16 wk
control	4	4	-10.0	0	0
control	4	4	-10.0	4	4
control	4	4	-10.0	4	4
control	4	4	-10.1	4	4
-7.1	4	4	-11.0	1	0
-7.1	4	4	-11.0	3	4
-7.0	4	4	-10.9	4	4
-7.1	4	4	-10.9	4	4
-8.1	4	4	-12.0	0	0
-8.0	4	4	-12.0	0	0
-8.0	4	4	-11.9	0	0
-8.1	4	4	-12.0	0	0
-9.0	4	4	-13.0	0	0
-9.1	4	4	-13.0	0	0
-9.0	4	4	-13.0	0	0
-9.0	4	4	-13.0	0	0

Table VII. Regrowth Data for Control Tifgreen (To) Plants. Regrowth scores (O=dead, 1=very weak, 2=weak, 3=good, 4=excellent)were made 8 and 16 weeks after plants were equilibrated to ca. 1 °C decrements of freezing stress (excepting the 0.4 °C decrement between -3.6 and -4.0 °C).

Temp	8 wk	16 wk	Temp	8 wk	16 wk
control	4	4	-6.0	0	0
control	4	4	-6.0	0	0
control	4	4	-6.0	0	0
control	4	4	-6.0	0	0
-3.6	0	0	-7.0	0	0
-3.6	4	4	-7.0	0	0
-3.8	0	0	-7.0	0	0
-3.6	4	4	-7.0	0	0
-4.0	0	0	-8.0	0	0
-4.0	0	0	-8.0	0	0
-4.0	0	0	-8.1	0	0
-4.0	1	0	-8.1	0	0
-5.0	0	0	-9.0	0	0
-5.0	0	0	-9.1	0	3
-5.0	0	0	-9.0	0	0
-5.0	4	4	-9.0	0	0

Table VIII. Regrowth Data for Cold-acclimated Tifgreen (Ta) Plants. Regrowth scores (O=dead, 1=very weak, 2=weak, 3=good, 4=excellent) were made 8 and 16 weeks after plants were equilibrated to ca. 1 °C decrements of freezing stress.

Temp	8 wk	16 wk	Temp	8 wk	16 wk
control	4	4	-8.0	0	0
control	4	4	-8.1	4	4
control	4	4	-8.0	4	4
control	4	4	-8.1	4	4
-5.0	4	4	-9.0	4	1
-5.0	4	4	-9.1	0	0
-5.0	4	4	-9.0	0	0
-5.0	4	4	-8.9	0	0
-6.0	4	4	-10.0	1	0
-6.0	4	4	-10.0	0	0
-6.0	4	4	-10.0	0	0
-6.0	4	4	-10.0	4	4
-7.0	4	4	-11.0	1	0
-7.0	4	4	-11.1	0	0
-7.0	4	4	-10.9	0	0
-7.0	4	4	-11.0	0	0

Figure 6. Estimations of LT_{50} Values by Linear Interpolation Using Regrowth Data Taken after Eight Weeks.

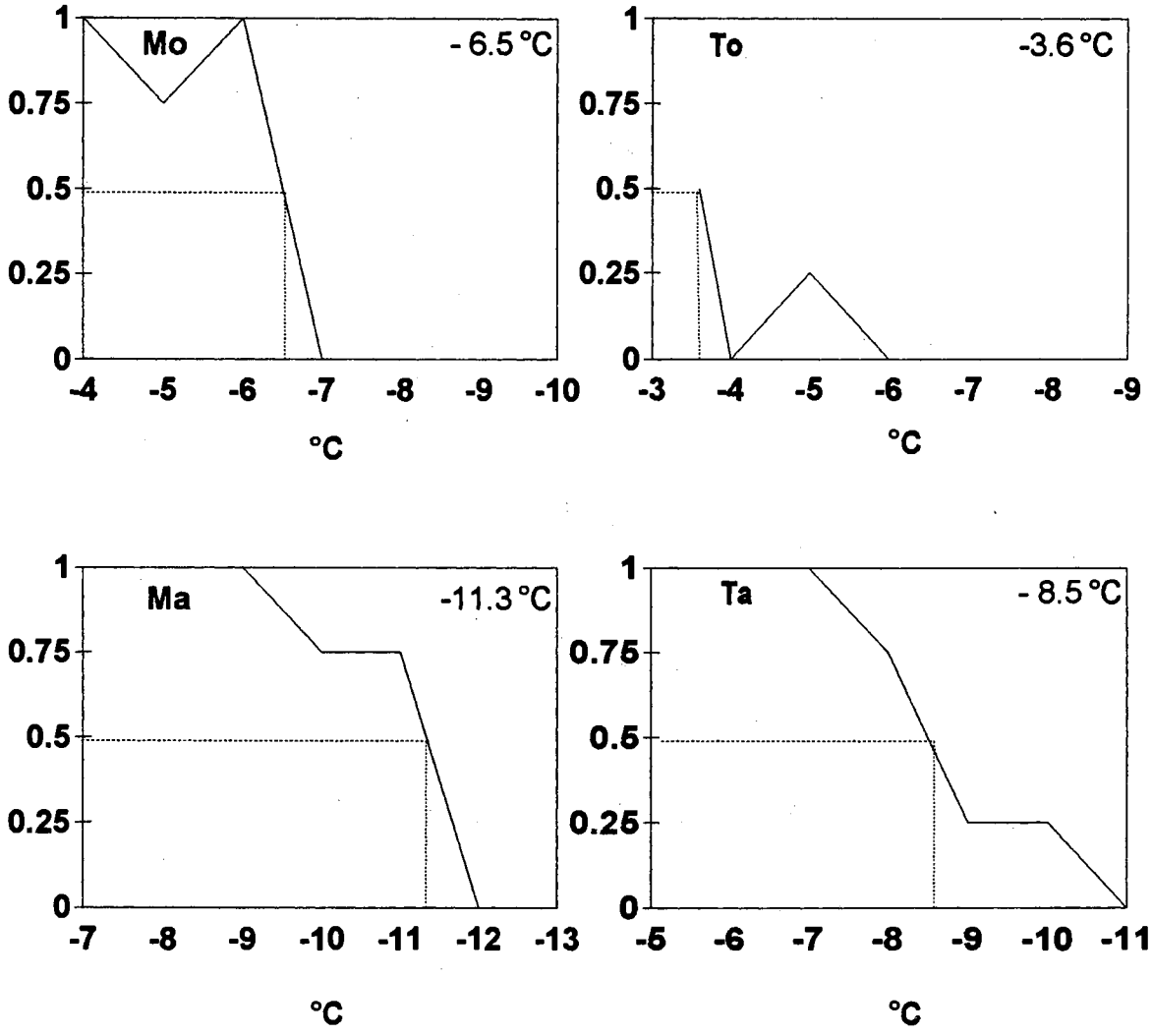
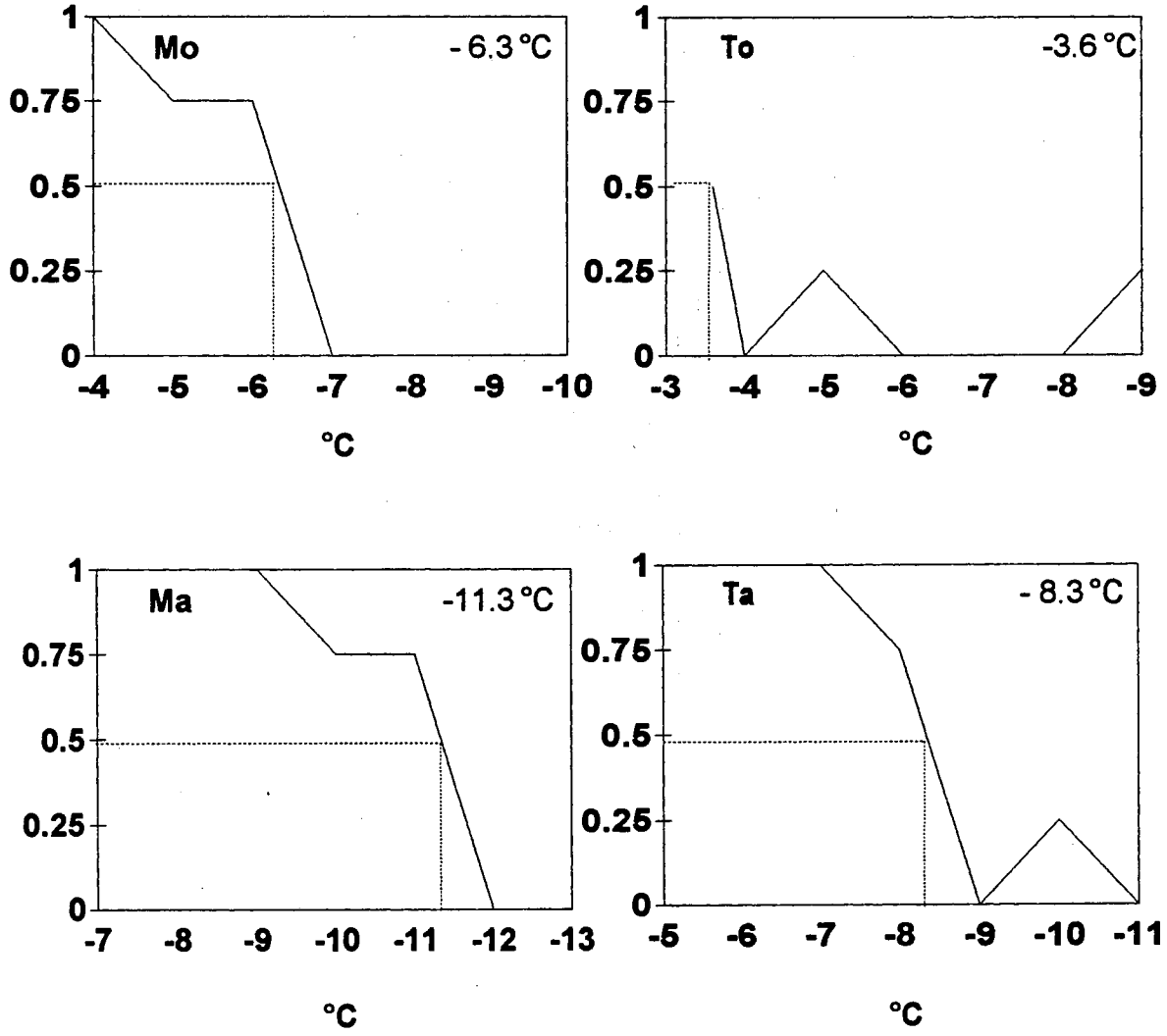

Mo
- 6.5 °C
1
0.75
0.5
0.25
0
-4 -5 -6 -7 -8 -9 -10
°C
To
-3.6 °C
1
0.75
0.5
0.25
0
-3 -4 -5 -6 -7 -8 -9
°C
Ma
-11.3 °C
1
0.75
0.5
0.25
0
-7 -8 -9 -10 -11 -12 -13
°C
Ta
- 8.5 °C
1
0.75
0.5
0.25
0
-5 -6 -7 -8 -9 -10 -11
°C

Figure 7. Estimations of LT_{50} Values by Linear Interpolation Using Regrowth Data Taken after Sixteen Weeks.


Mo
- 6.3 °C
1
0.75
0.5
0.25
0
-4 -5 -6 -7 -8 -9 -10
°C
To
-3.6 °C
1
0.75
0.5
0.25
0
-3 -4 -5 -6 -7 -8 -9
°C
Ma
-11.3 °C
1
0.75
0.5
0.25
0
-7 -8 -9 -10 -11 -12 -13
°C
Ta
- 8.3 °C
1
0.75
0.5
0.25
0
-5 -6 -7 -8 -9 -10 -11
°C

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APPENDIX C

PROTEIN METHODOLOGIES

In Vivo Radiolabeling of Proteins

The following solutions, adapted from laboratory notes of Dr. David R. Porter, were used for *in vivo* radiolabeling proteins of crowns:

20 mM Tris Buffer

242 mg Trizma Base
90 ml Nanopure H_2O
Adjust pH to 7.5 using concentrated HCl. Add Nanopure H_2O to bring buffer to 100 ml. Autoclave, or filter sterilize, and store refrigerated.

Chloroamphenicol (CAM) Solution (5 mg CAM/ml) (prepare fresh)

5 mg Chloramphenicol
1 ml 95% Ethanol

Tris-CAM Solution (50 μg CAM/ml) (prepare fresh)

5 ml 20 mM Tris Buffer
50.5 ul CAM Solution (5 mg CAM/ml)

Collect non-necrotic crown tissue cores lacking chlorophyll from several plant phytomers with green shoots. Carefully, remove roots, shoots, and

browning tissues surrounding crowns with scissors and forceps. Thoroughly wash collected crown tissue cores in distilled water, then swab cores in paper towels to remove excess water. Place washed crown tissues from 2 or more plants in pre-weighed 1.5 ml microcentrifuge tubes. Add crown tissue cores from other plant phytomers so that tissue totals per tube approximate 90 or 180 mg.

Tran^{35}S-Label from ICN Biomedicals (Irvine, CA)

Store at -70 °C until used. Tran^{35}S-label is produced from a cellular hydrolysate of *E. coli* grown in the presence of carrier free $^{35}SO_4^{-2}$. Tran^{35}S-label stock contains 70% L-Methionine[^{35}S], 15% L-Cysteine [^{35}S], and various nonlabeled amino acids in 50 mM lysine (pH 7.4) with 10 μmoles β-mercaptoethanol/ml. Vials of stock Tran^{35}S-label used in this research had total activities of 7.1 mCi on stock reference dates and volumes of 0.448 $\pm$ 0.024 ml.

After storage (e.g., for several days or weeks after shipment), use the exponential decay function for ^{35}S ($y = y_o \cdot e^{k \cdot t}$, where '$y_o$' is radioactivity on the reference date, 'k' for ^{35}S is ca. -0.007921 [for time ('t') measured in units of days], and 't' is days since the reference date) to calculate radioactivity on the date of radiolabeling. Use Tran^{35}S-label stock to make the following solution for radiolabeling 180 mg crown tissue:

Radiolabeling Solution (ca. 0.61 mCi, or 1.52 mCi, /ml Tris-CAM solution, for control and acclimated crowns, respectively)

350 μl Tris-CAM Solution
0.236 mCi, or 0.67 mCi, Tran^{35}S-label stock for control crowns or cold-acclimated crowns, respectively (e.g., 34 μl, or 97 μl, of 6.94 μCi/μl stock, respectively. Radioactivity of 6.94 μCi/μl would be expected after 98 d storage of stock with 7.1 mCi/0.471 ml on its reference date. Radioactivity values for the Tran^{35}S-label, e.g., 0.236 mCi for labeling proteins of control crowns, refer to total radioactivity, not simply L-Met[^{35}S] radioactivity.

Cool radiolabeling solution having 2.5 X radioactivity in a refrigerator ca. one hour before *in vivo* radiolabeling of cold-acclimated crowns. Add 350 μl radiolabeling solution to each 180 mg crown sample in 1.5 ml microcentrifuge tubes, or, 175 μl to each of two 90 mg crowns samples. Place radiolabeling solution having 1X radioactivity in tubes containing control crowns and cooled radiolabeling solution having 2.5 X radioactivity in tubes containing cold-acclimated crowns. Keep tubes of cold-acclimated crowns on ice.

Infiltrate radiolabeling solutions into control, and cold-acclimated crown tissues, under mild vacuum for 3 x 10 min at room temp., and on ice, respectively. Incubate both control and cold-acclimated crowns in radiolabeling solution for 16 h. Incubate mixtures of control crowns and radiolabeling solution at room temp. (22 to 26 °C), and keep mixtures of cold-acclimated crowns and radiolabeling solution with 2.5 X radioactivity refrigerated (2 to 6 °C). After 16 h, remove radiolabeling solutions and briefly wash control, or cold-acclimated, crowns once in Tris-CAM buffer maintained at room temp., or refrigerated, respectively. After washing, store crowns at -70 °C.

Discard excess and used radiolabeling solutions, and Tris-CAM buffer washes, in containers for liquid radioactive waste. Discard pipet tips and plastic tubes contaminated with ^{35}S in containers for solid radioactive waste.

For sources of non-radiolabeled crown proteins, prepare 180 mg crown samples and incubate crowns for 16 h as described above in Tris-CAM buffer lacking Tran^{35}S-label at room temp. (22 to 26 °C), or refrigerated (2 to 6 °C), for

control and cold-acclimated crowns, respectively. After 16 h, wash control, or cold-acclimated, crowns once in Tris-CAM buffer maintained at room temp., or refrigerated, respectively. After washing, store crowns at -70 °C.

Isolation of Proteins from Crowns

Isolation Solutions (after Damerval et al., 1986)

10% Trichloroacetic Acid (TCA) in Acetone (100 ml)

10.0 g Trichloroacetic Acid (TCA)
Add Acetone to 100 ml, then add 70 µl β-Mercaptoethanol (β-ME)

Store at -20 °C.

Acetone with 0.07% β-Mercaptoethanol (100 ml)

To 100 ml Acetone, add 70 µl β-ME

Store at -20 °C.

Protein Extraction (modified from Damerval et al., 1986)

Place 180 mg crown samples that had been stored at -70 °C in a mortar. Thoroughly grind crown tissue to a fine powder with a pestle using several washes (e.g., 6 or 7) of liquid nitrogen. Add 12 ml of chilled (-20 °C) 10% TCA in acetone with 0.07% β-ME. (10% TCA in acetone may freeze in a liquid-nitrogen-cooled mortar. Simply wait until the 10% TCA in acetone thaws before continuing the protein extraction. The 10% TCA in acetone usually thaws in ca. 10 min). Mix and suspend powdered crown tissue in solution of 10% TCA in

acetone and transfer suspension to 8 pre-weighed 1.5 ml microcentrifuge tubes. Place tubes in a -20 °C freezer for 45 min, then microfuge at 12,000x *g* for 15 min. Remove supernatants by pipet and discard. Thoroughly resuspend each pellet in 1.5 ml chilled (-20 °C) acetone with 0.07% β-ME (12 ml acetone total). Place tubes at -20 °C for 1 h, then microfuge at 12,000x *g* for 15 min. Again, remove supernatants by pipet and discard.

Tightly cover each 1.5 ml microcentrifuge tube containing pelleted acetone-insoluble material with a strip of parafilm. After making a hole in the center of each parafilm cover, vacuum dry pellets in tubes for 3 X 10 min under a mild vacuum. Stretched parafilm remaining around the lip of each 1.5 ml microcentrifuge tube will limit potential loss, by vacuum aspiration, of loose pieces of dried acetone-insoluble pelleted material. After vacuum drying, remove parafilm and determine the mass of each dried acetone-insoluble pellet remaining in each tube by weighing each tube/pellet and subtracting the mass of the pre-weighed tube. Store dried acetone-insoluble pellets at -70 °C.

Protein Solubilization

SDS-PAGE Solubilization Solutions (modified from Leammli, 1970)

SDS-Solubilization Buffer Compatible with the DC Protein Assay (8 ml)

Amount	Component	Final Concentration
1.0 ml	0.5 M Tris (pH 6.8)	62.5 mM
1.6 ml	10% (w/v) SDS	2% (w/v) SDS
1.01 g (0.8 ml)	Glycerol	10% (v/v) glycerol
4.6 ml	Nanopure H_2O	

This SDS-solubilization buffer is compatible with the Bio-Rad DC Protein Assay procedure described below. However, since this solution lacks a reducing agent, it is not intended for solubilization of protein samples to be left at ambient temperatures for extended periods after solubilization. Solubilize protein samples in SDS-solubilization buffer compatible with the DC Protein Assay prior to protein quantification as described below. After removing aliquots for protein quantification using the DC Protein Assay, store samples at -70 °C. Add equal volumes of SDS-solubilization buffer with 2X β-ME and BP Blue before freezing, or after thawing, to reconstitute protein samples solubilized in SDS-solubilization buffer with 1X β-ME and BP Blue suitable for SDS-PAGE.

SDS-Solubilization Buffer with 2X β-ME and BP Blue (8ml)

Amount	Component	Final Concentration
1.0 ml	0.5 M Tris (pH 6.8)	62.5 mM
1.6 ml	10% (w/v) SDS	2% (w/v) SDS
1.01 g (0.8 ml)	Glycerol	10% (v/v) glycerol
0.8 ml	β-Mercaptoethanol	10% β-ME
0.4 ml	0.05% Bromphenol Blue	0.0025% BP Blue
3.4 ml	Nanopure H_2O	

After protein quantification, add SDS-solubilization buffer with 2X β-ME and BP Blue 1:1 to (e.g., a sample of proteins solubilized in) SDS-solubilization buffer compatible with the DC Protein Assay. Again, protein samples solubilized in SDS-Solubilization Solution with 1X β-ME and BP Blue suitable for SDS-PAGE will be reconstituted.

SDS-Solubilization Buffer with 1X β-ME and BP Blue (8ml)

Amount	Component	Final Concentration
1.0 ml	0.5 M Tris (pH 6.8)	62.5 mM
1.6 ml	10% (w/v) SDS	2% (w/v) SDS
1.01 g (0.8 ml)	Glycerol	10% (v/v) glycerol
0.4 ml	β-Mercaptoethanol	5% β-ME
0.2 ml	0.025% Bromphenol Blue	0.00125% BP Blue
4.0 ml	Nanopure H_2O	

Proteins may be solubilized directly in SDS-solubilization buffer with 1X β-ME and BP Blue and used for SDS-PAGE. The radioactivity of labeled sample proteins solubilized in this solution may be determined by scintillation counting, but protein quantification of such samples may require a method other than one utilizing the Bio-Rad DC Protein Assay, since SDS-solubilization buffer with 1X β-ME and BP Blue is not compatible with protein quantification procedures which use the Bio-Rad DC-Protein Assay as outlined later.

2D-PAGE Solubilization Solutions (Modified from Damerval et al., 1986)

UST Buffer (5 ml)

Amount	Component	Final Concentration
2.85 g	Urea	9.5 M Urea
1.25 ml	10% (w/v) SDS	2.5% (w/v) SDS
125 mg or 125 μl	Triton X-100	2.5% (w/v or v/v) Triton X-100
1.7 ml	Nanopure H_2O	

UST buffer is a solubilization solution which is compatible with the Bio-RAd DC Protein Assay procedure described later. However, since this buffer also lacks a reducing agent, it is not intended for solubilization of protein samples to be left at ambient temperatures for extended periods after solubilization. Solubilize protein samples in UST buffer prior to protein quantification using the DC Protein Assay. After removing aliquots for protein quantification using the DC Protein Assay, store remaining samples at -70 °C. Add equal volumes of revised ampholyte buffer before freezing, or after thawing, to reconstitute UKS buffer-solubilized protein samples suitable for IEF-PAGE.

Revised Ampholyte Buffer (5 ml)

Amount	Component	Final Concentration
2.85 g	Urea	9.5 M Urea
475 mg or 475 μl	Triton X-100	9.5% (w/v or v/v) Triton X-100
50 mg	K_2CO_3	10 mM K_2CO_3
50 mg	DTT	1% (w/v) DTT
200 μl	Servalyt 3-10 Stock	4% Servalyt 3-10 Stock
2.3 ml	Nanopure H_2O	

After protein quantification, add revised ampholyte buffer 1:1 to (e.g., a sample of proteins solubilized in) UST buffer. Again, protein samples solubilized in UKS buffer and suitable for IEF will be reconstituted.

UKS Buffer (5 ml)

Amount	Comoponent	Final Concentration
2.85 g	Urea	9.5 M Urea
300 mg or 300 μl	Triton X-100	6.0% (w/v or v/v) Triton X-100
25 mg	K_2CO_3	5 mM K_2CO_3
25 mg	DTT	0.5% (w/v) DTT
625 μl	10% (w/v) SDS	1.25% (w/v) SDS
100 μl	Servalyt 3-10 Stock	2% Servalyt 3-10 Stock
2.0 ml	Nanopure H_2O	

Proteins may be solubilized directly in UKS buffer and used for IEF-PAGE. The radioactivity of labeled sample proteins solubilized in UKS buffer may be determined by scintillation counting, but protein quantification may require a method other than one utilizing the Bio-Rad DC Protein Assay, since UKS buffer is not compatible with protein quantification (as outlined later) utilizing the Bio-Rad DC-Protein Assay.

Solubilize proteins of dried pelleted material remaining after vacuum desiccation of acetone-precipitated material from crowns by adding 10 μl of

either SDS-PAGE or 2D-PAGE solubilization buffer per mg dried pelleted material in a 1.5 ml microcentrifuge tube. For example, if UST buffer were to be used as a 2D-PAGE solubilization buffer for 6.0 mg dried pelleted material, add 60 μl UST buffer to the 6.0 mg dried pelleted material. Thoroughly mix UST buffer and dried pelleted material with a pipet tip and solubilize proteins for 1 h at 22 to 26 °C with agitation by hand every 15 min. After 1 h, microfuge at 12,000x *g* for 15 min and transfer supernatants to new 1.5 ml microcentrifuge tubes. Microfuge supernatants at 12,000x *g* for 15 min to further clarify solubilization mixtures. Aliquots of UST buffer-solubilized proteins from samples of several supernatants within treatments may be pooled prior to microfuging samples for clarification.

About 30 μl of UST buffer-solubilized protein sample will likely be recoverable after this final centrifugation for clarification of supernatant from the original 60 μl amount of UST buffer added to solubilize proteins of the 6.0 mg dried pelleted material.

Protein Quantification

Stock Solutions

BSA Standard

Amount	Component	Final Concentration
100 µl	30 mg Bovine Serum Albumin (BSA) per ml Nanopure H_20	3 mg BSA/ml Nanopure H_2O
900 µl	Nanopure H_2O	

Reagent A'(for 10 spectrophotometer tubes)

Amount	Component	Final Concentration
100 µl	Reagent S*	
5 ml	Reagent A*	

* Provided by Bio-Rad with DC Protein Assay Kit

Reagent B (provided by Bio-Rad with DC Protein Assay Kit)

DC Protein Assay Standard Procedure for Four Solubilized Protein Samples

Number fourteen clean spectrophotemeter tubes '1' through '14'. To each of the fourteen clean tubes, add 60 µl Nanopure H_2O. Use tubes '1' through '6' for establishing a standard curve. Add the following solutions to tubes '1' to '6' to bring the total volume of diluted protein samples in each to 100 µl:

Tube #	BSA Standard (µl)	UST Buffer (µl)	Diluted [BSA] (µg/µl)	Corrected A_{750}
1	0	40	0	.000
2	0	40	0	.002
3	20	20	0.6	.162
4	20	20	0.6	.172
5	40	0	1.2	.309
6	40	0	1.2	.303

Add 15 µl volumes of UST buffer-solubilized protein sample and 25 µl volumes of UST buffer to spectrophotometer tubes '7' to '14' as follows:

Tube #	Sample in UST (µl)	UST (µl)	Corrected A_{750}	Original [Protein] (µg/µl)
7Mo	15	25	.143	3.381
8 "	"	"	.148	
9Ma	"	"	.266	6.877
10 "	"	"	.268	
11 To	"	"	.100	2.144
12 "	"	"	.105	
13 Ta	"	"	.162	3.784
14 "	"	"	.157	

A column for the diluted concentration of BSA in the 100 µl volumes of each standard spectrophotometer tube is included in the first table, and of sample protein concentrations (original) in the second table. These second values were calculated from a standard curve generated by data in the first table and typical absorbance at 750 nm readings (to be discussed later) for crown protein samples included in the second table.

To mix solutions to generate color reactions for absorbance at 750 nm readings, add 500 µl Reagent A' to each 100 µl standard solution listed in the above tables. Mix by gently shaking spectrophotometer tubes. At one minute

intervals, add 4 ml Reagent B to each mixture, covering each spectrophotometer tube with parafilm and mixing by inversion. After allowing a blue color to develop for 20 min, measure absorbance at 750 nm. Mixtures of tubes '1' and '2' will remain yellow; simply use mixtures in tubes '1' and '2' to zero the spectrophotometer. According to this 20 min development time, after addition of Reagent B at one minute intervals, zeroing the absorbance at 750 nm with tube '1' should follow on addition of Reagent B to tube '14' by 7 min.

Absorbances at 750 nm of solutions developed using the DC Protein Assay Standard Protocol are advertised as being linearly related to protein concentrations from 0.2 (or 0.3) mg/ml to 1.5 mg/ml (= μg/ μl). Generate a standard curve from the average A_{750} value of tubes '3' and '4' (0.6 μg/ μl = y_1), and of tubes '5' and '6' (1.2 μg/ μl = y_2), as follows:

Average A_{750} at 0.6 μg/ μl = (.162 + .172) ÷ 2 = .167 = x_1

Average A_{750} at 1.2 μg/ μl = (.309 + .303) ÷ 2 = .306 = x_2

Slope (= m) of the linear relationship between average A_{750} values (x_i) and protein concentrations (y_i) is calculated as follows:

$$m = (y_2 - y_1) / (x_2 - x_1) = (1.2 - 0.6) / (.306 - .167)$$

$$= 0.6 / .139 = 4.31655$$

The 'y' intercept (= b) of the linear relationship between average A_{750} values (x_i) and protein concentrations (y_i) is calculated as follows:

$y = mx + b$, or $b = y - m \cdot x$

but $m = 4.31655$ at $x_1 = .167$, $y_1 = 0.6$, so

$$b = 0.6 - 4.31655 \cdot (.167) = - 0.1209$$

similarly, at $x_2 = .306$, $y_2 = 1.2$, and

$$b = 1.2 - 4.31655 \cdot (.306) = - 0.1209$$

Hence, for this group UST buffer-solubilized protein samples assayed according to the same procedure, the concentration of protein diluted (15 μl in 100 μl) ([diluted protein] = y) is related to average A_{750} values (x) by the equation:

$$y = m \cdot x + b = 4.31655 \cdot (x) - 0.1209.$$

To calculate the concentration of protein in the original UST Buffer-solubilized sample, the correction factor of (100/15) is required to compensate for dilution of original protein sample (15 μl in the 100 μl). So, protein concentration in original UST buffer-solubilized Mo sample would be calculated as follows:

Ave. A_{750} value = (A_{750} value of tube '7' + A_{750} value of tube '8') $\div$ 2

= (.143 + .148) $\div$ 2 = .1455

Diluted Mo protein concentration = y = $4.31655 \cdot (x) - 0.1209$

$= 4.31655 \cdot (.1455) - 0.1209$

= .507158 μg/ μl.

Mo protein concentration in the original UST buffer-solubilized sample

= (100 / 15) $\cdot$ .507158 = 3.381 μg/ μl.

The protein concentrations in the Ma, To, and Ta protein samples solubilized in UST buffer may be calculated similarly from the average A_{750} values for these samples.

IEF-PAGE

Gels for IEF-PAGE

4% Polyacrylamide Gel Solution (for Mini-PROTEAN II IEF tubes) (5 ml) (in order of mixing) (other ampholyte combinations may be used)

Amount	Component	Final Concentration
2 ml	Nanopure H_2O	
2.75 g	Urea	9.2 M Urea
200 mg	CHAPS	4% (w/v) CHAPS
40 µl	Servalyt 3-10 Stock	0.8% (v/v) 3-10
80 µl	Servalyt 5-7 Stock	1.6% (v/v) 5-7
80 µl	Bio-lyte 7-9 Stock	1.6% (v/v) 7-9
630 µl	30% Acrylamide	3.78% Acrylamide
11 mg	Bis	0.22% (w/v) Bis

Mix thoroughly, syringe-filter through a 0.2 µm membrane, degas (urea may crystallize in a vacuum), and, to polymerize, add 15 µl 10% (w/v) ammonium persulfate (APS), and 6 µl TEMED. (Because of losses on filtering, to add 9 µl 10 % APS, and 3.6 µl TEMED to a 3 ml aliquot after filtering and degassing may be preferred). After mixing briefly, pour gel solution into a casting tube (a modified 3cc syringe tube) containing 15 to 20 clean mini-PROTEAN II glass tubes for IEF gel polymerization. Tap the casting tube containing the glass tubes and liquid IEF gel solution immediately after pouring the gel solution, in order to release trapped air pockets from the gel mixture before polymerization. Polymerization is generally complete in 20 min if the 10% APS and TEMED were mixed throughout the gel solution uniformly. After polymerization, in order to loosen polyacrylamide tube gels from glass surfaces of surrounding tubes, refrigerate the polymerized gels in glass tubes until urea (?) crystallizes in a thread along the length of the IEF tubes.

Solutions for IEF in Mini-PROTEAN II Units

Catholyte Buffer (500 ml) (for Mini-PROTEAN II IEF unit upper chamber)

2 g NaOH
500 ml Nanopure H_2O

Degas, or use after having refrigerated.

Anolyte Buffer (800 ml) (for Mini-PROTEAN II IEF unit lower chamber)

6 mM Phosphoric Acid (544 μl 85 % Phosphoric Acid)
800 ml Nanopure H_2O

Equilibration Buffer (O'Farrell, 1975)

0.292 M Sucrose
2.3% (w/v) SDS
62.5 mM Tris·HCl (pH 6.8)

Load protein samples (32-μg) in ca. 20-μl UKS buffer through 0.1 M sodium hydroxide catholyte buffer onto IEF tubes immersed in 6 mM phosphoric acid anolyte buffer. Overlay samples with 20-μl UKS-overlay buffer (2:1 / H_2O:UKS buffer / v:v) and isoelectric focus for 10 min at 500 volts, then for 3.5 h at 750 volts and maximum current using a ISCO (Lincoln, NE) Model # 470 power supply. Extrude gels (Porter et al., 1992) onto Parafilm strips, soak gels in equilibration buffer for 45 min, and store IEF gels on Parafilm at -70 °C.

SDS-PAGE

Gels for SDS-PAGE

12% Polyacrylamide Separation Gel (40 ml, for 4 Mini-PROTEAN II gels)

Amount	Component	Final Concentration
13.33 ml	1.5 M Tris (pH 8.8)	0.5 M
600 µl	10% SDS	0.15%
120 mg	Bis	0.3%
15.6 ml	30% Acrylamide	11.7%
400 mg	Sucrose	1.0%
10.0 ml	Nanopure H_2O	

After mixing thoroughly, syringe filter the mixture through a 0.45 µm membrane, de-gas it, and, for polymerization, add 200 µl 10% APS (ammonium persulfate) and 20 µl TEMED to the gel solution.

14% Polyacrylamide Separation Gel (80 ml, 2 gels 16 cm x 20 cm x 1 mm)

Amount	Component	Final Concentration
26.67 ml	1.5 M Tris (pH 8.8)	0.5 M
1200 µl	10% SDS	0.15%
280 mg	Bis	0.35%
36.4 ml	30% Acrylamide	13.65%
800 mg	Sucrose	1.0%
14.65 ml	Nanopure H_2O	

After mixing thoroughly, syringe filter the mixture through a 0.45 µm membrane, de-gas it, and, for polymerization, add 400 µl 10% APS (ammonium persulfate) and 40 µl TEMED to the gel solution. Mix gently for about a min, avoiding the introduction of air into the de-gassed gel solution. Pour or pipet the gel mixture of 14 % polyacrylamide into large-format PROTEAN II xi gel plates to within approximately 1.5 cm of the top edge of the shorter glass plates. Layer isobutanol on the gel surface. Allow 30 min or more for polymerization of acrylamide. Wash isobutanol from the gel surface with Nanopure H_2O and replace with SDS-overlay buffer [which is made by mixing 14% polyacrylamide gel solution (80ml), but substituting 36.7 ml Nanopure H_2O for 280 mg Bis and 36.4 ml 30% Acrylamide]. Allow acrylamide monomer to polymerize overnight.

4% Polyacrylamide Stacking Gel (10 ml)

Amount	Component	Final Concentration
2.5 ml	0.5 M Tris (6.8)	0.125 M
100 µl	10% SDS	0.1%
11 mg	Bis	0.11%
1.3 ml	30% Acrylamide	3.9%
6.1 ml	Nanopure H_2O	

After mixing thoroughly, syringe filter the mixture through a 0.45 µm membrane, de-gas it, and, for polymerization, add 50 µl 10% APS (ammonium persulfate) and 10 µl TEMED to the gel solution. Mix gently for about 15 sec, avoiding the introduction of air into the de-gassed gel solution. Pour or pipet the gel mixture of 4% acrylamide to capacity into large-format PROTEAN II xi casting stands onto polymerized acrylamide separation gels with well combs in place above separation gels (With thorough de-gassing, polymerization is complete within 20 min)

To avoid losing the pH gradient between the separation gel (pH 8.8) and the stacking gel (pH 6.8), load samples solubilized in SDS-solubilization buffer with 1X β-ME and BP Blue immediately after polymerization of the stacking gel. Electrophoresis is at 16 mAmps per gel and maximum voltage with an ISCO Model #470 power supply.

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VITA

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