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EVALUATION OF DROUGHT AND SALINITY TOLERANCE IN TRANSGENIC CREEPING BENTGRASS

By

Susan M. Redwine

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ABSTRACT

EVALUATION OF DROUGHT AND SALINITY TOLERANCE IN TRANSGENIC CREEPING BENTGRASS

By

Susan M. Redwine

Drought and high salinity are abiotic conditions that incur water stress in plants. These conditions are becoming increasingly important in turfgrass management due to more regulated water use. Creeping bentgrass (*Agrostis palustris* L. Huds. cv Penncross) transformed with the mannitol-1-phosphate dehydrogenase (mtlD) gene was evaluated for its ability to withstand drought and salinity conditions imparted by mannitol accumulation catalyzed by MTLD. Plants used in these experiments had been previously transformed using the biolistic method. Polymerized chain reaction (PCR), southern blot, and slot blot analysis confirmed the presence of both bar gene and mtlD DNA insertions in the transformed plants. Additionally, densiometer readings of the slot blot showed that each of the transformed plants contained several copies of the inserted genes. Northern blot analysis of the transformed plants confirmed the presence of the mRNA transcripts for both the bar gene and the mtlD gene. Nine clones were used in these studies; seven with the mtlD gene, Penncross grown from seed, and Penncross which had gone through regeneration through tissue culture. Plants were subjected to drought by withholding irrigation for 12 days, and saline conditions (0, 100, 250, and 500 mM NaCl) for 66 days in the

greenhouse. Measurement of growth included collection of clipping mass, root mass, and mass of the thatch layer. Next, the plants were assessed for accumulation of mannitol, the product of the reaction catalyzed by MTLD using high performance liquid chromatography (HPLC). Finally, the presence of MTLD was evaluated in the transformed plants using western blotting techniques to see if the mRNA message was being translated into protein. Growth measurements indicated no increased stress resistance; no mannitol accumulation was detected by HPLC; MTLD was not detected by western blots. These results show that the transformed clones are not expressing the mtlD gene, which may implicate the occurrence of post transcriptional gene silencing

To my parents

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LIST OF ABBREVIATIONS

ATP – adenosine triphosphate

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

DNA – deoxyribonucleic acid

ET – evapotranspiration

HPLC – high performance liquid chromatography

MTD – mannitol dehydrogenase

MtlD – mannitol-1-phosphate dehydrogenase

Mp – Penncross plants grown from seed

MRNA – messenger ribonucleic acid

NADH – hydrogenated nicotinamide adenine dinucleotide

NADP – nicotinamide adenine dinucleotide phosphate

NADPH – hydrogenated nicotinamide adenine dinucleotide phosphate

NBT – nitro blue tetrazolium

PBST – phosphate buffered saline + 0.02% Tween 20

PTGS –post-transcriptional gene silencing

RNA – ribonucleic acid

Rp – Penncross plants regenerated from callus tissue

SDS-PAGE -- sodium dodecyl sulfate-polyacrilamide gel electrophoresis

TEMED – N, N, N', N' - tetramethylethylenediamine

I. INTRODUCTION

Creeping bentgrass (*Agrostis palustris* L. Huds.) is widely used on golf courses to provide a highly desirable playing surface on putting greens, tees and fairways. This species is adapted to cooler climates and requires high cultural intensity, including extensive irrigation when subjected to drier conditions. Because creeping bentgrass is very desirable for golf courses, yet not well adapted for warmer climates or regions that might use effluent water for irrigation, or be cultivated in saline soils, the species would benefit from varietal improvement in these respects. Genetic manipulation is a viable way to develop creeping bentgrass that is adapted to more diverse habitats.

Genetic engineering is a recent technological advancement in biology that has practical applications for the improvement of cultivated plant species. Novel genes can be isolated and cloned, then inserted into the genome of a completely different plant. Very specific responses can be targeted through such techniques. It is this method that has been used to approach the problem of limited adaptation of creeping bentgrass.

A mechanism that has been identified to incur salinity and drought tolerance in various organisms is osmotic adjustment. Accumulation of solutes in a cell could result in the osmotic requirement that would keep enough water inside the cell to keep it alive, and more hopefully, thriving. The mannitol-1-phosphate dehydrogenase gene cloned from *Escherichia coli* has been demonstrated to ultimately result in osmotic adjustment. This gene was engineered into creeping bentgrass to improve the stress tolerance of the species. The primary purpose of this research was to discover whether or not creeping bentgrass engineered with the mannitol-1-phosphate dehydrogenase gene is more salt and/or drought tolerant than non-transformed plants within the species. Drought and salt stress tolerance of the whole plant was evaluated by measuring growth parameters in greenhouse experiments. Detection of a functional metabolic pathway was performed by looking for the end-product formed by the mtlD gene, mannitol. Mannitol accumulation was assessed by using high performance liquid chromatography (HPLC). Finally, expression of the gene was evaluated by looking for MTLD using immunodetection techniques.

II. REVIEW OF LITERATURE

Importance of water in turf culture

The venerable Dr. James Watson stated, 'Every turfgrass manager in America, in the entire world for that matter, must begin immediately to plan and implement a program of water conservation and wise management of this finite resource.' (Watson, 1994). Water use is a major concern in western states where shortages occur in urban areas, which have led to restricting the use of potable water for irrigating landscaped areas. Both Arizona and California have adopted legislation that require the use of effluent or other secondary, often saline, water for irrigation of turfgrass areas (Marcum, 1999). Carrow (1994) noted that some causes of the need to better utilize water resources comes from increasing competition for scarce water resources, water shortages from periodic drought, increasing costs associated with obtaining water, and turf managers' greater awareness that excessive irrigation is not necessary to maintain a good quality turf and often leads to other management problems. This reflects the growing concern and need to monitor and effectively manage the water used on golf courses.

Not only is water use a concern, but water quality is now an issue for turf managers. With a rising demand for playable turf areas, development is pushing land-use into areas where salinity might have an adverse effect on the physiology of the turf. Increased development into arid and semiarid regions has caused salinity problems in turf management, since in these areas salinity problems already frequently exist in soil and water. In areas that are limited in water resources, fresh water is reserved for human consumption and effluent water is often used for irrigation. Construction of turf areas near salt water and the addition of salt to turf areas as run-off from deicing highways, sidewalks, and airport runways are also factors which cause the increased problems with salinity in turfgrass areas (Harivandi et al., 1992). The severity of the salinity problem in agricultural production is illustrated by the fact that it affects 2 million km^2 of land used for production agriculture and 30-50% of irrigated land (Franco and Melo, 2000).

In light of the apparent need for more efficient water usage, Carrow (1994) recommended several methods that would help optimize water use on golf courses: 1) develop/use grasses with better drought resistance; 2) develop/use grasses with salt resistance; 3) use wastewater; 4) alter management practices; 5) improve irrigation systems; 6) improve irrigation scheduling; 7) use water harvesting; and 8) develop water conservation and drought contingency plans. Research at Michigan State University has been conducted with goals 1) and 2) in mind. Genetic engineering methods were used to alter a specific metabolic pathway in order to make creeping bentgrass (*Agrostis palustris* Huds.) more drought and salt tolerant. With such metabolic engineering, this highly desirable grass species will be more amenable to growth in adverse environmental conditions that result from changed management practices and development onto marginal lands. The viability and potential for these plants to withstand drought and salt stress is discussed.

Importance of water in plants

Water quantity and quality are profoundly important in plant growth and metabolism. This fundamental compound constitutes 80-95% of the mass in growing tissues (Taiz and Zeiger, 1991; Sebanek, 1992). Water is the most abundant and best solvent known and comprises the medium in which most metabolic processes in plants are conducted. Molecules move through and are affected by water, and it is important in biochemical reactions (Hopkins, 1999).

The most limiting resource for agricultural productivity is water; it also limits productivity of natural ecosystems. Alteration of the water status in the plant system can be detrimental for the health of the plant. Drought stress has been documented to decrease leaf area, stimulate leaf abscission; cause root extension into deeper, moist soil; cause stomatal closure; limit photosynthesis; induce osmotic adjustment; inhibit seed set; alter energy dissipation from leaves; increase resistance to liquid-phase water flow; and increase wax deposition on the leaf surface (Taiz and Zeiger, 1991; Passioura, 1994).

Water movement in plants

Water moves in the water-conducting tissue in plants, the xylem, through the process of transpiration. Transpiration keeps water moving continually through the plant and so aids in providing nutrients, in the form of inorganic ions, from the soil. Indeed, close to 99% of water taken in by plants is transpired (Raven et al., 1992). This movement of water is responsible for controlling the degree of saturation of cells with water. Transpiration also provides a mechanism by which plants can maintain a suitable temperature for metabolism.

Due to the high capacity for water to hold heat, its presence and movement ensures that temperature fluctuations in plants occur slowly (Taiz and Zeiger, 1991).

The concept of water potential

Transpiration is caused by a water potential gradient in the soil-plant-air continuum. There are at least three mutually independent components that comprise water potential. Each component represents a quantity dependent on concentration, pressure, and the interaction of water with the surface of a solid phase, i.e., a cell wall or a soil particle. This relationship is expressed in the equation:

$$\Psi_w = \Psi_s + \Psi_p + \Psi_m,$$

where Ψ_s is the osmotic potential, Ψ_p the pressure potential and Ψ_m the matric potential.

The osmotic potential (Ψ_s) describes the pressure associated with the amount of solute dissolved in the cytoplasm. The presence of solutes reduces the water potential of a solution and so the quantity in the equation is usually negative. Pressure potential (Ψ_p) is due to the hydrostatic pressure that builds up on the rigid walls of the cell. This is generally referred to as turgor pressure. Turgor pressure is the internal pressure of the cell pushing outward, and thus usually contributes a positive quantity to the water potential equation. Matric potential (Ψ_m) represents the component of specific free energy of water which is associated with water status on interfacial borders. In the soil-plant continuum, this pressure would be due to the colloidal structures of cytoplasm, micellar structures in cell walls, and in capillary systems in soil. The matric potential generally contributes negative numerical values in the equation describing water potential. However, the interfacial effects are often grouped within the osmotic and pressure potential values. Therefore, this component of the equation can be ignored for discussions of plant water relations (Slavik, 1974; Taiz and Zeiger, 1992; Hopkins 1999).

Water moves from high to low water potential. Pure water has the greatest water potential, which is zero. Within plant cells, osmotic potential causes the overall water potential of the cell to be more negative than neighboring cells, and thus water is drawn into the cell. Additionally, if a cell is flaccid, this means that the pressure potential of the cell is more negative than neighboring cells and also would create an overall influx of water. Movement of water within plant cells continues until the water potential within a given cell equals the water potential outside the cell, that is, equilibrium is achieved.

The effect of drought stress on plant physiology

Drought stress leads to a decrease in leaf area. This is an early biophysical response to drought stress that results from the loss of turgor. The difference between turgor (P) and the yield threshold (Y) multiplied by cell wall extensibility (*m*), affects the growth rate (GR) of cells:

$$GR = m(P - Y)$$

Generally, the difference between cell turgor and the yield threshold is very small, so slight changes in turgor can inhibit the growth rate of cells (Taiz and Zieger, 1992; Passioura, 1994).

Drought stress has also been recognized to stimulate the growth of roots. This mechanism allows the plant to mine water from deeper soils. Assimilates can be distributed more readily to the roots during drought stress due to the decrease in assimilate use for leaf expansion. Breeders have recognized deep root extension as a characteristic of plant species that are able to endure reduced water conditions and have sought it as a trait in drought resistant crops.

Protein stability is compromised under drought stress. In young tissues, protein loss may be irreversible after rapidly imposed drought stress. In mature tissues, the breakdown of proteins has been hypothesized to be a mechanism to provide N to growing tissues because uptake of soil nitrogen may be hindered due to water stress. The N from the proteins would in effect be recycled in the plant once the stress is relieved (Hanson and Hitz, 1982).

Drought stress is known to lead to a decrease in photosynthesis. Researchers have hypothesized that the decrease in photosynthesis is due to increased diffusive resistance in the stomata that occurs with drought stress (Hanson and Hitz, 1982; Sullivan and Eastin, 1974; Tezara et al., 1999). The reduction in photosynthesis would thus be due to changes in carbon dioxide (CO_2) availability. Recently, researchers have demonstrated that the decrease in photosynthesis is due not merely to a reduction in diffusive resistance, but an inhibition of CO_2 assimilation caused by inhibition of the Calvin Cycle. Inhibition of the Calvin Cycle was concluded to result from a decrease in adenosine triphosphate (ATP), which was correlated to a decrease in ATP synthase content

(Tezara et al., 1999). Not only might the decrease in ATP inhibit photosynthesis, but it also could impede protein synthesis.

Drought and turfgrass

Because water use is such an important factor in the maintenance of turf areas, a considerable amount of research has been conducted to examine mechanisms of adaptation to water stress by various turf species (Bonos and Murphy, 1999; Ervin and Koski, 1998; Fry and Butler, 1989; Huang, 1999; Lehman and Engelke, 1993; Lehman et al., 1993; Salaiz et al., 1991; White et al., 1992;). However, these studies tend to focus on drought avoidance resulting from deep, expansive root systems of the turfgrasses studied.

Tall fescue (*Festuca arundinaceae* Schreber) is widely recognized as one of the more drought tolerant turfgrass species. Ervin and Koski (1998) found that when compared to Kentucky bluegrass (*Poa pretensis* L.), tall fescue is better adapted to avoid drought by extending roots deeper into soil, thus enabling it to extract moisture from these deeper regions. White et al. (1992) suggest that tall fescue may be able to osmotically adjust in certain tissues, the youngest tissues having the greatest magnitude of adjustment.

In Kentucky bluegrass, Bonos and Murphy (1999) note that total root mass wasn't a good indication of tolerance or susceptibility to water stress in different cultivars. Rather, the characteristic that was found to be important as an indicator of more tolerant cultivars in the study was the depth of the root activity. The authors note that continued uptake and transpiration of water may not be as beneficial in semiarid regions where total water use would hopefully be reduced.

Huang (1999) found that 'Prairie' buffalograss (*Buchloe dactyloides* (Nutt.) Engelm.), an acknowledged drought tolerant species, had a deeper root system than 'Meyer' zoysiagrass (*Zoysia japonica* Steud.) after subjection to drying of the soil surface. In the buffalograss plots, surface soil water status was increased at night in the soils where only the bottom half of the soils were receiving water, thereby suggesting that there may be an efflux of water from the deep roots. This mechanism would enhance water uptake in the upper roots, thereby allowing the plant to use more of its root resources.

Creeping bentgrass is a cool season species, yet is the preferred grass for use on golf course tees, greens, and fairways, and is increasingly being sought for use in warmer climates. Additionally, on a golf course, this species is typically grown on sandy soils with little water holding capacity and is mown several times a week. These conditions make frequent irrigation of such stands of creeping bentgrass necessary.

Fry and Butler (1989) showed that there were differences in ET between creeping bentgrass and annual bluegrass, and the ET of two different mowing heights of creeping bentgrass was different. Creeping bentgrass mown at 12 mm used more water than creeping bentgrass mown at 6 mm. However, the authors suggest that the difference in ET found between the grasses mown at different heights was not enough to merit a different watering regime.

Salaiz et al. (1991) conducted a study with ten creeping bentgrass cultivars to determine if there were differences between rooting and evapotranspiration (ET) characteristics of these plants. A cultivar that is able to produce deep roots and also maintain a low ET would be well suited as a grass able to withstand reduced water conditions. Among these cultivars, five were shown to produce roots below 600 mm. Pennlinks, a cultivar with deep roots and also the lowest ET promised to have the highest potential for drought avoidance.

Lehman et al. (1993) used thirteen clones of creeping bentgrass to determine whether or not variation existed in leaf water potential existed within the species. The researchers compared leaf water potential measurements to relative water content of the same plants and compared them with soil moisture measurements to determine which measurement would be the best predictor of maintained growth under water stress conditions. They determined that relative water content was the best indicator of drought tolerance in creeping bentgrass. It was also determined that variation existed in the species so that plants could be chosen for their response to water deficits and then be incorporated into breeding programs for improved varieties.

The effect of salinity on plant physiology

An excess of salts in the soil solution can impede plant growth and development. The water potential of the soil solution is decreased in saline conditions, possibly leading to osmotic inhibition of water uptake. Halophytes represent the classification of higher plants that are tolerant to high levels of salt, whereas glycophytes are the classification for plants that are intolerant to high salt levels; the growth of glycophytes is inhibited when there is ion accumulation in the shoot. Many halophytes are able to take in salts, usually in the form of NaCl. Research has suggested that this intake of salt is compartmentalized, perhaps in the vacuole, because enzymes and protein-synthesizing systems are sensitive to salt ions (Hellebust, 1976; Marcum and Murdoch, 1992). This observation implies that there should be an equal amount of osmotic pressure produced in the cytoplasm, which would have to come from solutes that are compatible with the enzymes in the cell.

Many monocots, including grasses, respond to water stress by restricting shoot ion accumulation, which is associated with reduced shoot water content (Marcum and Murdoch, 1992; Marcum, 1999). Another commonly reported effect of salinity stress in glycophytes is growth inhibition associated with tissue dehydration and the resulting loss of turgor and an inadequate osmotic adjustment. Growth limitation at high salinity might stem from an inadequate respiratory system to provide the energy for active transport of ions, or there may be an insufficient number of carriers required for the fast rate of ion uptake for cell elongation under saline conditions (Marcum and Murdoch, 1992).

Protein synthesis can be adversely affected by salt stress. Enzyme systems in plants are sensitive to Na^+ and Cl^- concentrations above 100 to 200 mM (Marcum, 1999). Protease inhibitors have been shown to be synthesized after extreme salt stress (Franco and Melo, 2000). This would assist in preventing the breakdown of proteins, which would contribute to maintaining the osmotic balance, as well as protection of membranes that might be disrupted during dehydration.

Salinity Stress and Agrostis palustris

Several studies have found variable levels of salt tolerance within various populations of creeping bentgrass (Kik, 1989; Ahmad and Wainwright, 1976, 1977; Tiku and Snaydon, 1971; Ashraf et al., 1986; Venables and Wilkins, 1977; Ahmad et al., 1981; Hodson et al., 1981; Wu, 1981). Tiku and Snaydon (1971) found that the salt tolerance of the population sampled was correlated with the salinity in the soil where the plants were found. This suggests that the potential for salt tolerance is a trait within the species, and that selection pressure of the region (such as a salt marsh) will cause a population to become predominantly, if not completely composed of salt tolerant individuals.

Ahmad et al. (1981) studied ecotypes of creeping bentgrass to determine the effect of salinity on the species. Results from these studies indicated that the ecotype from a salt marsh showed greater salt tolerance relative to a coastal spray zone ecotype and an inland ecotype. All ecotypes increased Na^+ and Cl^- content with exposure to increase salinity. However, the salt marsh ecotype didn't accumulate these ions in shoot tips as readily as the other ecotypes, which could contribute to better growth in salt conditions. Accumulation of proline, asparagine, glutamine, serine, and blycine betaine were markedly increased in the salt marsh ecotype. The accumulation of these compatible solutes is suggested to provide salinity tolerance by this ecotype. These studies show that the ability to tolerate salinity is present within the genetic background of the *Agrostis palustris* species.

Osmoregulation for water stress tolerance

Tolerance of water stress usually involves the development of low osmotic potential (Morgan, 1984). Osmoregulation, the accumulation of a solute or solutes in response to water stress, helps to maintain turgor potential, and therefore continued growth (Morgan, 1984; Yancey et al., 1982; Nuccio et al. 1999). Osmoregulation also includes the protection of organic compounds such as proteins and membrane lipids; compounds that are produced or accumulate in the process of osmoregulation are capable of stabilizing these structures during high temperatures, chemical denaturation, and dehydration (Franco and Melo, 2000).

Specific examples of osmoregulation as a defense mechanism have been reported in numerous organisms, including fungi, algal cells, and higher plants (Zimmerman, 1978). However, it should be noted that osmoregulation is a mechanism that is involved in processes other than water stress tolerance. Such mechanisms include ion uptake, long-distance translocation of ions, or the function of specialized organs such as salt glands or stomata. Stomatal action is dependent on osmoregulation and has been studied extensively. These mechanisms have proven to be extremely complicated; guard cells rely on ion concentrations and fluxes, metabolic energy, pH, CO_2, compartmentation of ions and metabolites, hormones, and interactions of these factors (Hellebust, 1976). It is likely that osmoregulation in other plant cells is very complicated as well.

Compatible solutes are compounds that allow the organic molecules in the cell to remain functional through retention of conformation. Among the classes of compatible solutes are sugar alcohols, quaternary ammonia compounds, proline, and tertiary sulfonic compounds (Stoop et al., 1996; Shen et al., 1997). Of these compounds, only glycinebetaine and proline are reported to accumulate in grasses (Marcum, 1999).

Sugar alcohols, or polyols, closely resemble sugars; it has been suggested that they may mimic the structure of water with the hydroxyl groups they contain. These may aid in protection of macromolecules by maintaining an artificial sphere of hydration around the molecules. Sugar alcohols may also be beneficial in plant metabolism by scavenging activated oxygen species and therefore prevent peroxidation of lipids and cell damage (Stoop et al, 1996; Shen et al., 1997).

Mannitol metabolism

Many organisms contain mannitol, including numerous families of higher plants. Stoop et al. (1996) reported mannitol in the following plant families: Apiaceae, Areacaceae, Brassicaceae, Bromeliaceae, Buxaceae, Cactaceae, Combretaceae, Compositae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Gnetaceae, Gramineae, Lilaceae, Oleaceae, Platanaceae, Rosaceae, Tubiaceae, and Scrophulariaceae. Several research groups are currently using celery (*Apium graveolens*) as a model organism to study mannitol metabolism in higher plants.

Reports of mannitol metabolism in celery reflect the usage of mannitol for an increased NADP-NADPH turnover compared to plants that do not produce sugar alcohols. Mannitol may also provide more efficient carbon use resulting from energy production in the initial step of mannitol catabolism. This step generates NADH and therefore a higher ATP yield than the catabolism of an equal amount of sucrose (Stoop et al., 1996). Chemically reduced sugars, that is, sugar alcohols, contain more energy than the corresponding oxidized form of the sugar. Therefore, sugar alcohols are higher energy storage compounds than their corresponding sugars.

The pathway for production of mannitol in higher plants typically follows from mannose-6-phosphate to mannitol-1-phosphate through the action of NADPH mannose-6-phosphate reductase. After mannitol-1-phosphate is synthesized, a phosphatase dephosphorylates the compound to produce the end product, mannitol. The celery model provides evidence of the catabolism of mannitol with an NAD-dependent mannitol dhydrogenase (MTD). This enzyme catalyzes the oxidation of mannitol to mannose (Stoop et al, 1996).

Genetic engineering for crop improvement

In recent years, technological advances in the area of genetics and molecular biology have increased the possibilities for improving plants of economic importance. This technology is a very useful way to enhance traditional breeding efforts. By having the ability to incorporate a single gene of interest into an economically important crop, years of hit-and-miss germplasm searches can be side-stepped, and useful plants developed.

Several methods exist for delivering a specific gene into the genome of a plant. Among the many common methods are transformation mediated by *Agrobacterium tumefaciens*, and particle bombardment. Transformation with *A.*

tumefaciens is the most widely used mechanism for genetic transformation. This naturally occurring bacteria was originally described as a tumor-forming pathogen responsible for crown gall in 1907 by Smith and Townsend (Hooykaas and Schilperoort, 1992). Modern research of the pathogenesis of this bacterial strain showed that the so-called Ti plasmid transferred two segments of DNA into the nuclear genome of plant cells during tumor induction. The genes that are inserted into the plant during tumor induction cause infected cells to produce the auxin indole acetic acid (IAA), and the cytokinin isopentenyl-AMP. These phytohormones are responsible for the proliferation of cells that constitute the crown gall. The other gene inserted by the *Agrobacterium* is one that causes the infected cell to produce opines, the carbon source necessary for utilization by the bacterium (Hooykaas and Schilperoort, 1992).

Since the discovery of the natural genetic manipulation of the host plant by *Agrobacterium*, researchers have extensively developed the technology to use this as a medium for genetic engineering. Many useful traits have been transferred to crop plants using the newer *Agrobacterium* systems: resistance to viruses, herbicide tolerance, altered shelf life of tomato, male sterility, cold tolerance, altered source-sink relationships, altered starch composition, and resistance to pathogenic bacteria (Hooykaas and Schilperoort, 1992).

Agrobacterium-mediated transformation has several advantages over other methods. *Agrobacterium*-transformed plants often contain single-or low-copy number of transgene cassettes, which are reported to be less prone to cosuppression of gene-silencing effects than plants that contain several copies of the transgene cassettes (Chai, 1999).

The 'biolistic' method (biological ballistics) is the process that employs microprojectiles – small particles of gold or tungsten – to deliver substances into cells. The biolistic process was invented in 1984 by Sanford et al. (1988) at Cornell University. The method employs shooting DNA coated particles directly into cells with physical force generated from compressed hydrogen or gun powder. This technology was hoped to be the 'universal' transformation method that could be used regardless of tissue or species, and an ingenious way to circumvent the host range restrictions of *Agrobacterium tumefaciens* (Klein et al., 1987). Transformations were reported for a variety of tissue types, including embryos, hypocotyls, cotyledons, leaf disks, callus and cell suspensions. Also reported were transformations of tobacco, petunia, soybean, pea, *Arabidopsis*, sunflower, onion, corn, rice, and wheat (Sanford, 1988).

Background of mtlD

The mannitol-1-phosphate dehydrogenase enzyme (EC 1.1.1.17) was first reported in *Escherichia coli* by Wolff & Kaplan in 1956 (Chase, 1986). This enzyme has also been found in various bacteria: *Aerobacter aerogenes, Bacillus subtilis, Streptococcus mutans,* and *Aspergillus niger* (Chase, 1986); *Aspergillus parasiticus* (Foreman and Niehaus, 1985); as well as aglae: *Caloglossa leprieurri* (Montagne) J. Agardh (Karsten et al., 1997). In these organisms, mannitol-1-phosphate dehydrogenase is reported to be involved in NADPH regeneration.

Interest in the potential for this gene to be used for drought and salinity stress began with researchers at the University of Arizona. Tarczynski et al. (1992) used a clone of the mtlD gene to genetically transform tobacco (*Nicotiana tabacum* L.). The transformed plants were shown to accumulate mannitol, which was not normally produced in tobacco. Studies comparing the mtlD-transformed plants with non-transformed control plants showed that the transformation event did not effect the physiological characteristics of the plants without exposure to NaCl. After 30 days of exposure to 250 mM NaCl, the mtlD-transformed tobacco increased in height a mean of 80% whereas the non-transformed plants only increase in height a mean of 22%. Other groups have transformed tobacco with the same gene to achieve similar results (Karakas et al., 1997; Liu et al., 1995).

The proposed pathway of the enzyme in the transformed plants begins with cytoplasmic fructose-6-phosphate (which is a substrate of glycolysis) and NADH used as substrates for MTLD (Figure 1). The products of this reaction are mannitol-1-phosphate and NAD^+. A non-specific phosphatase is hypothesized to be naturally occurring in the tobacco, which cleaves the phosphate group from the mannitol-1-phosphate to form mannitol (Tarczynski et al., 1992).

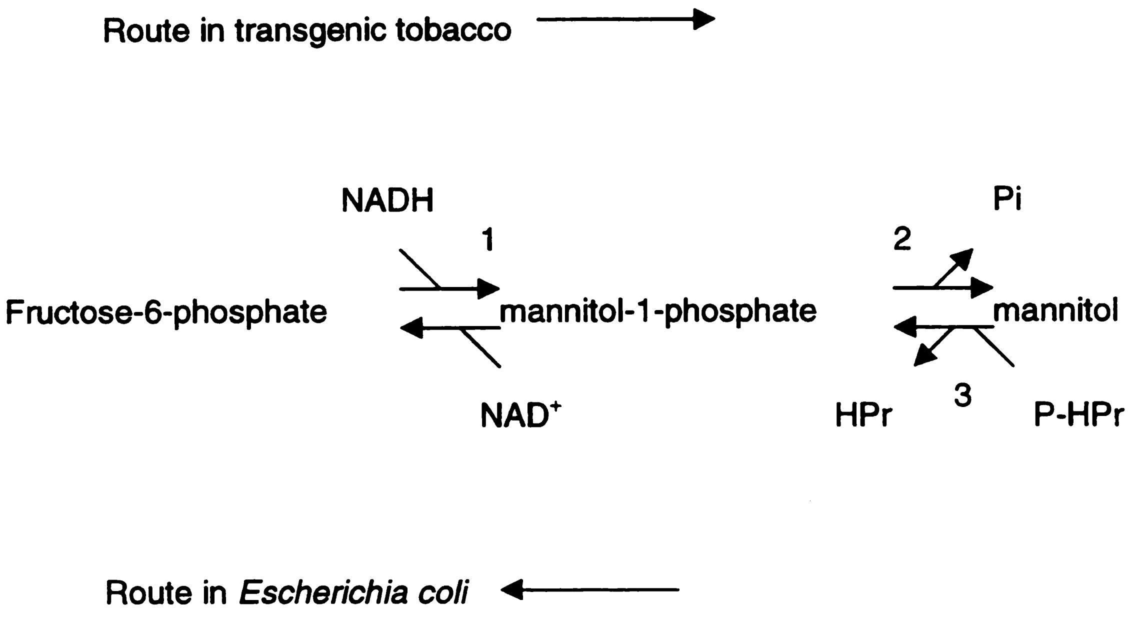

Figure II.1. Mannitol metabolism in *E. coli* and transformed tobacco plants. Arrows: 1, mannitol-1-phosphate dehydrogenase; 2, nonspecific phosphatase(s); 3, mannitol-specific enzyme II. P-HPr, phosphorylated heat-stable protein (after Tarczynksi et al., 1992).

Introduction to the plants used in this study

The Penncross variety of creeping bentgrass was transformed in Sticklen's lab at Michigan State University, using the biolistic method as described by Chai (1999). Friable, yellowish embryogenic calli were selected from existing cultures and placed in a petri dish containing solid callus induction medium. Tungsten particles (Sylvania, Towanda, PA), 0.9-1.2 µm in diameter, were used as carriers for the DNA to be inserted. The particles were sterilely washed and the plasmid DNA was precipitated onto the particles. The DNA-coated microcarriers were ultimately re-suspended in 100 µl of 100% ethanol. Ten µl of this suspension was loaded into a macrocarrier and the macrocarrier was loaded into the Biolistic® PDS-100/He system. For each callus preparation, bombardment was repeated three times within 5 minutes. *In vitro* selection for transformed plants was carried out by *bar* gene resistance to the herbicide bialaphos.

The pJS101 plasmid used for the biolistic transformation of creeping bentgrass was 8.5 kb and contained the mtlD gene for drought and salinity tolerance, and also the bar gene for bialaphos resisitance (Figure 2). This construct was provided by Dr. Ray Wu of Cornell University. The mtlD gene was under the control of the rice actin 5' promoter, while the bar gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

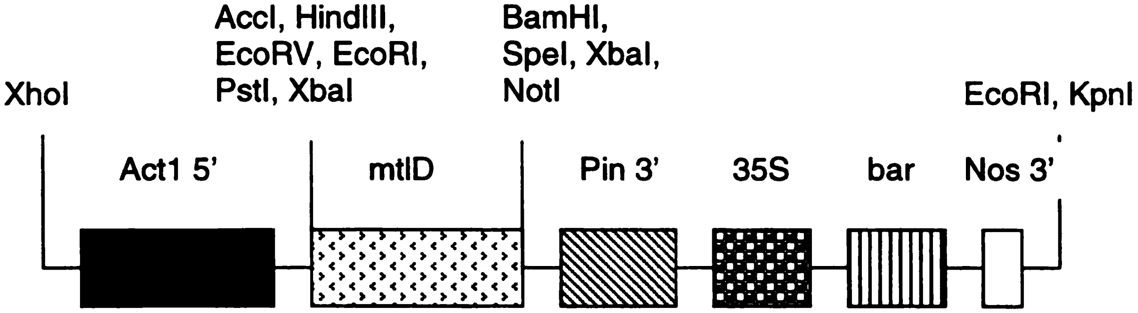

Figure II.2. Plasmid JS101 showing promoters (Act1, 35S); genes of interest (mtlD, bar); termination sequences (Pin 3', Nos 3'); and restriction sites of interest.

Studies on the transformed plants were conducted to detect the presence of the inserted genes (Chai, 1999). PCR, southern blot, and slot blot analysis confirmed the presence of both bar gene and mtlD DNA insertions in the transformed plants. Additionally, densiometer readings of the slot blot showed that each of the transformed plants contained several copies of the inserted genes. Northern blot analysis of the transformed plants confirmed the presence of the mRNA transcripts for both the bar gene and the mtlD gene. No immunodetection of the mannitol-1-phosphate dehydrogenase enzyme was carried out in these studies. Therefore, it was not known if the protein was being translated from the mRNA, and if so, if it is stable enough to accumulate and be physiologically active. Further, assuming the enzyme is physiologically active, thus enabling the accumulation of mannitol in the transgenic plants, whether or not the accumulation of mannitol would provide salinity and drought stress tolerance in the *Agrostis palustris* species is not known.

The transformed plants were reared *in vitro* until they were mature enough to be transferred to soil, and subsequently, greenhouse conditions. The plants used in these studies were vegetative clones of the original transformants, and not the offspring of the transformed plants.

Chapter III

GROWTH RESPONSES OF MtlD-TRANSFORMED CREEPING BENTGRASS UNDER DROUGHT AND SALINITY STRESS CONDITIONS

ABSTRACT

There is growing interest in the development of plants that are tolerant to a number of stresses. We examined whether creeping bentgrass (*Agrostis palustris* L. Huds. cv Penncross) genetically transformed with the manntitol-1-phosphate dehydrogenase (mtlD) gene would be more drought and salt tolerant due to osmotic adjustment from an accumulation of mannitol in plant tissue induced by the transgene. Assuming the mtlD gene was being expressed, MTLD would accumulate and assist the transformed plants in osmotic adjustment, and thus drought and salinity stress tolerance. Greenhouse experiments were conducted to evaluate whole-plant responses to drought and salt stress conditions. A drought experiment was conducted on transformed and non-transformed clones of creeping bentgrass. Water was withheld from the plants for 12 days while leaf extension and gravimetric water measurements were taken. A salinity experiment was conducted by irrigating the clones with 0, 100, 250, and 500 mM NaCl for 66 days. Clippings were taken every two weeks, and dry mass of roots, thatch, and aboveground tissue was measured at the conclusion of the experiment. Each experiment was conducted once. Statistical analysis showed few differences in any of the growth characteristics that were measured; no differences were detected that would provide a trend indicative of

increased tolerance. This is evident as the non-transformed controls generally showed the same trends in growth as the transformants. These results demonstrate the need to approach the analysis of the transformants using more sophisticated methodology, such as assessment of mannitol accumulation using chromatographic techniques, and detection of the MTLD with immunological techniques.

INTRODUCTION

Drought and salinity stress adversely affect growth of plants. Leaf expansion, root expansion, and dry matter accumulation are reduced when plants are exposed to water stress conditions. Reduced turgor pressure of cells resulting from either a lack of water or osmotic stress, leads to an inability to engage in expansive growth. Dry matter accumulation is inhibited under stress conditions because there is an increase in respiration of the stressed plants accompanied by a decrease in photosynthesis.

Reduced leaf expansion has been noted as one of the first effects from water deficit stress, and is the result of a loss of turgor (Taiz and Zeiger, 1992; Passioura, 1994). Cells are capable of expansion because their cell walls become slightly flexible due to cell wall loosening. This expansion takes place due to an increase in turgor pressure inside cells. In the absence of enough water, turgor pressure drops. When turgor decreases, cell expansion ceases when the cell wall loosens, and therefore, when cell walls loosen, plants wilt.

Several researchers have noted a decrease in photosynthesis for plants under drought stress (Hanson and Hitz, 1982; Sullivan and Eastin, 1974; Tezara et al., 1999). A reduction in photosynthesis would ultimately result in a decrease in dry matter accumulation for plants under this type of stress. In the case of turfgrass, this is evidenced in a reduction of clipping mass over time.

Excess salts in the soil or irrigation water can cause plants to undergo ‘physiological drought’. This is because the osmotic potential of the soil solution becomes less than that of the plant, making the plant unable to take in water.

This osmotic stress has also been reported to result in inhibited growth, similar to that of drought stress (Marcum and Murdoch, 1992).

Creeping bentgrass has been the target of drought and salinity tolerance evaluation because it is such a desired species for golf courses and other finely mowed conditions. Research has been conducted to determine the average water use for the species (Fry and Butler, 1989), as well as to identify characteristics of stress tolerant creeping bentgrass so that plants displaying these characteristics can be incorporated into breeding programs. Lehman et al. (1993) showed that there was variance among creeping bentgrass varieties in water potential, but that it could be best estimated by relative water content. Additionally, Ahmad et al. (1981) found salinity tolerance in ecotypes of creeping bentgrass.

In this study, several clones of creeping bentgrass were used to determine if an engineered metabolic pathway would enable the plants to become more drought and salt tolerant. The plants were transformed with the mannitol-1-phosphate dehydrogenase (mtlD) gene that would allow the plants to produce mannitol. An accumulation of this compound would allow the osmotic potential of the plant to adjust, and therefore allow the plants to sustain growth in a stressful environment. In this study plants transformed with the mtlD gene were subjected to drought and salt stress in the greenhouse, and several growth parameters were measured to evaluate whether or not they were more stress resistant compared to non-transformed creeping bentgrass.

MATERIALS AND METHODS

Plant material

Nine lines of creeping bentgrass (*Agrostis palustris* L. Huds. cv. Penncross) were propagated in the Plant Science Greenhouses, Michigan State University, East Lansing, MI in 1998 and 1999. Clones 9604, 9605, and 815-6 contained the mtlD gene; clones 815-7, 9603, 9606, and 711 contained the mtlD gene and a chitinase gene cloned from American elm (*Ulmus americana*). Two of the clones were non-transformed controls: non-transformed creeping bentgrass regenerated from callus under tissue culture conditions (Rp); creeping bentgrass grown from seed (Mp). These plants were established in Conetainers (Stuewe and Sons, Inc. Corvallis, Oregon, USA). Conetainers were 1.67 cm in diameter and 11.67 cm in depth, with holes for water drainage. A mixture of sand-peat (85:15 v/v) conforming to United States Golf Association (USGA, 1993) specifications was used for establishment. Temperatures in the greenhouse ranged from 11°C to 29°C. Light was supplemented by using two high-pressure sodium bulbs suspended above the greenhouse bench. Light intensity was 650 μmol m^{-2} s^{-1} as measured with an integrating quantum sensor (Licor 190-S; Lincoln, NE). Clones were mown regularly at 5 mm above the rim of the Conetainer and topdressed with the same sand-peat used for establishment. Peters (Scotts Co., Allentown, PA) 20-20-20 fertilizer (7.3 g/3.785 l water) was applied once per week. Plants were allowed to grow until a uniform stand of bentgrass filled the Conetainer.

Drought experiment

The experiment was conducted from 11 Sept 1998 to 23 Sept 1998, and was set up in a completely randomized design with four replications per clone. Soil moisture content at field capacity was measured gravimetrically for each clone at the beginning of the experiment. Drought stress was incurred by withholding irrigation. Water was withheld from the plants for 12 days until the plants were desiccated. Water status of the Conetainers was measured gravimetrically. Leaf expansion measurements were taken every other day by randomly sampling five blades of grass per Conetainer. Dry clipping yield was measured twice during the experiment.

Gravimetric water content of the Conetainers was used to calculate the evapotranspiration rate (ET). ET was calculated by first recording the weight of the Conetainer at field capacity, then subsequently subtracting the weight of the Conetainer as it dries down. The difference in the weights is divided by the weight of the Conetainer at field capacity to give ET.

$$ET = (\text{conewet} - \text{conedry})/\text{conewet}$$

Salinity experiment

The experiment was set up in a two-way factorial arrangement of salt concentration and clone type in a completely randomized design with four replications for each treatment. Salinity stress was incurred from 11 Nov, 1998 until 8 Jan, 1999 by daily irrigation to field capacity with solutions of NaCl. Each of the clones received the following levels of treatment with NaCl solution: 0, 100, 250, and 500 mM. Clipping mass was taken over the course of the experiment.

Clippings were collected once every two weeks, placed in an envelope, and then dried at 37C for four days. Dried clipping weights were recorded. Upon termination of the experiment, Conetainers were frozen at -20°C until they could be processed for separation of roots. Once the roots were thawed and separated from the sand-peat medium, each was placed in an envelope and dried, whereupon the mass was measured and recorded. The remaining above ground tissue and thatch tissue was also separated, dried, and the mass was recorded.

Data analysis

Data for the drought experiment were analyzed using the analysis of variance and SAS general linear models procedures (SAS Institute, Inc., Cary, NC). Means were compared by computing Fischer's least significant difference with an alpha level of 0.05.

Data for the salinity experiment were analyzed using analysis of variance and SAS general linear models procedures. Least squares means with an alpha level of 0.05 were computed for significant effects. The physiological parameters that were measured for each clone were only compared to different salt levels of the same clone. This was done because each clone was morphologically different, and thus had different growth characteristics, even under optimal environmental conditions. Neither the drought nor the salinity experiment were repeated.

RESULTS

Drought experiment

Clipping data were not very useful in determining tolerance to reduced water conditions. This is because the dry-down period of the experiment was only 12 days, which was only long enough to collect two sets of clippings; not enough to identify a trend among the clones.

Leaf length data were the most useful tool in evaluating the performance of the clones for the drought experiment. Significance levels were calculated for these data (Tables III.1-6). The differences between clones varied considerably, not allowing a clear trend to become evident. As such, these data were compiled in a chart showing leaf lengths as a percentage of the non-stressed control for each clone (Figure III.1). This indicates that all clones decreased in leaf length over the course of the experiment relative to the well-watered plants. None of the clones seemed to perform better than any of the others. Also, the non-transformed control plants showed the same downward trend in leaf length, indicating that they tolerated the stress comparably to the mtlD-transformed clones. Calculations of ET showed that each of the plants was transpiring a similar amount of water throughout the study, showing that this mechanism was not altered in any of the clones, and therefore would not be the cause of altered response to water stress (Figure III.2).

Salinity experiment

Statistical analysis of the clipping mass data showed few significant differences. At ten days after treatment with the salt irrigation, nine out of the ten clones showed no differences when the average clipping masses of the clones were compared for the different salt treatments (Table III.7). Clone 9606 showed a decrease in clipping mass that was statistically lower for each of the treatments with NaCl. However, this was not an observed trend for the two subsequent samplings. Twenty-four days after treatment, there were no significant differences for any of the clones when the average clipping mass was compared (Table III.8). Thirty-eight days after treatment, there was one significant difference found in the clipping mass of the clones (Table III.9). Of all the comparisons, only clone 9604 showed a significant decrease in clipping mass for the 250 mM NaCl treatment. However, clipping mass for the same clone at 500 mM was not different from the 0 mM and 100 mM clipping masses. Therefore, it cannot be concluded that the increased salt concentration in the irrigation was responsible for the difference in clipping mass. The clipping masses for the clones 52 days after treatment reflect the degree of severity that the 500 mM NaCl concentration had on the growth of the plant (Table III.10). None of the clones produced any growth that could be quantified through clipping. This is not surprising because this salt concentration is approximately that of sea water, which is extremely harsh for a glycophyte. The clones that were subjected to the 0 mM NaCl treatment had the highest average clipping weight for this sampling date. The 100 mM treatment was not different from the 0 mM treatment for each of the clones. Also, the clipping mass for clones 815-6 and 815-7 were not different from the higher concentration salt treatments. The 250 mM treatment was not different from the 500 mM treatment for all of the clones. This shows that there is a general decrease in the growth of all of the clones as the concentration of NaCl in the irrigation water is increased past 250 mM. The final sampling date for the clipping mass data (Table III.11) indicates decreasing growth of the plants subjected to the 250 mM NaCl treatment. However, only one of these clipping masses, that of clone 815-7, was significantly different than the clipping mass of the controls. From these data, it is difficult to see any clear trend that would support the hypothesis that the mtlD-transformed clones maintain growth under salt stress.

After 66 days of irrigation with the varying levels of NaCl, the whole plant was sampled for root, thatch, and remaining aboveground dry mass. The only significant difference in aboveground dry tissue mass was found for clone 711 (Table III.12). This clone had significantly less aboveground tissue upon termination of the experiment for the 0 and 100 mM NaCl treatments than the 250 and 500 mM NaCl treatments.

The average thatch tissue after 66 days of irrigation with NaCl only shows one difference among any of the clones (Table III.13). Clone 9606 had a higher amount of thatch in the 100 mM treatment than for any of the other three treatments. This is probably due to one or two of the replications in this treatment having unusually excessive amounts of thatch, or a unique morphology of this particular clone.

The average root masses for the clones after 66 days of subjugation to NaCl treatments showed an interesting trend for some of the transformed clones (Table III.14). In all cases, the 100 mM treatments were among those with the highest rootmass. For non-transformed Penncross, Penncross grown from tissue culture, clone 815-6, and clone 815-7, there were no differences among any of the rootmasses. For clones 9604 and 9603, there was significantly less rootmass for the 250 and 500 mM treatments, which reflects a decrease in growth resulting from a stressful environment. Clones 711, 9605, and 9606 had significantly lower rootmass for the 0, 250, and 500 mM treatments compared to the 100 mM NaCl treatment.

DISCUSSION

The original work with plants transformed with the mtlD gene by researchers at the University of Arizona did not examine whether or not the transformants were more drought tolerant than non-transformed plants (Tarczynski et al., 1992; Tarczynski et al., 1993; Shen et al., 1997). Tolerance of plants to reduced water conditions is not simple to quantify due to the highly integrated function of water in plants. Researchers have noted that a decrease in leaf expansion is one of the earliest plant responses to water deficit stress (Taiz and Zeiger, 1992; Passioura, 1994). This is because water is needed to create adequate turgor pressure to push out cell walls once they loosen in order to achieve cell elongation. Measuring leaf length from a certain reference (such as the rim of the Conetainer) seemed to be a reasonable approach to the problem of quantifying a drought response.

By subjecting the plants to quick, severe drought stress, it was hoped that the transformants' altered metabolism would allow the mechanism of osmoregulation to help the plants better tolerate the reduced water conditions. Unfortunately, there was no clear trend observable in the leaf length data that was collected. This lack of trend among the clones may have been due to the severity of the drought stress that the plants were subjected to. Had the experiment been repeated, the approach would likely have been to measure the soil moisture gravimetrically and maintain a stressfully low level of water in each of the Conetainers. Under sustained stress conditions, the plants would have been allowed to grow at a reduced rate for a longer period of time, and any trend that existed in the growth patterns of the clones may have been detected in the increased amount of data. As it is, each sampling date represents a different level of stress; on each date, there is less water than the previous date. These plants used up the available water resources very quickly. Better results may have been obtained by measurement and observation of the growth of clones that were provided with only a meager supply of water. However, the drought experiment was not repeated in favor of testing the clones' tolerance to salinity.

Tarczynski et al. (1993) used mtlD-transformed tobacco exposed to 250 mM NaCl for 30 days to demonstrate salinity tolerance imparted by the altered metabolism. These plants were grown hydroponically for 6 weeks before NaCl was added. After the NaCl was added, the plants were assessed at 14 and 30 days for height and fresh weight. The growth of all of the plants was impaired, however, the transformed plants maintained significantly greater growth than the control plants as exemplified by % change in height and % change in fresh weight.

In the salinity study with mtlD-transformed creeping bentgrass, each NaCl level of each clone was compared to every other NaCl level for that clone. In essence, this is comparing the growth for an individual clone, and not a cumulative salt effect, as was compared by Tarczynski et al (1993). However, in any of the growth characteristics of creeping bentgrass clones that were measured, no differences were detected that would provide a trend indicative of increased tolerance. This is evident as the non-transformed controls generally showed the same trends in growth as the transformants.

The greenhouse experiments were carried out first because the mtlD-transformed clones were hypothesized to generate mannitol-1-phosphate dehydrogenase, which in turn catalyzes the reaction for the formation of mannitol. If this is the case, then the apparent lack of increased stress tolerance calls the nature of the mechanism of osmotic adjustment into question. Osmotic adjustment as a phenomenon has been well documented and extensively discussed (Franco and Melo, 2000; Hanson and Hitz, 1982; Hellebust, 1976; Morgan, 1984; Nuccio et al., 1999; Zimmerman, 1978). However, the role of osmolytes as an *adaptive* mechanism for stress tolerance has been questioned, that is, the instances of osmotic adjustment that have been reported may be the side effect of the stressed metabolism of the plant (Hanson and Hitz, 1982).

If it is assumed that osmotic adjustment is an adaptive mechanism that enables plants to withstand greater salt and/or drought stress, then we are caused to wonder why mannitol accumulation does not impart increased salt or drought tolerance to these transformed creeping bentgrass. Ahmad et al. (1981) studied ecotypes of creeping bentgrass and found that some of the plants, those found in a salt marsh, were more tolerant to salt conditions that some of the other ecotypes. This research also showed that several types of compatible solutes accumulated in the tolerant ecotype. These findings suggest that salt tolerance is present in the genetic background of the species. As such, it might be that there is a threshold tolerance level that has already been achieved in the species. Water is so fundamental to plant well-being that some limits for tolerance exist, even with adaptive mechanisms in place.

With this in mind, the data from the salinity experiment suggest that salinity tolerance is already manifest in the plants used for this study, the cultivar Penncross. The dry tissue mass of the leaf tissue, which was collected over the course of the experiment showed no differences among the clones until 52 days after treatment. This suggests that these creeping bentgrass plants are salinity tolerant, as opposed to the tobacco plants, which showed decreased growth as soon as 14 days after treatment (Tarczynski et al.,1993).

Despite evidence for salinity tolerance in all of the plants grown in the study, visual observation showed that plants exposed to higher salt levels were undergoing stress. By the end of the experiment, plants that were subjected to 250 mM NaCl showed leaf firing compared to lower salt levels, and all plants exposed to 500 mM NaCl were completely brown.

Table III.1. Average leaf length of clones 2 days after cessation of irrigation.

Clone	**Irrigation regime**	
	Well watered	None
	Average length (cm)	
Penncross	$0.383^{CD\dagger}$	0.463^{ABC}
TC Penncross	0.363^{d}	0.485^{ab}
815-6	0.433^{abcd}	0.463^{abc}
815-7	0.400^{bcd}	0.468^{abc}
711	0.405^{bcd}	0.450^{abcd}
9605	0.513^{a}	0.433^{abcd}
9606	0.513^{a}	0.443^{abcd}
9604	0.423^{abcd}	0.465^{abc}
9603	0.380^{cd}	0.475^{abc}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.2. Average leaf length of clones 4 days after cessation of irrigation.

Clone	**Irrigation regime**	
	Well watered	None
	Average length (cm)	
Penncross	0.748^{bcde}	0.715^{bcdef}
TC Penncross	0.663^{defg}	0.820^{abc}
815-6	0.633^{defg}	0.603^{efg}
815-7	0.553^{g}	0.708^{cdef}
711	0.578^{fg}	0.723^{bcdef}
9605	0.768^{bcd}	0.730^{bcde}
9606	0.935^{a}	0.858^{ab}
9604	0.670^{defg}	0.648^{defg}
9603	0.633^{defg}	0.623^{defg}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.3. Average leaf length of clones 6 days after cessation of irrigation.

Clone	Irrigation regime	
	Well watered	None
	Average length (cm)	
Penncross	1.028^{abc}	0.658^{g}
TC Penncross	0.918^{bcd}	0.968^{bcd}
815-6	0.683^{fg}	0.685^{fg}
815-7	0.710^{efg}	0.890^{bcde}
711	0.688^{fg}	0.873^{bcdef}
9605	0.948^{bcd}	0.953^{bcd}
9606	1.175^{a}	1.053^{ab}
9604	0.973^{bcd}	1.005^{abc}
9603	0.838^{cdefg}	0.785^{defg}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.4. Average leaf length of clones 8 days after cessation of irrigation.

Clone	Irrigation regime	
	Well watered	None
	Average length (cm)	
Penncross	0.465^{a}	0.225^{de}
TC Penncross	0.418^{abc}	0.193^{e}
815-6	0.465^{a}	0.403^{abc}
815-7	0433^{ab}	0.350^{abcd}
711	0.299^{bcde}	0.335^{abcde}
9605	0.480^{a}	0.358^{abcd}
9606	0.463^{a}	0.182^{e}
9604	0.417^{abc}	0.293^{bcde}
9603	0.458^{a}	0.265^{cde}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.5. Average leaf length of clones 10 days after cessation of irrigation.

	Irrigation regime	
Clone	Well watered	None
	Average length (cm)	
Penncross	0.678^{a}	0.300^{bcd}
TC Penncross	0.655^{a}	0.118^{d}
815-6	0.683^{a}	0.318^{bc}
815-7	0.630^{a}	0.333^{b}
711	0.575^{a}	0.188^{bcd}
9605	0.653^{a}	0.253^{bcd}
9606	0.665^{a}	0.135^{cd}
9604	0.575^{a}	0.195^{bcd}
9603	0.685^{a}	0.155^{bcd}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.6. Average leaf length of clones 12 days after cessation of irrigation.

	Irrigation regime	
Clone	Well watered	None
	Average length (cm)	
Penncross	0.768^{ab}	0.293^{c}
TC Penncross	0.863^{a}	0.050^{d}
815-6	0.793^{ab}	0.193^{cd}
815-7	0.815^{ab}	0.210^{cd}
711	0.643^{b}	0.115^{cd}
9605	0.830^{ab}	0.160^{cd}
9606	0.875^{a}	0.080^{d}
9604	0.745^{ab}	0.150^{cd}
9603	0.710^{ab}	0.098^{cd}

[†] Means followed by the same letter are non-significant at $\alpha = 0.05$.

Table III.7. Effect of NaCl concentration on dry clipping mass of creeping bentgrass clones 10 days after treatment.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	Average mass (g)			
Penncross	0.018^{a†}	0.016^{a}	0.019^{a}	0.019^{a}
TC Penncross	0.017^{a}	0.018^{a}	0.011^{a}	0.012^{a}
815-6	0.022^{a}	0.025^{a}	0.019^{a}	0.018^{a}
815-7	0.017^{a}	0.023^{a}	0.020^{a}	0.021^{a}
711	0.018^{a}	0.011^{a}	0.015^{a}	0.020^{a}
9605	0.017^{a}	0.013^{a}	0.013^{a}	0.011^{a}
9606	0.096^{a}	0.016^{b}	0.015^{b}	0.022^{b}
9604	0.024^{a}	0.014^{a}	0.015^{a}	0.013^{a}
9603	0.033^{a}	0.017^{a}	0.021^{a}	0.018^{a}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.8. Effect of NaCl concentration on dry clipping mass of creeping bentgrass clones 24 days after treatment.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	Average mass (g)			
Penncross	0.056^{a†}	0.036^{a}	0.034^{a}	0.039^{a}
TC Penncross	0.069^{a}	0.049^{a}	0.035^{a}	0.036^{a}
815-6	0.037^{a}	0.036^{a}	0.022^{a}	0.024^{a}
815-7	0.030^{a}	0.031^{a}	0.017^{a}	0.021^{a}
711	0.061^{a}	0.042^{a}	0.034^{a}	0.039^{a}
9605	0.065^{a}	0.055^{a}	0.049^{a}	0.059^{a}
9606	0.059^{a}	0.047^{a}	0.042^{a}	0.045^{a}
9604	0.070^{a}	0.057^{a}	0.045^{a}	0.042^{a}
9603	0.077^{a}	0.054^{a}	0.044^{a}	0.060^{a}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.9. Effect of NaCl concentration on dry clipping mass of creeping bentgrass clones 38 days after treatment.

Clone	Concentration NaCl			
	0 mM	100 mM	250 mM	500 mM
	Average mass (g)			
Penncross	0.067^{a}[†]	0.058^{a}	0.052^{a}	0.041^{a}
TC Penncross	0.083^{a}	0.080^{a}	0.060^{a}	0.050^{a}
815-6	0.048^{a}	0.050^{a}	0.042^{a}	0.031^{a}
815-7	0.040^{a}	0.048^{a}	0.032^{a}	0.027^{a}
711	0.083^{a}	0.079^{a}	0.067^{a}	0.054^{a}
9605	0.082^{a}	0.102^{a}	0.079^{a}	0.072^{a}
9606	0.076^{a}	0.074^{a}	0.068^{a}	0.055^{a}
9604	0.092^{a}	0.095^{a}	0.023^{b}	0.062^{a}
9603	0.091^{a}	0.095^{a}	0.077^{a}	0.066^{a}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.10. Effect of NaCl concentration on dry clipping mass of creeping bentgrass clones 52 days after treatment.

Clone	Concentration NaCl			
	0 mM	100 mM	250 mM	500 mM
	Average mass (g)			
Penncross	0.057^{a}[†]	0.047^{a}	0.031^{ab}	0.000^{b}
TC Penncross	0.066^{a}	0.056^{a}	0.032^{ab}	0.000^{b}
815-6	0.046^{a}	0.033^{ab}	0.010^{ab}	0.000^{b}
815-7	0.041^{a}	0.031^{ab}	0.009^{ab}	0.000^{b}
711	0.050^{a}	0.043^{a}	0.020^{ab}	0.000^{b}
9605	0.053^{a}	0.056^{a}	0.037^{ab}	0.000^{b}
9606	0.053^{a}	0.051^{a}	0.025^{ab}	0.000^{b}
9604	0.056^{a}	0.055^{a}	0.030^{ab}	0.000^{b}
9603	0.063^{a}	0.060^{a}	0.027^{ab}	0.000^{b}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.11. Effect of NaCl concentration on dry clipping mass of creeping bentgrass clones 66 days after treatment.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	Average mass (g)			
Penncross	0.039^{a}[†]	0.025^{ab}	0.011^{ab}	0.000^{b}
TC Penncross	0.036^{a}	0.027^{a}	0.012^{a}	0.000^{a}
815-6	0.027^{a}	0.010^{a}	0.002^{a}	0.000^{a}
815-7	0.020^{ab}	0.041^{a}	0.002^{b}	0.000^{b}
711	0.029^{a}	0.019^{a}	0.005^{a}	0.000^{a}
9605	0.030^{a}	0.022^{a}	0.012^{a}	0.000^{a}
9606	0.041^{a}	0.027^{ab}	0.009^{ab}	0.000^{b}
9604	0.032^{a}	0.020^{a}	0.009^{a}	0.000^{a}
9603	0.040^{a}	0.027^{ab}	0.012^{ab}	0.000^{b}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.12. Average Above Ground Tissue Mass of Clones After Subjugation to Salt Stress.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	(g)			
Penncross	0.371^{a}[†]	0.539^{a}	0.456^{a}	0.382^{a}
TC Penncross	0.432^{a}	0.468^{a}	0.407^{a}	0.413^{a}
815-6	0.614^{a}	0.746^{a}	0.616^{a}	0.537^{a}
815-7	0.708^{a}	0.598^{a}	0.789^{a}	0.580^{a}
711	0.281^{b}	0.273^{b}	0.790^{a}	0.856^{a}
9605	0.337^{a}	0.399^{a}	0.556^{a}	0.642^{a}
9606	0.325^{a}	0.398^{a}	0.483^{a}	0.541^{a}
9604	0.410^{a}	0.397^{a}	0.695^{a}	0.410^{a}
9603	0.401^{a}	0.308^{a}	0.545^{a}	0.449^{a}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.13. Average Thatch Tissue Mass of Clones After Subjugation to Salt Stress.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	(g)			
Penncross	0.733^{a}[†]	0.577^{a}	0.463^{a}	0.560^{a}
TC Penncross	0.745^{a}	0.677^{a}	0.645^{a}	0.538^{a}
815-6	0.205^{a}	0.191^{a}	0.129^{a}	0.156^{a}
815-7	0.208^{a}	0.225^{a}	0.113^{a}	0.129^{a}
711	0.399^{a}	0.307^{a}	0.448^{a}	0.276^{a}
9605	0.753^{a}	0.609^{a}	0.376^{a}	0.408^{a}
9606	0.828^{b}	1.468^{a}	0.800^{b}	0.799^{b}
9604	0.490^{a}	0.336^{a}	0.439^{a}	0.252^{a}
9603	0.412^{a}	0.352^{a}	0.232^{a}	0.326^{a}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

Table III.14. Average Root Mass of Clones After Subjugation to Salt Stress.

	Concentration NaCl			
Clone	0 mM	100 mM	250 mM	500 mM
	(g)			
Penncross	0.281^{a}[†]	0.313^{a}	0.261^{a}	0.270^{a}
TC Penncross	0.275^{a}	0.241^{a}	0.282^{a}	0.228^{a}
815-6	0.074^{a}	0.072^{a}	0.066^{a}	0.054^{a}
815-7	0.069^{a}	0.126^{a}	0.060^{a}	0.058^{a}
711	0.224^{b}	0.329^{a}	0.199^{b}	0.186^{b}
9605	0.315^{b}	0.410^{a}	0.276^{b}	0.238^{b}
9606	0.180^{b}	0.377^{a}	0.189^{b}	0.174^{b}
9604	0.270^{b}	0.358^{a}	0.220^{bc}	0.176^{c}
9603	0.388^{b}	0.677^{a}	0.289^{c}	0.232^{c}

[†] Means followed by the same letter in each row are non-significant at $\alpha = 0.05$.

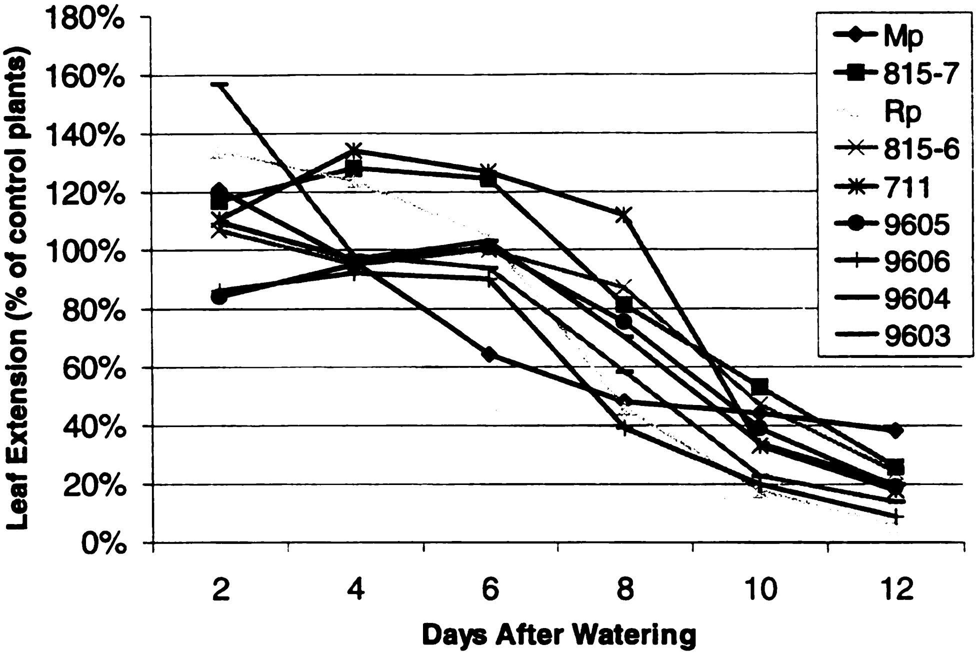

Figure III.1. Average leaf length measurements for drought stressed clones reported as a percentage of the average leaf length of the corresponding well watered clone.

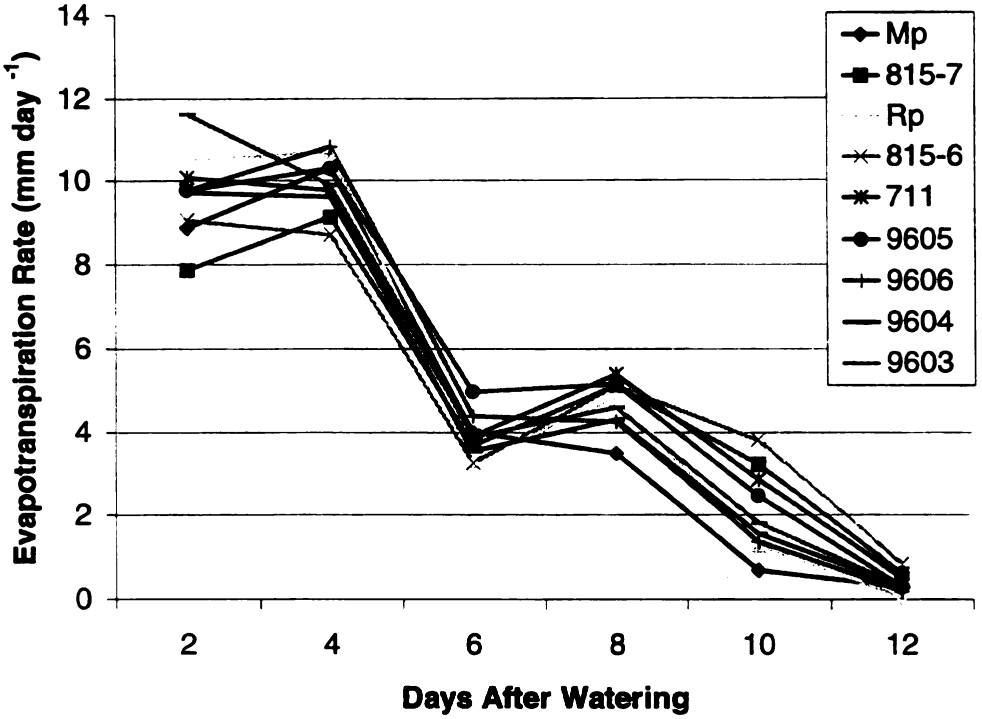

Figure III.2. Evapotranspiration calculations for each of the clones in the drought experiment.

Chapter IV

MANNITOL ACCUMULATION IN MANNITOL-1-PHOSPHATE DEHYDROGENASE-TRANSFORMED CREEPING BENTGRASS

ABSTRACT

Expansion of human development is causing an increase in the use of land and water that has salinity problems, or where water may be very limiting. We examined whether creeping bentgrass (*Agrostis palustris* L. Huds. var. Penncross) genetically transformed with the mtlD gene would be more drought and salt tolerant due to osmotic adjustment from an accumulation of mannitol in plant tissue induced by the transgene. Mannitol accumulation was measured to assess the functionality of the inserted gene. Tissue of transformed bentgrass and non-transformed control plants was subjected to salt stress, sampled, then soluble sugars were extracted using 80% ethanol. Celery (*Apium graveolens*), a plant known to contain mannitol was extracted and used as a positive control. Extracts were run through normal phase high performance liquid chromatography (HPLC) and sugars were detected using a refractometer. Elution time of mannitol was 11 minutes. Mannitol was present in the celery extract, but no mannitol was detected in the bentgrass. This would indicate that either the protein is not being properly expressed or that the metabolic pathway for the formation of mannitol is incomplete.

INTRODUCTION

Central to the theory of molecular biology is the concept that deoxyribonucleic acid (DNA), the genetic message of all organisms, is the template for synthesis of ribonucleic acid (RNA). The arrangement of bases in the messenger RNA (mRNA) molecule then determines the sequence of amino acids as it is translated, resulting in protein synthesis. Proteins are vital to organisms because they catalyze reactions that would not normally be able to take place in the ambient thermal environment of an organism.

Tarczynski et al. (1992) used the mannitol-1-phosphate dehydrogenase (mtlD) gene that was isolated from *Escherichia coli* in genetic transformation experiments of tobacco. The mtlD gene encodes the mannitol 1-phosphate dehydrogenase enzyme (E.C.1.1.1.17). These researchers found that the insertion of the mtlD gene altered the metabolic pathway of the transformants, which enabled the plants to form mannitol. Fructose 6-phoshate and NADH are substrates that the enzyme uses to form mannitol 1-phosphate and NAD^+. A nonspecific phosphatase is hypothesized to cleave the phosphate group from the mannitol 1-phosphate, therefore resulting in the formation of mannitol.

Creeping bentgrass (*Agrostis palustris* L. Huds., cv Penncross) was transformed using particle bombardment. Embryogenic calli were bombarded with plasmid coated tungsten particles using the Biolistic PDS-100/He system (Bio-Rad Laboratories, Inc.) with the mtlD gene (Chai, 1999). Based on the tobacco studies, it was hypothesized that the transformed creeping bentgrass plants would be able to synthesize mannitol. Non-transformed creeping bentgrass is not known to produce mannitol. Our study was designed to determine the presence of mannitol in the normal and transformed plants. Presence of mannitol would indicate the presence of the novel enzyme in the transformed creeping bentgrass. We used high performance liquid chromatography (HPLC) to determine whether mannitol was present in the transformed plants, which would indicate that the inserted mtlD gene was causing the formation of the compound in this species.

MATERIALS AND METHODS

Plant material

Nine transformed clones and two non-transformed clones were propagated in the greenhouse in Conetainers using a sand-peat (85:15 v/v) medium. Clones 815-7, 9601, 9603, 9606, and 711 were clones of *A. palustris* (Huds.) cv. 'Penncross' that had been transformed with the bar gene for glufosinate resistance, a chitinase gene, and the mtlD gene. Clones 9604, 9605, 9104, and 815-6 were transformed with the bar gene and the mtlD gene. A Penncross control clone that had been subjected to the same tissue culture techniques as the transformants, as well as Penncross grown from seed were also included in the study. Average temperatures in the greenhouse ranged from 24°C to 13°C. Celery (*Apium graveolens* L.), a plant known to contain detectable amounts of mannitol, was used in the study as a positive control. O Boy! brand celery (Van Solkema Produce, Byron Center, MI) was purchased at a local grocery store.

Treatments and sampling

Two separate HPLC analyses were performed each for healthy creeping bentgrass clones, and clones under salinity stress. Salt stress was induced on the plants with 250 mM sodium chloride (14.61 g of NaCl/1 liter of double deionized water). Conetainers were irrigated with this solution daily to field capacity for 4 d. After the 4 d irrigation with salt solution, the plants were allowed to recover for 3 d by irrigation with fresh water prior to harvesting. Each clone was sampled by removing the entire plant from the Conetainer. The roots were washed clean of soil particles, and a 1-2 g sample was collected. Celery was cut then sampled and weighed to 1.99 g.

Extraction

For each clone and the celery sample, fresh tissue (leaf, and root material for the grass, and stem tissue for the celery), was put into a 16 x 125 mm test tube with 3.5 ml 80% ethanol. The test tubes were covered with foil and placed in a 70°C water bath. After 30 min, the ethanol was removed and collected into a separate test tube. Another 3.5 ml of 80% ethanol was put into the test tube, and the heating process was repeated. The ethanol was again collected after 30 min, and added to ethanol removed from the first heating. The ethanol heating/extraction procedure was repeated a third time and collected, resulting in 10.5 ml of ethanol for each tissue sample. One additional control extraction was prepared by adding 100 µg of mannitol to the grass sample as it was being heated in methanol. This would ensure that mannitol does not degrade during the extraction.

The test tubes were placed in a vacuum rotary evaporator to evaporate the liquid, leaving extracted compounds collected in residue on the bottom of each of the test tubes. These were stored at 4°C until processing for HPLC analysis.

HPLC analysis

To prepare the samples for HPLC analysis, 1 ml of millipore water was added to each test tube to redissolve the samples. Each solution was drawn up into a sterile syringe and passed through a sterilizing filter unit (Millex-GS).

Purified samples were placed into HPLC injection vials and set in the autosampler. Injections of 200 µl were sent through the HPLC column. The two analyses in which the samples were from healthy plants were run on a Shodex SugarSC 1101 column heated to 86°C with a flow rate of 0.6 ml/min and a run time of 24 min. The two analyses of the stressed plants were run in an Alltech 700CH Carbohydrate Column heated to 86°C with and a flow rate of 0.6 ml/min. The run time for this column was 15 min. Both systems used a refractive index detector to identify different types of sugar molecules as they elute at different times. Resulting chromatograms of each sample were used to examine the presence of mannitol. Chromatograms from the stressed plants using the Alltech column are used in the Results section to represent the findings of the HPLC studies.

RESULTS

The celery extract showed the elution of several sugars (Fig. IV.1). Mannitol was found to elute from the column at 11 minutes. Other sugars common to plants were also detected to elute from the column. Sucrose was retained until 6.5 minutes, glucose 8.1 minutes, and fructose 9.6 minutes. A 200 µM mannitol solution eluted from the column at 11 minutes (Fig. IV.2). Creeping bentgrass that was not transformed with the mtlD gene showed a peak on the chromatogram indicating the presence of sucrose (Fig. IV.3). MtlD-transformed clone 9604 showed a similar chromatogram to the non-transformed control (Fig. IV.4). The peaks indicate that there are detectable amounts of sugars, however there is no peak indicating the presence of mannitol. Clone 9604 was chosen to represent the findings of the HPLC studies. For each clone, stressed or non-stressed, there were no detectable amounts of mannitol shown in any of the resulting chromatograms.

DISCUSSION

The hypothesis that mannitol would be produced in the transformed clones of creeping bentgrass was not supported by studies to detect mannitol accumulation. HPLC methodology was not able to identify the novel compound. The reasons for this could be due to one of several possibilities resulting from the incorporation of the transgene into the genome of creeping bentgrass. The Act1 promoter controls the mtlD gene (Fig. II.2). This promoter should express the gene constitutively. Therefore, mannitol should be produced in transformed creeping bentgrass in a non-stress environment. However, if the promoter requires stress-induction, exposure to 250 mM NaCl should shock the plants and induce them to produce mannitol. In both of these cases, no mannitol was detected in any of the plants.

A possible explanation for lack of mannitol accumulation could be that other key steps are missing from the pathway leading up to mannitol production. Mannitol-1-phosphate dehydrogenase leads to the formation of mannitol-1-phosphate (Fig. II.1). To complete the formation of mannitol, a phosphatase enzyme is needed to remove the phosphate from the molecule. In the tobacco studies, it was assumed that a phosphatase that could serve this purpose was already present in the species (Tarczynski et al., 1992). It is possible that a phosphatase that would fill this function is lacking from creeping bentgrass.

Lack of mannitol accumulation could stem from degradation of the compound due to mannitol-degrading enzymes present in the cytoplasm of certain species. Such enzymes have been found in the cytoplasm of celery

(Stoop et al., 1996). If similar enzymes exist in creeping bentgrass, any production of mannitol by the transgene could readily be catabolized.

Another possible explanation is that the gene is not being expressed to the point of translation, meaning that MTLD is not being synthesized in the transformed plants. This could be due to methylation of the DNA or post-transcriptional gene regulation. Also, if the protein is being produced, it may not be synthesized correctly. An enzyme that is not composed of the proper amino acid sequence could change conformation and therefore not function. The topic of genetic regulation will be addressed in Chapter V.

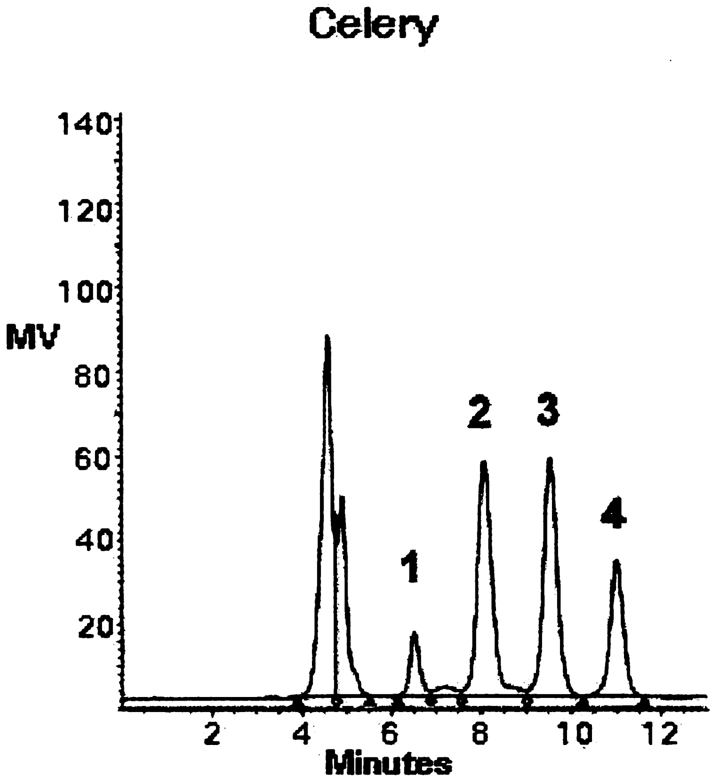

Figure IV.1. Chromatogram of celery extract. Peaks 1, 2, 3, and 4 indicate the elution of sucrose, glucose, fructose, and mannitol, repsectively.

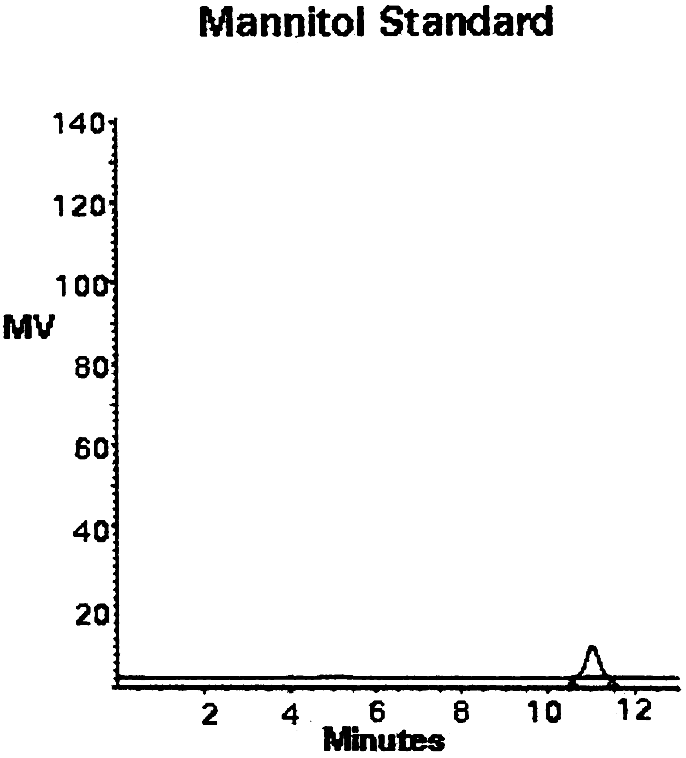

Figure IV.2. Chromatogram of 200 µM mannitol.

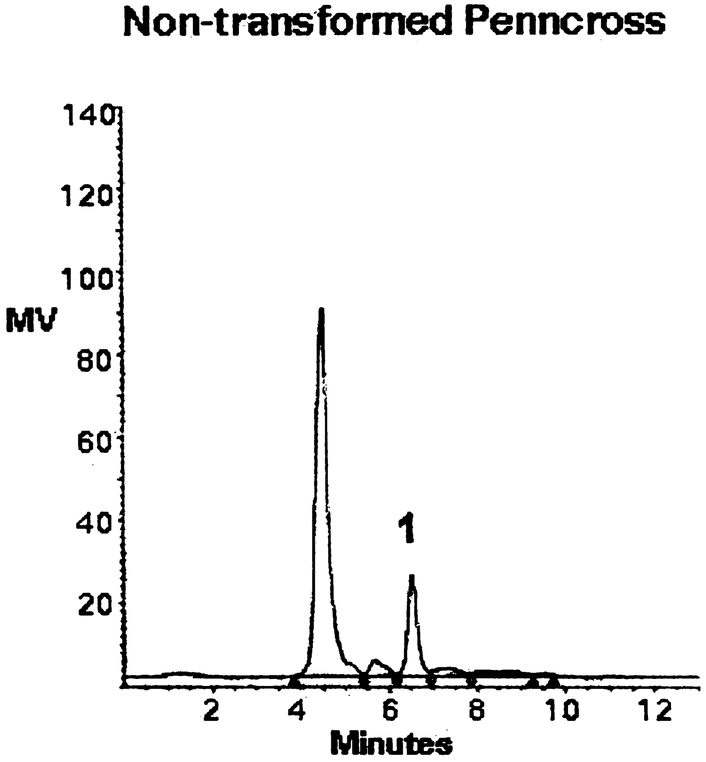

Figure IV.3. Chromatogram of Penncross creeping bentgrass regenerated from tissue culture, grown under non-stress conditions. Peak 1 indicates presence of sucrose.

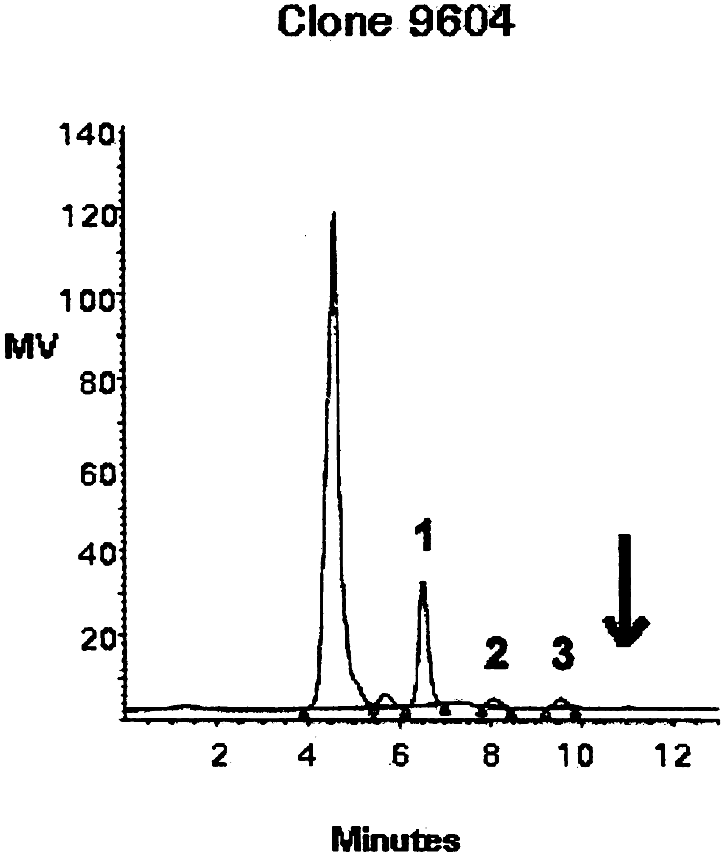

Figure IV.4. MtlD-transformed Penncross creeping bentgrass, clone 9604. Peaks 1, 2, and 3 show elution of sucrose, glucose, and fructose, respectively. The arrow indicates where mannitol would be detected.

Chapter V

IMMUNOLOGICAL DETECTION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE IN TRANSFORMED CREEPING BENTGRASS

Abstract

Previous studies with mtlD-transformed creeping bentgrass indicated no increase in drought and salinity stress tolerance, and no accumulation of mannitol, the product of the reaction catalyzed by MTLD (chapters III and IV). This study was designed to examine whether or not MTLD is present in the transformed plants. An antibody for MTLD was produced by identifying a suitable epitope from the amino acid sequence of the protein and creating a synthetic peptide to serve as the antigen for injection into rabbits. Testing the blood serum from the rabbits drawn on several different days, an antibody was identified that could possibly be used in the detection of MTLD. Proteins were extracted from tissue of the transformed creeping bentgrass, as well as *E. coli*, which was used as a positive control. The proteins were then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were immobilized on a nitrocellulose membrane, incubated with the antibody, and visualized with alkaline phosphatase. The band appearing on the membrane indicated that the antibody detected MTLD in *E. coli*, but not in any of the creeping bentgrass plants.

INTRODUCTION

Among the many techniques of molecular biology that are now at our disposal is the detection of a specific protein using antibodies. Antibodies against specific proteins can be generated by harnessing the immune response of certain animals. This technique is based on the principle that a foreign antigen injected into an animal will be recognized by the immune system, which in turn will react by producing antibodies against the various epitopes found within this antigen. Such antibodies are known as polyclonal, referring to the different possible antibodies found within the blood of the animal, each a result of the recognition by the immune system of a single epitope within the antigen.

In order to visualize the protein that is being detected by the antibodies, total proteins from a tissue need to be separated and immobilized on a support. Separation of total proteins is done through sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE), and then immobilized by transferring them onto a nitrocellulose membrane. Incubation of the primary antibody with the membrane allows the antibody to bind to the protein of interest. Through an additional series of incubations, the specific antibody can be visualized, and therefore the presence of the protein of interest can be assessed.

MATERIALS AND METHODS

Antibody production

There was no mannitol-1-phosphate dehydrogenase protein in our possession or obtainable from a commercial source for the production of the antibody. Therefore, we chose to synthesize a peptide based on the sequence of the mannitol-1-phosphate dehydrogenase protein in order to inject into rabbits. For this, we searched for the mannitol-1-phosphate dehydrogenase protein in the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database as accessed through SeqWeb Sequence Analysis (http://www.bch.msu.edu:8080/gcg-bin/seqweb.cgi). Using the amino acid sequence as found through this database, we were able to predict which sections of the polypeptide sequence would best serve as our region to use for the antigens. Two peptides were chosen to synthesize in order to achieve a greater likelihood that a suitable antibody would be produced. The regions of the amino acid sequence used for creation of the synthetic peptide were those that were calculated to be most hydrophilic, and thus more likely to be on the outside of the conformation of the protein. This estimation was determined using PepPlot from the SeqWeb database, and is based on the Kyte and Doolittle hydropathy measure (J. Mol. Biol. 157; 105-132 (1982)). The amino acid sequence for MTLD is given:

Sequence of mannitol-1-phosphate dehydrogenase (E.C.1.1.1.17):

mkalhfgagn igrgfigkll adagiqltfa dvnqvvldal narhsyqvhv vgeteqvdtv

sgvnavssig ddvvdliaqv dlvttrvgpv vleriapaia kgqvkrkeqg nesplniiac

enmvrgttql kghvmnalpe dakawveehv gfvdsavdri vppsasatnd plevtvetfs
ewivdktqfk galpnipgme ltdnlmafve rklftlntgh aitaylgkla ghqtirdail dekiravvkg
ameesgavli krygfdadkh aayiqkilgr fenpylkddv ervgrqplrk lsagdrlikp
llgtleyglp hknliegiaa amhfrseddp qaqelaalia dkgpqaalaq isgldansev
vseavtayka mq

The sequences of the antigen regions are given:

Peptide sequence #1 (DW-1):

akgqvkrkeqgnespl

Peptide sequence #2 (DW-2):

rseddpqaqe

Dr. Joseph Leykam at the Macromolecular Structure, Sequencing and Synthesis Facility located in the Department of Biochemistry at Michigan State University, synthesized the peptides. The peptides were then confirmed through mass spectrometry. The synthetic peptides were conjugated to keyhole limpet hemocyanin.

Once the synthetic peptides were synthesized, they were given to Mr. Don Herrington at University Laboratory Animal Resources, Michigan State University, for injection into rabbits (New Zealand white females). The antigens were dissolved in 31 mM sodium phosphate buffer (0.46 m NaCl and 41 mM sucrose, pH 7.4). The antigen concentration was 0.1 mg/ml. Ten µl of this antigen solution containing 1 µg protein was mixed with 0.5 ml water and 0.5 ml TiterMax adjuvant (CytRx Corp., Norcross, GA). The emulsion was formed by passing the mixture through a double-sided needle between two 3cc syringes until the viscosity made the passage of the mixture through the syringe difficult. The emulsion was then left in one of the 3cc syringes, and a 22-guage needle was attached. The rabbits (rabbits no. 142 and 143) were injected subdermally on the back in ten sites, 0.1 cc of the antigen/adjuvant emulsion per site. The first injection was performed on 6/2/99. Booster injections of the antigens into the rabbits were performed on 7/9/99 and 8/12/99, as described above.

Prior to the injections, blood was drawn from the rabbits to provide pre-immune serum to use as a negative control when various antiserums were tested. Bleedings of the rabbits were taken approximately every two weeks, 20 ml per rabbit. The blood was centrifuged at full speed for two min in a tabletop clinical centrifuge. The blood cells were precipitated to the bottom of the test tube, and the serum was removed with a 5 ml pipette. The serum was collected in a new test tube and stored at -20°C. Bleedings were taken on 7/9/99, 7/16/99, 7/30/99, 8/12/99, 8/26/99, 9/13/99, and 9/24/99.

The sera were then tested for antibody activity by reacting the antisera with the antigen, and performing the subsequent visualization procedures. Nitrocellulose membrane was cut into 8 cm x 1 cm strips and sectioned off into 1 cm segments by marking with a pencil. Each strip was numbered for reference. Each antigen was diluted to the following concentrations: 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg. Two µl of each dilution was spotted onto every membrane in decreasing concentration into each of the 1 cm sections. The membranes were then blocked by incubating them in 2% nonfat dry milk (2 g dry milk in 100 ml PBST). The strips were allowed to incubate with agitation for 1.5

h. Each antiserum was thawed and diluted to 1/1000, 1/10,000, and 1/100,000. Each dilution was allowed to incubate with one strip for 1h at 37°C. After incubation with the antisera, the membranes were washed three times in PBST for 15 minutes per washing. The membranes were then incubated with the secondary antibody (antirabbit IgG alkaline phosphatase conjugate) for one hour with agitation at 37°C. The membranes were then washed three times, as above with PBST for 15 minutes per washing. Membranes were then soaked in the detection solution: 50 µl NBT and 25 µl BCIP in 7.5 mls alkaline phosphatase buffer. Once spots were visible, the reaction was stopped by removing the strips from the detection solution.

Western blotting

SDS-PAGE gels with a 10% acrylamide concentration, which is best for separating proteins between 16-68 kD, were prepared for protein separation. The polyacrylamide solution was prepared in a 100 ml beaker over ice (7.9 ml water; 30% acrylamide mix; 1.5 M Tris pH 8.8; 110% SDS; 10% ammonium persulfate; 0.008 ml TEMED) and poured into the gel mold. The solution was allowed to polymerize at room temperature for 30 min. A stacking gel of 5% acrylamide was prepared in a 100 ml beaker over ice (4.1 ml water; 30% acrylamide mix; 1.0 M Tris pH 6.8; 10% SDS; 10% ammonium persulfate; 0.006 ml TEMED). This was poured over the polymerized gel to about 1/10th of the final length of the first gel. A plastic comb was placed in the stacking gel to mold wells for loading the protein buffers. The gel was allowed to polymerize at room temperature for 30 min. The prepared gel was placed in a vertical electrophoresis box, and surrounded in a bath of running buffer (25 mM Tris; 250 mM glycine; 0.1% SDS).

Protein was extracted from the creeping bentgrass tissue (approximately 0.2 g) by grinding it to a fine powder under liquid N, then adding 100 µl protein extraction buffer (50 mM Tris, pH 7.5; 0.25 M sucrose; 0.2% EDTA, 0.016 µM PMSF, 1 mM DTT). Protein samples were subjected to centrifugation for 5 min at top speed on clinical tabletop centrifuge. The supernatant was pipetted off and collected in a new microfuge tube. Five µl loading buffer (for 5x: 250 mM Tris, pH 6.8; 500 mM dithiothreitol; 10% SDS; 0.5% bromophenol blue; 50% glycerol) was added to 20 µl protein extract. The protein extracts/loading buffer solutions were denatured by incubating them for 10 min in a boiling water bath. The microfuge tubes were centrifuged for 30 seconds, and then the entire 25 µl of the protein extracts/loading buffer solutions were load onto the gel.

The electrophoresis unit was run at 100 V for approximately 3.5 hours, or until the dye had run to ¾ of the way down the gel; then the gel was removed from the electrophoresis unit. The gel was placed in contact with a 6.5 cm x 8.5 cm nitrocellulose membrane, cut to the dimensions of the gel and pre-soaked in transfer buffer (39 mM glycine; 48 mM Tris; 0.037% SDS; 20% methanol). The gel and membrane were placed between two pieces of Whatman filter paper, pre-soaked in transfer buffer. The filter paper-membrane-gel sandwich was set in the Trans-blot Semi-dry blotting system (Bio-Rad Laboratories, Hercules, CA). The sandwich was set up so that the membrane was closer to the anode end of the transfer unit. The transfer was run at 15 V for 15 min.

After the membrane was removed from the transfer unit, the mannitol-1-phosphate dehydrogenase enzyme could be visualized on the membrane. The membrane was blocked in 5% nonfat dry milk (5 g dry milk in 100 ml PBST) overnight at room temperature. The blocking solution was discarded, and was replaced with a solution of PBS containing the antibody (10 ml PBS; 10 µl serum from rabbit # 142, bled on 9-13-99). Incubation of the primary antibody was carried out for one hour, after which time, the membrane was washed three times in PBST for 15 min each. The membrane was then incubated in solution with the secondary antibody (10 ml PBS; 1 µl antirabbit IgG alkaline phosphatase conjugate) for one hour. The membrane was then washed three times in PBST for 15 min each. Finally, the membrane was incubated in alkaline phosphatase buffer (100 mM NaCl; 5 mM $MgCl_2$; 100 mM Tris, pH 9.5; 66 µl NBT; 33 µl BCIP). Once bands were visible, the reaction was stopped by discarding the buffer and soaking the membrane in ddH_2O.

RESULTS

Antibody production

Two separate efforts were made to identify a rabbit blood serum that contained a suitable antibody for detection of MTLD. The sera were tested by subjecting them to visualization against the antigens on 3 Aug 1999 and 27 Sep 1999. From the second testing, a bleeding that provided good detection of several dilutions of the antigen was identified. The 13 Sep 1999 bleeding gave visualization of the DW-2 antigen (Fig. V.1). Spots with the following concentrations of antigen were visible from the 1:1,000 dilution of the antiserum: 100, 10, and 1 ng. The 100 ng spot was very dark, indicating strong affinity for the antibody to the antigen. The 10 ng spot was dark, but noticeably lighter than the spot for the 100 ng detection, while the spot for the 1 ng detection was faint. Spots with the following concentrations of antigen were visible from the 1:10,000 dilution of the antiserum: 100, 10, and 1 ng. Color intensities for the spots were similar to those for the 1:1,000 dilution. The 1:100,000 dilution of the antiserum resulted in detection of a spot for 100 ng of antigen. However, this spot was not very intense.

Detection of the several different amounts of the antigen with the varying concentrations of the antibody indicate that this bleeding would be a good candidate for detecting MTLD. It should be noted, however, that good affinity for the antigen does not guarantee good affinity to the protein. The antigen that was used to raise this antibody was a peptide that was synthesized based on predicted behaviors of regions of the protein based on its amino acid sequence.

Western blot analysis failed to detect MTLD in any of the creeping bentgrass plants (Fig. V.2). No band was detected in the lane for non-transformed Penncross (lane 2). Additionally, there was a band detected in the lane with *E. coli*, which produces the enzyme naturally (lane 3). This band indicates that the antibody was able to detect the naturally occurring enzyme, in it's native conformation. However, there were no bands detected in any of the lanes containing the mtlD-transformed creeping bentgrass. This indicates that MTLD is not present in any of the transformed clones.

DISCUSSION

Numerous questions arise due to the fact that MTLD was not detected in any of the transformed clones. Chai (1999) showed evidence for the presence of both the DNA and mRNA for the inserted mtlD gene. In an ideal situation, the mRNA would simply be translated to a functional protein that would catalyze the desired reaction. However, there could be several possible reasons why this situation is less than ideal.

The transformed clones that were used in this study were the actual plants that were regenerated from tissue culture after biolistic bombardment. The transformants were not raised through several generations to develop a line of plants in which the expression of the transgene was stabile. Generating progeny and testing them for presence of the transgene by using the selectable marker or performing PCR is a common practice in genetic engineering. It is a mechanism by which researchers can make sure that the plants they are working with are expressing the gene of interest. It would have been useful in these studies to have carried out some breeding to insure that the plants were known to contain a stable transgene of a certain copy number. By doing this, more control of the genetic material in the plants would have been exercised, and confirmed our knowledge of the background of the plants.

Another possible mechanism for the absence of mannitol-1-phosphate dehydrogenase in the transgenic plants is post-transcriptional gene silencing (PTGS). This is a hypothesized phenomenon that describes a lack of gene expression even though nuclear transcription of the gene is occurring

(Baulcombe, 1996; Vaucheret et al., 1998; Wassenegger and Pelissier, 1998). In this case, steady state mRNA might still be present in reduced amounts, and therefore detectable. PTGS is not well understood, but several models exist that describe how such a mechanism may work. Baulcombe (1996) suggested that regulation at this level is most probably carried out by production of antisense RNA of the RNA targets by the plant, and would be specific to the target RNA. Antisense RNA could be transcribed directly from the gene, it could be produced as a response to over expression of a transgene, or as a response to aberrant sense RNA. Aberrant RNA could be formed from irregular termination of transcription, irregular processing, or mRNA degradation. Depending on where the irregularity lies in aberrant RNA, it is possible that the RNA would be detectable. This could possibly explain why the previous research showed a mRNA signal, whereas this research shows lack of translation into MTLD.

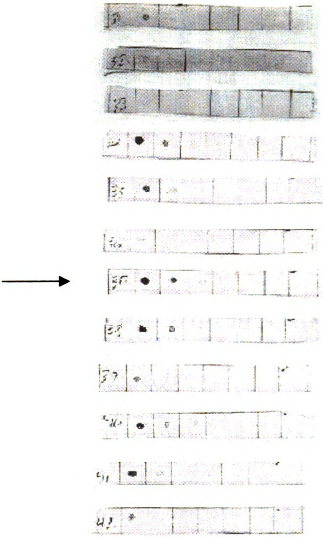

Figure V.1. Antibody testing from 27 Sept, 1999. Membrane no. 37 (arrow) indicated good detection from the 13 Sept, 1999 bleeding at a 1:1000 dilution of antiserum. This serum was used to provide the polyclonal antibody for the western blot analysis.

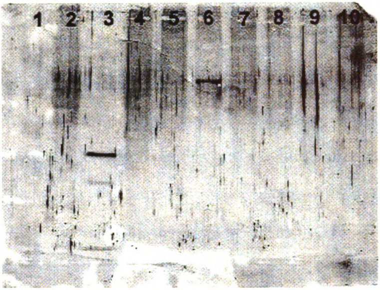

Figure V.2. Western blot analysis for MTLD. Lane 1: molecular weight marker. Lane 2: non-transformed plant. Lane 3: *E. coli* preparation. Lanes 4-10: MtlD-transformed clones 815-7, 9604, 9603, 711, 601, 9605, and 815-6, consecutively.

SUMMARY

SUMMARY

In the drought experiment we saw that there was no clear trend that any of the clones seemed to be able to withstand water deficit longer than any of the other clones. The salinity experiment showed that the plants used in the study seemed to have a degree of salt tolerance already in the species. However, the results from the study also provided no evidence that any of the clones could better tolerate exposure to differing concentrations of NaCl than any other clones. Analysis of the clones with HPLC showed that the transformed clones did not produce detectable amounts of mannitol. Celery controls and mannitol standards that were also run through the HPLC system showed clear peaks in the chromatograms when mannitol eluted from the column. Immunoblot detection techniques showed that there was not any mannitol-1-phosphate dehydrogenase in the transformed clones.

Each experiment, taken on its own, could be criticized for having various inadequacies that would offer ambiguous results. However, the results of each of the experiments contradicted the hypothesis that the mtlD-transformed clones have an altered metabolic pathway that allows them to be more drought and salt tolerant than non-transformed creeping bentgrass. Taken as a whole, each experiment reinforces the other. The conclusion that should be drawn from the sum of this research is that clones 9604, 9605, 815-6, 815-7, 9603, 9606, and 711 did not produce mannitol-1-phosphate dehydrogenase, and therefore did not produce mannitol. Additionally, these plants were no more stress tolerant than non-transformed plants.

Even though the results seem to be quite clear, they are somewhat surprising given that Chai (1999) reported evidence of transformation through Southern and Northern blot analysis. These experiments show that the genetic code is present in the transformed plants, and that the code is being transcribed into the message necessary for the production of MTLD. Evidently, there is a problem with translation of the mRNA into mannitol-1-phosphate dehydrogenase. The idea that there is some form of gene silencing would be a very plausible explanation. However, the means to characterize the mechanism of gene silencing is a long way off, and constitutes a complex genetic problem.

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