PHYSIOLOGICAL AND GENETIC CONTROL OF WATER STRESS TOLERANCE IN ZOYSIAGRASS

A Dissertation

by

DANIEL WADE DEWEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Molecular and Environmental Plant Sciences

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ABSTRACT

Physiological and Genetic Control of Water Stress

Tolerance in Zoysiagrass. (December 2005)

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Significant cultivar difference in many water stress responses of zoysiagrass (*Zoysia japonica* (Steud.) and *Zoysia matrella* (L.) Merr.) are shown in this study. Of the four cultivars, Palisades was the most water stress tolerant, had the most negative turgor loss point, and leaf rolled after loss of full turgor pressure. On the other end of the spectrum, Diamond was the least water stress tolerant, had the lowest full turgor pressure, the least negative turgor loss point, and leaf rolled at full turgor. Differences between Diamond, Cavalier, Palisades, and DALZ 8504 in leaf rolling, loss of full turgor, water release curve parameters, root characteristics and gene expression make zoysiagrass a prime candidate for further investigation into the mechanisms of water stress avoidance/tolerance. Enhanced antioxidant activity and stomatal control, along with root characteristics, most likely explain the cultivar difference in water stress tolerance of zoysiagrass. Palisades and DALZ 8504 maintained full turgor for significantly longer than Diamond and Cavalier, which may be associated with root characteristics and/or enhanced stomatal control as only those two cultivars showed enhanced expression of a stomatal control gene (phospholipase D). The apparent response (most apparent in turgid weight/dry weight ratios (TWDW)) of well watered plants to water stressed neighbor

plants will likely be the most novel finding of this study. Well watered zoysiagrass and Kentucky bluegrass responded to water stressed neighbors by reducing TWDW. Significant increases in gene expression of a systemin degrading enzyme and of an integral membrane protein (signal receptor) were also observed in well watered plants. Results from this study indicate that this phenomenon is occurring and expose a dearth in scientific understanding that must be filled. Improving water stress tolerance through breeding for parameters like those discussed in this paper (delayed leaf rolling or loss of full turgor, enhanced stomatal control, enhanced antioxidant activity, deep rooting characteristics, etc.) may very likely produce turfgrasses that can survive and maintain desired aesthetic qualities using significantly less water.

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INTRODUCTION

This study examines the physiological responses and gene expression patterns of two water stress tolerant and two water stress susceptible cultivars of zoysiagrass (*Zoysia japonica* (Steud.) and *Zoysia matrella* (L.) Merr.) under water stressed and non-stressed conditions. The goal of this work is to lay a foundation for future research in water stress tolerance of zoysiagrass with the ultimate goal being to improve water stress tolerance of zoysiagrass and other turfgrasses through molecular approaches.

Water Conservation

Quality water is a valuable and limited natural resource as it is necessary for maintenance and growth of any population. The population of the United States has increased steadily since its foundation. The United States 2000 Census reported that the largest population increase in United States history occurred in the 1990's (32.7 million) (Hobbs and Stoops, 2002). The census also reported that the U.S. population has grown increasingly metropolitan with each decade (80% in 2000), and that "suburbs, rather than central cities, accounted for most of the metropolitan growth" (Hobbs and Stoops, 2002). Suburban growth in metropolitan areas may indicate that people are buying homes close to work, but still want the privacy and recreation afforded by a home surrounded by turfgrass, shrubs and trees. Areas that provide for outdoor recreational activities such as soccer, baseball, football, golf and picnicking will accompany suburban growth because

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This dissertation follows the style and format of Crop Science.

people want green space in their neighborhoods (Beard and Green, 1994). As the United States population increases and suburban areas replace farmland, landscape water conservation will become increasingly important. This is especially true in the western United States, much of which is semi-arid or arid, where from 1940 through 2000 the population grew at least twice as fast as any other region (Midwest, Northeast, and South) in the United States (Hobbs and Stoops, 2002).

The amount of quality water is relatively constant and population growth on a fixed volume of water is dependent on an array of indoor and outdoor conservation measures. The United States uses 340 billion gallons per day of fresh water with 80 percent of the water consumed in the United States being used for agricultural purposes (Schaible, 2004; United States Geological Survey, 1995a). Twenty-six billion gallons of water per day (8% of total use) is used for domestic purposes (most residential irrigation would fall under this category) (United States Geological Survey, 1995a). Outdoor water use constitutes 30 to 50 percent of total domestic water use in the southwestern United States (Gerston et al., 2002; Center for Urban Water Conservation, 2003; Gelt et al., 2003), resulting in an estimated 2 to 3.5 billion gallons per day being used for domestic outdoor water use in the southwestern United States (United States Geological Survey, 1995b).

Outdoor water conservation can be achieved through many methods including education, water-wise landscaping, water pricing, and proper irrigation management. Research focused on understanding plant-water relations and water stress tolerance is another avenue of water conservation that will assist end users in conserving water, through development of landscape plants with enhanced water stress tolerance and/or

reduced water requirements. Water conservation measures must be taken to reduce the amount of water being used for landscape irrigation because landscapes are important, but not essential (drinking water is essential). If water is limited, landscapes are usually the first areas to feel the effects. This is reflected in many southwestern communities where landscape irrigation is restricted or prohibited during extremely dry years. Arizona and Utah, along with communities in California, Nevada, New Mexico, and Texas are also giving rebates for, or encouraging, the replacement of turfgrass with more water efficient species (Arizona Department of Water Resources, 2004; City of Albuquerque, 2004; City of Austin, 2004; El Paso Water Utilities, 2004; San Diego County Water Authority, 2004; Southern Nevada Water Authority, 2004; Utah Division of Water Resources, 2004). As populations continue to rise, water will become increasingly limited and turfgrass may be drastically reduced, or eliminated, from landscapes in much of the southwestern United States. To prevent this from occurring, a concerted effort must be made to improve water stress tolerance of turfgrasses because turfgrass provides not only aesthetic appeal, but enhances the environment in which we live. Turfgrass prevents soil erosion, provides dust control, is much cooler than hardscapes because it is transpiring water into the air, absorbs CO_2 and releases O_2, and provides a filter for purifying irrigation water (Turfgrass Producers International, 1999).

Plant Water Stress Responses

A thorough understanding of how turfgrasses respond to water stress is necessary to facilitate improvement of turfgrasses to water stress. In general, plants respond to water stress by first decreasing growth, followed by reproduction (yield), and finally death if the stress persists (Boyer, 1982; Yancey et al., 1982; Li and Chen, 2000; E.

Schupp, personal communication, 2001; Pantuwan et al., 2002). As a plant experiences water stress, its stomata close which then decreases or stops transpiration and photosynthesis depending on the severity of the stress (Taiz and Zeiger, 2002). On a cellular level, water loss can concentrate solutes, decrease cell volume, disrupt membranes and gradients across those membranes, and cause the cell to lose turgor pressure (Bray, 1997). Turgor pressure is the hydrostatic pressure within cells and must be positive for viable cells to function. As soil dries, cellular turgor pressure declines. Eventually the turgor pressure will fall below the permanent wilting point. The permanent wilting point is when the soil water potential is so low that the plant will not regain turgor pressure even if water is applied.

Plants are not defenseless against water stress. They have sensitive and complex pathways to signal water deficit and initiate cellular water stress responses. Some physiological water stress responses include cessation of leaf expansion, stomatal closure, leaf rolling or folding, leaf or branch senescence, increased root growth, and hydraulic lift. Cessation of leaf expansion prevents expansion of transpiring surface area, while stomatal closure and leaf rolling or folding prevent transpiration from existing leaf surfaces. Leaf or branch senescence and root growth increase the root to shoot ratio and/or root depth which increases a plant's ability to supply water to viable above-ground tissues. Leaf senescence and increased root growth are well-documented water stress responses in turfgrasses (Carrow, 1996a; Ervin and Koski, 1998; Carrow and Duncan, 2003; Bonos et al., 2004; Ebdon and Kopp, 2004). Hydraulic lift refers to a plant's ability to move water from areas of high soil water potential (deep soils) to areas of lower water potential (shallow soils) through passive flow. This was first observed by Richards

and Caldwell (1987), who showed that the water potential of surface soils under sagebrush (*Artemisia tridentate* Nutt.) increased at night due to water movement from moist, deep soil into deep roots and then traveling up to shallow roots and was released into dry, shallow soil. Richards and Caldwell (1987) estimated that in sagebrush 25-50% of transpired water was lifted water. Hydraulic lift is more prevalent in deep rooted shrubs and trees, but has been shown in grass species including Indian ricegrass (*Achnatherum hymenoides* (Roemer & J.A. Schultes) Barkworth), desert wheatgrass (*Agropyron desertorum* (Fisch. ex Link) J.A. Schultes), and wiregrass (*Aristida stricta* Michx.) (Caldwell, 1990; Yoder and Nowak, 1999; Espeleta et al., 2004).

On a cellular level, plants may increase osmotic potential (osmotic adjustment) (Bray, 1997; Taiz and Zeiger, 2002; White et al., 2001), increase production of protectionary or stabilizing proteins (Dure, 1993; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Wolkers et al., 1999; Ramanjulu and Bartels, 2002), and/or increase enzymatic activity associated with reactive oxygen species (Shinozaki and Yamaguchi-Shinozaki, 1997; Neill and Burnett, 1999; Ramanjulu and Bartels, 2002). Osmotic adjustment is the active accumulation of solutes and allows a cell to maintain turgor pressure at increasingly negative leaf water potentials (Taiz and Zeiger, 2002). Some solutes used in osmotic adjustment are glycerol, glycine betaine, potassium, proline, sugars, and sugar alcohols like mannitol and sorbitol (Kramer and Boyer, 1995; Taiz and Zeiger, 2002). Osmotic adjustment is a common water stress response in many turfgrasses including zoysiagrass (West et al., 1990; Qian and Fry, 1997; Jiang and Huang, 2001a; White et al., 2001). Another mechanism to deal with water stress is the production of proteins that protect or stabilize cellular components. Two common

proteins are the LEA proteins and heat shock proteins. Some LEA proteins (group 1) are thought to protect macromolecules from desiccation, while other LEA proteins (groups 2 and 3) are thought to sequester ions that would otherwise accumulate in the cytoplasm to unacceptable levels (Buitink et al., 2002). Heat shock proteins function as molecular chaperones in preventing protein misfolding and to correct misfolded proteins (Taiz and Zeiger, 2002). Increases in group 1 LEA protein and heat shock protein levels in response to water stress have been documented in turfgrasses (Jiang and Huang, 2000). Another common response to water stress is the production of reactive oxygen species (ROS). When stomata close, energy dissipation through latent heat transfer (water changing from a liquid to a vapor) is severely reduced. Reactive oxygen species are formed when excess energy reduces water molecules to superoxide, hydroxyl radicals, or hydrogen peroxide (collectively known as ROS) (Taiz and Zeiger, 2002). Reactive oxygen species are toxic but plants have ROS detoxifying enzymes to oxidize ROS and prevent cell death. Some detoxifying enzymes associated with ROS are aldehyde dehydrogenase, ascorbate peroxidase, catalase, glutathione peroxidase, glutathione transferase, hydrogen peroxidase, and superoxide dismutase (Mittler, 2002; Ramanjulu et al., 2003; Xu and Huang, 2004).

Measuring Plant Responses to Water Stress

Many techniques are available for studying plant responses to water stress. One commonly used method is to measure leaf water and osmotic (solute) potentials from which pressure potential (turgor pressure) can be calculated. Water potential (Ψ_W) is the sum of osmotic potential (Ψ_S), pressure potential (Ψ_P) and gravitational potential (Ψ_L). Gravitational potential is typically negligible and is generally ignored in plant water

potential calculations (Taiz and Zeiger, 2002). A generally accepted tool for taking water and osmotic potential measurements is the psychrometer. Psychrometers measure the difference in electrical current, due to temperature differences, passing through the junction of two wires (typically, a chromel wire and a constantan wire). A leaf is inserted into an air tight chamber containing the psychrometer. A cooling current is passed through the wires to create a water droplet on the junction. After a water droplet has formed, the cooling current is stopped and the droplet evaporates, causing the junction to cool. The rate of droplet evaporation is determined by the leaf water content. A leaf with low water content will cause the droplet to evaporate more rapidly which causes a large difference in temperature (current) between the junction and ambient temperature (Comstock, 2000). On the other hand, a leaf with high water content will cause the droplet to evaporate more slowly which will decrease the electrical current flowing through the junction. Water potential is then calculated from the electrical current flowing through the junction. Osmotic potential is measured the same way except that the leaf is frozen and thawed prior to taking measurements so as to compromise cellular integrity and eliminate pressure potential. The pressure potential is then obtained by subtracting the osmotic potential from the leaf water potential. Psychrometers are widely used in assessing plant responses to water stress as well as showing osmotic adjustment to water stress.

Water release curves are another useful tool in assessing plant responses to water stress. Water release curves are created by weighing samples subjected to incremental pressure increases (White et al., 1992a). Plant parameters such as cell wall bound water, elasticity, relative water content at zero turgor, leaf water potential at zero turgor, osmotic

potential at full turgor, and turgid weight/dry weight ratios can then derived from the data in water release curves. Cell wall bound water is the proportion of water present in cell walls and is unavailable for turgor maintenance (Taiz and Zeiger, 2002). Elasticity is a measure of cell rigidity with high elasticity values indicative of more rigid cells (White et al., 2001). Elasticity values tend to increase due to stress and are associated with increases in cell wall thickness (White et al., 2001, Wilson et al., 1980). Relative water content at zero turgor is another measurement associated with water stress. Low values of relative water content at zero turgor, as well as cell wall bound water and elasticity, are indicative of poor water stress tolerance (White et al., 2001). Leaf water potential at zero turgor reflects how well a plant can deal with water stress, as more negative leaf water potentials at zero turgor will allow a plant to maintain turgor longer (White et al., 2001). Osmotic potential at full turgor is associated with leaf water potential at zero turgor as osmotic adjustment under water stress allows for leaf water potential to decrease without reaching zero turgor (Nilsen and Orcutt, 1996). Turgid weight/dry weight ratios measure the change in cell wall thickness or constituents (Cutler et al., 1977; Wilson et al., 1980; Liu and Stutzel, 2002). Although cessation of cell growth (expansion) is a primary response to water stress (Taiz and Zeiger, 2002), cells do respond to water stress by increasing cell wall thickness which is reflected in lower turgid weight/dry weight ratios (Cutler et al., 1977; Wilson et al., 1980; Liu and Stutzel, 2002; Martinez et al., 2004).

Advances in molecular biology have made it possible to analyze genetic responses of plants to water stress on a genome scale. Using array techniques, a researcher can look at gene expression over time between control and treatment plants for thousands of genes. Lee et al., (2004) looked at differences in expression of over 26,000

genes of *Arabidopsis thaliana* (L.) Heynh. using an array system. Arrays rely on the ability of single-stranded RNA to bind to single-stranded DNA (Lewin, 2000). A labeled RNA solution is added to a membrane or glass slide with DNA segments (genes or oligonucleotides) attached to its surface (Hays and Skinner, 2001). Labeled RNA binds to complementary DNA and the labeling can then be visualized (Hays et al., 1999). DNA segments attached to the membrane or slide are typically synthesized DNA fragments or DNA complements of RNA transcripts present in a plant at a given time. DNA segments of interest from the array (whose RNA expression matches some physiological response, for example) can then be targeted for further characterization. One widely utilized method for evaluating importance or function of a specific gene is to insert a long segment of DNA, called T-DNA, into the gene of interest. The inserted DNA stops full length transcription of the gene of interest resulting in a truncated, non-functional protein. Then plants with the functional and non-functional gene can be subjected to a treatment of interest, such as water stress, to assess whether the gene of interest confers an advantage to the plants with the functional gene product (Koiwa et al., 2003).

Improving Water Stress Tolerance

Improving water stress tolerance of agronomically important crops has been the focus of a vast quantity of research as water stress is a major environmental factor affecting yield (Boyer, 1982; Yancey et al., 1982; Pantuwan et al., 2002; Rohila et al., 2002). From 1939 to 1975, restitution by the United States government for crop losses due to water stress was almost three times higher than for crop losses due to any other factor (Boyer, 1982). Extensive research has been done to assess the genetic control of water stress tolerance in cereal crops (corn, barley, wheat, and rice) as they account for

70% (1.9 billion tons in 2001) of the world food production (Chandler and Brendel, 2002; Food and Agriculture Organization of the United Nations, 2003). Cereal crop research has identified a large number of water stress tolerance genes (Curry et al., 1991; Hong et al., 1992; Curry and Walker-Simmons, 1993; Holappa and Walker-Simmons, 1995; White and Riven, 1995; Li and Chen, 2000; Saijo et al., 2000; Nakamura et al., 2001; Choi et al., 2002; Malatrasi et al., 2002). Once water stress tolerance genes are identified they can be cloned, transformed and constitutively expressed in the same, or other, species, with improved water stress tolerance of transgenic plants having been observed (Xu et al., 1996; Kishitani et al., 2000; Saijo et al., 2000; Sivamani et al., 2000; Cheng et al., 2002; Rohila et al., 2002). Improving water stress tolerance through transfer of water stress tolerance genes to water stress sensitive species, or over-expressing those genes in water stress sensitive species, has enormous potential for improving or maintaining crop yield with reduced irrigation. These same principles could be used in turfgrasses to maintain turf quality with reduced irrigation (water stress tolerance genes) or to maintain turf quality under high salt conditions (low-quality water) by transferring or over-expressing salt tolerance genes.

One reason that advances in cereal crop genetics can be used to accelerate genetic research in related grass species like turfgrass is orthology. Orthology refers to genes that are conserved between related species (Gale and Devos, 1998). Based on orthology, researchers can look for genes in less extensively studied species, like turfgrass, that have been characterized in well-studied grasses, like corn and rice, with a relatively good chance of finding what they are looking for. An example of orthology that directly relates to water stress tolerance are the late embryogenesis abundant (LEA) genes. Late

embryogenesis abundant genes have been shown to be associated with water stress stress in barley (Hong et al., 1988; Nakamura et al., 2001), wheat (Curry et al., 1991;Curry and Walker-Simmons, 1993), corn (White and Riven, 1995), and rice (Takahashi et al, 1994). Other studies have shown increased water stress tolerance of transgenic plants constitutively expressing LEA genes (Xu et al., 1996; Sivamani et al., 2000; Cheng et al., 2002).

Turfgrass genetic research has a unique opportunity to take advantage of the research that has been done in well-studied relatives and the orthology that exists between grass species. Turfgrass is unique in that the majority of recent landscape water conservation research has focused on improving irrigation management and conventional breeding while little research on biotechnological approaches to identify water stress tolerance genes in turfgrass species appears in the literature (de los Reyes et al., 2001; B. Huang, personal communication, 2003). This is not to say that no one is working in turfgrass biotechnology (reviews by Lee, 1996; Chai and Sticklen, 1998; Sticklen and Kenna, 1998; Johnson and Riordan, 1999; Wang et al., 2001). The genome size of several turfgrass species has been reported (Arumuganathan et al., 1999) and transgenic turfgrasses have been successfully produced for herbicide resistance (Hartman et al., 1994; Lee et al., 1996; Liu et al., 1998; Asano et al., 1998), fungicide resistance (Liu et al., 1998), disease resistance (Chai et al., 2002), and decreased allergenic effects (Bhalla et al.,1999). The turfgrass biotechnology field is not being ignored, but is definitely underutilized especially with respect to improving water stress tolerance.

There are many reasons that biotechnological approaches to improve water stress tolerance of turfgrass should be the focus of more research. The need for more water

stress tolerant turfgrass is only going to intensify as populations grow and suburbs replace farmland. From 1980 to 2000, the acres of land in farms decreased by 36 million acres in Texas and the Western States (United States Department of Agriculture, 2003) while the total number of housing units in the United States increased by 28 million units for the same time period with the South and West growing fastest (Hobbs and Stoops, 2002). Turfgrass also offers the opportunity to evaluate water stress tolerance issues independent of food safety concerns. One concern with transgenic turfgrasses is cross-pollination with other species. This is of special concern with herbicide resistant genes as those genes may be transferred to weedy species (Johnson and Riordan, 1999). The risk associated with transferring a water stress tolerance gene from a transgenic turfgrass to weedy species would be no higher than the risk of gene transfer to weedy species from the tolerant turfgrass species from which gene of interest was inserted into the transgenic turfgrass. However, the risk of constitutive expression of the transgene in weedy species would have to be evaluated. There are risks associated with turfgrass water stress tolerance biotechnology so research should proceed with caution, but should proceed.

Zoysiagrass as a Model for Water and Other Abiotic Stresses

Many cool- and warm-season species have been used for turfgrass biotechnology research (Sticklen and Kenna, 1998; Wang et al., 2001). One species that has potential for researching water stress tolerance is zoysiagrass. Zoysiagrass is a heat and water stress tolerant C_4 grass (Beard, 1973; Inokuma et al., 1998; White et al., 2001). Zoysiagrass is an allotetraploid with a chromosome number of 2n = 40 (Yaneshita et al., 1999). Being an allotetraploid, it is surprising that zoysiagrass has a fairly small genome size (421 Mb for *Z. japonica*, which is similar to rice and about three times larger than

Arabidopsis thaliana) (Arumuganathan et al., 1999). Arumuganathan et al., (1999) also found that *Z. japonica* had a smaller genome than buffalograss, bermudagrass, centipedegrass, bahiagrass and St. Augustinegrass and that the warm-season grasses had smaller genomes than cool-season grasses. Significant genetic (Yaneshita et al., 1997) and physiological variation (White et al., 2001) is present among zoysiagrass cultivars, which makes it a prime subject for studying gene expression and identifying genes of interest. White et al. (2001) found significant differences in the water stress response of 13 zoysiagrass cultivars. This information can be used to study the genetic differences between water stress tolerant and susceptible cultivars of zoysiagrass. Having a relatively small genome with significant genetic and physiological variation makes zoysiagrass a prime target for a turfgrass model species. Another reason to target zoysiagrass for biotechnological studies is that zoysiagrass has no close turfgrass relatives (Turgeon, 2002) and is not native to the United States (Duble, 1989). This makes it highly unlikely that cross-pollination with undesirable species would be a problem in the United States. The argument may be made that zoysiagrass is a non-native, invasive turfgrass that should not be promoted. As many of the most common turfgrasses are not native to the United States (for example, Kentucky bluegrass (Duble, 2005), tall fescue (Applegate, 2005), and bermudagrass (Hale, 2005)), there is no reason to single out zoysiagrass as a non-native invasive species. Zoysiagrass is a commercially available turfgrass used throughout the Central and Southern United States and Eastern Asia for golf courses, athletic fields, parks and home lawns. Biotechnological approaches to identify and use water stress tolerant genes in zoysiagrass will have direct effects on water conservation and can be expanded to other turfgrass species. Increasing

turfgrass water stress tolerance through biotechnological approaches will help meet the increasing need for landscape water conservation in the South and Western United States and throughout the world.

Zoysiagrass has the potential to be more than just the model species for studying turfgrasses. Zoysiagrass has the qualities necessary to argue for its utilization as the model monocot species for studying abiotic stress and C_4 photosynthesis. As yield is the controlling factor in grain production and abiotic stresses have such drastic effects on yield, humans have tried to avoid these stresses by providing supplemental irrigation and growing crops on the best soils. This has lead to increased yields but may have been at the cost of abiotic stress tolerance. This is not to say that there have not been improvements in tolerance of cereal crops to abiotic stresses, but that improvements are so closely tied to yield that unless yield is relatively unaffected the economic feasibility of improvements are questionable.

The model species for molecular research in monocots is rice (*Oryza sativa* L.). Rice has a much smaller genome (about 430 Mb) than the other cereal crops and is used as a major food source for most of the world (United States Rice Genome Project, 2004). However, rice has many qualities that make it sub-optimal as a model species for studying how plants respond to abiotic stress. Rice (1) requires a lot of water, (2) is sensitive to water and salinity stress, (3) is a short-lived annual, and (4) is a C_3 species. Bhuiyan (1992) reported that rice has a water requirement of 7-12 mm/day of water during its 100-day growing season. The USDA Salinity Laboratory classifies rice as salt sensitive with a threshold of 3 dS m^{-1} (United States Department of Agriculture, 2005). As rice is a short-lived annual, it is most similar to spring annual species in semi-arid and

arid ecosystems that complete their lifecycle while water is plentiful in the spring and dry up once the hot, dry summer months arrive. If this short-lived, water stress avoidance lifestyle was reflected in all grass species the Western United States would be a dustbowl. Rice is a C_3 grass species which means that as temperature increases, rice does more photorespiration and less photosynthesis (Taiz and Zeiger, 2002). C_4 species, on the other hand, are well adapted to hot, dry environments because photosynthesis is relatively unaffected as temperature increases (Taiz and Zeiger, 2002). As *Arabidopsis thaliana* (also a C_3 species) is the other model species for genomics research, there is a need for a model species with a C_4 carbon cycle. The high water requirement, high sensitivity to water and salinity stress, short-lived lifestyle, and C_3 carbon cycle of rice make it less than ideal for studying abiotic stress and begs for a more appropriate alternative with potential application to a broader spectrum of species found in semi-arid and arid environments.

Corn (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) are C_4 species that have had more extensive genome maps than zoysiagrass. However, as both are grain crops, they, like rice, have been ultimately bred for yield. As yield is the controlling factor in grain production and abiotic stresses have such drastic effects on yield, humans have tried to avoid these stresses by providing supplemental irrigation and growing crops on the best soils. This has lead to increased yields but may have been at the cost of abiotic stress tolerance. This is not to say that there have not been improvements in tolerance of cereal crops to abiotic stresses, but that improvements are so closely tied to yield that unless yield is relatively unaffected the improvements are not economically feasible.

Zoysiagrass, on the other hand, has not been bred for yield and has been bred for abiotic stress tolerance. Zoysiagrass is ideal because (1) tolerant and susceptible cultivars are available for many abiotic stresses (water, cold, heat, salt, and shade), (2) there is significant genetic diversity between zoysiagrass species and cultivars, (3) zoysiagrass is a perennial grass like most of the grass species in the Western United States, (4) it is a C_4 species (an adaptation to living in hot, dry climates), (5) abiotic stress tolerance can be studied without concern for yield, (6) zoysiagrass is more applicable to urban settings, (7) results can most likely be applied to other perennial grass species (turf, pasture, and range) and cereal crops being used in the Western United States, and (8) zoysiagrass has a small genome (comparable to rice).

A lot of breeding and physiological work has been done in zoysiagrass as illustrated by the cultivar differences in abiotic stress tolerance (Marcum et al., 1995; Engelke et al., 1996; Marcum et al., 1998; Qian et al., 2000; White et al., 2001). Some preliminary molecular work has been done in zoysiagrass to look at genetic maps/markers. Yaneshita et al. (1999) created an RFLP linkage map of zoysiagrass with 115 loci in 22 groups and Ebina et al. (1999) created an AFLP/RFLP linkage map with 165 loci in 23 groups. Anderson (2000) identified 40 RFLP loci among several zoysiagrass species and current research by Krishna (2004) has identified 41 RFLP loci for insect resistance in zoysiagrass. As rice has over 2200 markers (Harushima et al., 1998), there is a lot of progress to be made in zoysiagrass. Chen et al. (2002) constructed a physical map of rice and integrated it with the above-mentioned genetic map and discussed how many of the markers are conserved across grass genomes. This concept would likely accelerate the genetic map construction in zoysiagrass.

As was mentioned previously, rice is a short-lived annual species, which is not reflective of the lifestyle of the majority of grass species in the United States. Most grass species found in urban, forage pasture, and native grassland environments are perennial species, which means they have to deal with abiotic stresses associated with hot, dry summers rather than try to avoid them. As zoysiagrass is a water stress and heat tolerant perennial turfgrass, it is better suited for studying abiotic stress under more representative conditions than rice. Improvements made in zoysiagrass could also likely be applied to other turfgrass, forage, and grassland perennial grasses, with the resultant improvements measurable in parameters such as water savings, improved plant growth on salt affected soils or with low quality water, improved soil stabilization, or increased biomass and/or forage quality for grazing.

Zoysiagrass also affords an opportunity to better understand how plants respond to abiotic stress through the study of C_4 photosynthesis. C_4 photosynthesis is a mechanism some plants use to reduce stomatal water loss. In C_3 plants, the Calvin cycle is occurring in mesophyll cells. The Calvin cycle converts CO_2 into carbohydrate, which can then be used for energy (Taiz and Zeiger, 2002). However, O_2 competes with CO_2 for the enzyme (Rubisco) that starts the Calvin cycle and temperature increases cause more rapid decline in CO_2 concentration than O_2 concentration. As temperature increases, C_3 plants have to keep their stomata open longer to harvest enough CO_2 to maintain carbohydrate levels. C_4 plants have adapted to hot, dry climates by concentrating CO_2 (and localizing the Calvin cycle) in bundle sheath cells and harvesting CO_2 (converted to HCO_3^-) in mesophyll cells with an enzyme (PEP carboxylase) that O_2 does not compete for, and that has a high affinity for its substrate (HCO_3^-) (Taiz and

Zeiger, 2002). PEP carboxylase adds the CO_2 to a three carbon sugar to create a four carbon sugar (malate) that is then transported to the bundle sheath cells where the fourth carbon is removed as CO_2. This CO_2 is then available for use in the Calvin cycle (Taiz and Zeiger, 2002). This strategy has an energy cost, but avoids losing excessive water at high temperatures, which is why C_4 plants are prevalent in hot, dry environments. As neither rice nor A. *thaliana* are C_4 species, zoysiagrass would fill the need for a C_4 model species to further elucidate how C_4 plants cope with the adverse environmental conditions common to the western United States.

Having zoysiagrass as a model species for studying abiotic stress and C_4 photosynthesis will help meet the challenges associated with sustaining a growing population on limited natural resources. Improvements in water stress and salinity tolerance of zoysiagrass will help reduce strain on municipal water resources and allow municipalities to irrigate turf with alternative water supplies that may have higher salt content. As zoysiagrass is a monocot, and significant orthology exists among monocots (Gale and Devos, 1998), it is likely that improvements in water stress or salinity tolerance in zoysiagrass can be applied to pasture and rangeland grass species as well as cereal crops. This would allow farmers to reduce pasture irrigation without negatively affecting forage quality or quantity. Improved salinity tolerance would allow farmers to irrigate pastures with lower quality water or to utilize saline soils that were previously unprofitable as pasture. Improved water stress and/or salinity tolerance in rangeland grass species would likely improve grazing quality/quantity, reduce noxious weed invasion, and reduce soil loss from saline soils. Improvements in water stress and salinity tolerance in zoysiagrass may also be applicable in cereal crops (especially C_4 species like

maize and sorghum) to reduce irrigation or use lower quality water or more saline soils. In the future, decreased yields may also be acceptable if the crop can be grown on significantly less water, with low quality water, or on saline soils. In studying a system, like zoysiagrass, that is stress tolerant and then transferring those improvements to less tolerant species, we are more likely to see measurable results than studying a system, like rice, that is not tolerant to abiotic stress.

Objectives

This study will examine the physiological responses and genetic expression of two cultivars of *Zoysia japonica* (DALZ 8504 and Palisades) and two cultivars of *Zoysia matrella* (Cavalier and Diamond) under water stressed and non-stressed conditions. We hypothesize that (i) differences in physiological responses exist between the two species and between the two cultivars within each species and (ii) that a gene, or several genes, are responsible for those differences. To test our hypotheses, we will (i) measure physiological responses of the four cultivars to water stress, (ii) identify genes that exhibit up-regulation correlated to differences in physiological responses, and (iii) label genes with previously ambiguous identifications ("hypothetical" or "unknown function" proteins) as water stress responsive genes.

MATERIALS AND METHODS

Water Relations Experiment

Plant Culture. Two *Zoysia japonica* (Steud.) cultivars (Palisades and DALZ 8504) and two *Zoysia matrella* (L.) Merr. cultivars (Cavalier and Diamond) were used based on previous results reported by White et al. (2001). Palisades is more water stress tolerant than DALZ 8504 (White et al., 2001) and Cavalier is more water stress tolerant than Diamond (R. White, personal communication, 2003). Five flats (51 x 33 cm) each filled with 10 kg of a mixed sand (70% United States Golf Association – Green Section specification sand and 30% Scotts Redi-Earth ®) were planted with sprigs for each of the four cultivars to produce a total of 20 flats The flats were watered as needed and received 57 g N 93 m^{-2} wk^{-1} from a 20:20:20 ($N:P_2O_5:K_20$) fertilizer for 12 wk. The same fertilizer source was used throughout the study.

After 12 weeks, sod plugs of each cultivar were transferred to 15.2 cm diameter by 6.3 cm deep open-ended tubes. Tubes were placed in flats of fritted clay and each flat received 4 L of water twice per week and 57 g N 93 m^{-2} wk^{-1}. After 4 wk, 12 uniform tubes of each cultivar were selected and one tube of each of the four cultivars was transplanted to a 43 cm by 43 cm by 28 cm deep plastic tub filled to within 3 cm of the top with about 24 kg of fritted clay. A total of 12 tubs were planted. The grasses were grown for 4 wk and received 4 L of water twice per week and 57 g N 93 m^{-2} wk^{-1}. All four cultivars were grown in the same tub to offset cultivar differences in water use that may affect rate of water stress development (Thomas, 1987).

Experimental Design. A randomized complete block split-plot experimental design with three replications was used. Main plots were water stress treatments of stressed and no stress (control). Irrigation was withheld from the stress treatment for 32 d while the control continued to receive 4 L of water twice per week. Percent soil moisture measurements were recorded each day from an "Ech2o" soil moisture probe (Decagon Devices, Inc., Pullman, WA) in the center of each tub. Sub-plots were species and sub-sub-plots were cultivars. The 12 previously established tubs were separated into two experiments (repetitions) with three stressed and three control tubs in each experiment.

Water Relations Characteristics. At the beginning (day 2) and end (day 28) of each repetition, three samples were collected from each treatment and leaf water potential (ψ_L), turgid weight, and fresh weight measured using a hydraulic press and sequential weighing technique as previously described by White et al., 2001. Water release curves were used to estimate cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor ($\psi_{\pi 100}$), elasticity (ε), and turgid weight/dry weight ratios (TWDW) for each treatment. Dry weight for each sample was measured after drying for 48 hours at 65 ^{o}C. Relative water content (RWC) was calculated for each ψ_L by the following equation:

$$RWC = [(FW\text{-}DW)/TW\text{-}DW)]$$

where FW, DW, and TW are fresh weight, dry weight, and turgid weight. The pressure-volume curve was plotted as $1/\psi_L$ versus RWC.

Leaf Water, Osmotic, and Turgor Potential. Leaf samples from each treatment were collected every 2 d. Leaf water potential was measured with calibrated

thermocouple psychrometers following the methods of White et al., 1992a with the only deviation being that measurements were taken every 2 d in this study instead of daily. Visual symptoms of stress (leaf rolling and permanent leaf rolling) were also recorded. Additional leaf tissue was collected from each treatment and frozen in liquid N_2 immediately upon excision and stored at –80 ^{o}C for use in assessing gene expression

Statistical Analyses. All statistical analysis was done using either standard error calculations and/or PROC MIXED in SAS version 8.2 (SAS Institute, Cary, NC) with cultivar (Cavalier, Diamond, Palisades, DALZ 8504), days (before and after stress for the water relations measurements and days 2 through 32 for the leaf water, osmotic, and turgor potential measurements), and treatment (control, water stressed, preconditioned) being main effects. Data that did not satisfy the tests for normality (normal quantile plot, tests for normality, and residual plots) were transformed by taking the square root or log of the raw data. If those transformations did not satisfy the tests for normality, and individual outliers were obvious, they were deleted as a last resort. For main effect interactions, interaction plots were constructed to see what caused the two- or three-way interaction.

Preconditioning Experiment

Plant Culture. An experiment was conducted to evaluate the effect of preconditioning on water relations characteristics under greenhouse conditions. Sod plugs of each cultivar were transferred to 15.2 cm diameter by 6.3 cm deep open-ended tubes. Tubes were placed in flats of fritted clay and each flat received 4 L of water twice per week and 57 g N 93 m^{-2} wk^{-1}. After 10 months, one tube of each of the four cultivars was transplanted to a 43 cm by 43 cm by 28 cm deep plastic tub filled to within 3 cm of

the top with about 24 kg of fritted clay. Plants were grown for ten weeks and received the same fertilization and irrigation schedules as mentioned previously.

Experimental Design. A randomized complete block split-plot experimental design with two replications was used. Main plots were water stress treatments of stressed (no irrigation for 28 d), preconditioned (irrigation withheld for 3 wk followed by a single irrigation to soil saturation and then 28 d of no irrigation) , and non-stressed (4L water tub^{-1} $week^{-1}$). Percent soil moisture measurements were recorded each day from an "Ech2o" soil moisture probe (Decagon Devices, Inc., Pullman, WA) in the center of each tub. Sub-plots were species and sub-sub-plots were cultivars. The 12 previously established tubs were separated into two experiments (repetitions) with two stressed, two preconditioned, and two non-stressed tubs in each experiment.

Water Relations Characteristics. At the beginning (day 2), middle (2 wk) and end (4 wk) of each repetition, three samples were collected from each treatment and leaf water potential (ψ_L), turgid weight, and fresh weight measured using a hydraulic press and sequential weighing technique as previously described by White et al., 2001. Water release curves were used to estimate cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor ($\psi_{\pi 100}$), elasticity (ε), and turgid weight/dry weight ratios (TWDW) for each treatment. Dry weight for each sample was measured after drying for 48 hours at 65 ^{o}C. Relative water content (RWC) was calculated for each ψ_L by the following equation:

$$RWC = [(FW\text{-}DW)/TW\text{-}DW)]$$

where FW, DW, and TW are fresh weight, dry weight, and turgid weight. The pressure-volume curve was plotted as $1/\psi_L$ versus RWC.

Statistical Analyses. All statistical analysis was done using PROC MIXED in SAS version 8.2 (SAS Institute, Cary, NC) with cultivar (Cavalier, Diamond, Palisades, DALZ 8504), days (before, 2 wk, and 4 wk), and treatment (stressed, preconditioned, and non-stressed) being main effects. Data that did not satisfy the tests for normality (normal quantile plot, tests for normality, and residual plots) were transformed by taking the square root or log of the raw data. If those transformations did not satisfy the tests for normality, and individual outliers were obvious, they were deleted as a last resort. For main effect interactions, interaction plots were constructed to see what caused the two- or three-way interaction.

Water Stress Signaling Experiment

An additional experiment was conducted to determine if well watered plants responded to water stress on neighboring plants. This experiment was conducted in the horticultural greenhouses at Brigham Young University-Idaho, in Rexburg, Idaho. Twelve 20.3 cm diameter pots were partially filled with Sunshine Mix 1 (Sun Gro Horticulture Distribution Inc., Bellevue, WA), and planted with Kentucky bluegrass (*Poa pratensis* L.) sod. The plants were watered once a week for about 6 wk before starting the water stress experiment. The twelve pots were randomly assigned to one of four treatments. The treatments included water stressed and non-stressed plants placed 0, 0.5, and 1 m away from water stressed plants. Three leaf samples were taken from each treatment prior to withholding water from the water stressed treatment. Leaves were re-cut underwater and placed in a dark refrigerator at 4 ^{o}C for 24 hr to allow full hydration. The samples were then patted dry and weighed to determine turgid weight. Sample dry weights were determined after drying 48 hr at 65 ^{o}C in a forced air oven. The TWDW for

each sample was then derived by dividing the turgid weight by the dry weight. After the before stress samples were taken, the pots were placed on a greenhouse bench in full sun with the water stressed pots in a row immediately adjacent to the 0 m row of well watered pots. Additional well watered plants were placed 0.5 m and 1 m away from the well watered pots adjacent to the water stressed plants. Water was withheld from the water stressed treatment for 21 d while all three groups of well watered plants remained on the irrigation schedule previously described. After 21 d, three samples from each pot were taken and re-cut underwater and placed in a dark refrigerator at 4 ^{o}C for 24 hours to allow full hydration. The TWDW for all samples were then derived as previously described. The water stressed pots were then discarded and all three groups of well watered plants were maintained on the same irrigation schedule for an additional seven weeks. After the seven additional weeks, the sampling and weighing techniques previously described were used to determine TWDW. The data were analyzed using standard error calculations to see significant differences in well watered plants between days (before stress, after stress, and after removal) and distance from water stressed plants (0, 0.5, and 1.0 m).

Gene Expression Experiment

Samples collected during the water relations experiment and stored at –80 ^{o}C were ground in liquid N_2 and total RNA isolated using TRI-zol ® (Gibco-BRL, Gaithersberg, MD) according to manufacturer's instructions. Subtracted cDNA libraries were made and cloned into pGEM-T Easy (Promega, Madison, WI) vectors (Hays and Skinner, 2001) with the control (non-water stressed) cDNA as the driver and the water stressed cDNA as the tester. Template preparation and sequencing was done as described by Hays and Skinner (2001), with the only deviations being that eight 96-well microtiter

plates were created for each species (768 clones) and that the sequencing was done using an ABI 3100 Genetic Analyzer (Norman E. Borlaug Center, Texas A&M University, College Station, TX). Ten ul of each of the 768 clones were dot blotted onto HybondN (Pharmacia, Piscataway, NJ) positively charged nylon membranes and immobilized by UV crosslinking. Radiolabeled cDNA probes for hybridization to dot-blotted subtracted cDNAs were then synthesized (Hays and Skinner, 2001). Briefly, probes containing the Superscript III enzyme (Clontech, Palo Alto, CA) were added to 10 ug of RNA at 47 ^{o}C for 1 hr. Two ul of dCTP and 0.2 ul of Superscript III were then added to each reaction and continued at 47 ^{o}C for an additional 30 min. The reaction was then terminated by heating to 75 ^{o}C for 10 min. Probes were then run through G50 Sephadex columns with 55 ul of water (2000 g for 2 min) to isolate ^{32}P incorporated probes. Probes were then boiled for 5 min, quenched on ice for 2 min, and then added to membranes that had been spinning at 60 ^{o}C for one hr in 20 ml of denatured salmon sperm prehybridization buffer. Probes were spun at 60 ^{o}C for 24 hrs to allow for membrane hybridization and then rinsed with 40 ml of a 1X SSPE and 0.5% SDS solution. Membranes were then washed with 40 ml of a 1X SSPE, 0.5% SDS solution for 20 min at 60 ^{o}C, followed by two more washings with 0.5X SSPE, 0.5% SDS and 0.1X SSPE, 0.5% SDS solutions, respectively. Membranes were then placed in a 0.5X SSPE, 0.5% SDS solution and transferred to imaging plates and exposed for 7, 12, or 24 hrs. Differential hybridization was quantified using a Fujifilm BAS-1800 II phosphoimager (Fujifilm Medican Systems USA, Inc., Stanford, CT) according to manufacturer's instructions. Initial macroarrays were created with all 768 clones and hybridizations done as described by Hays and Skinner (2001) with probes from pooled samples (day 8, 14, and 20) for well watered and water stressed

treatments of all four cultivars. DNA clones corresponding to spots with more than five fold induction levels under water stress were sequenced by the Laboratory for Crop Genome Analysis at Texas A&M University using an ABI 3100 Genetic Analyzer (Norman E. Borlaug Center, Texas A&M University, College Station, TX) and putatively identified (BLASTx and PSI-BLAST). Differentially expressed cDNAs with high sequence similarity to known water stress tolerance genes or which correlate with physiological changes were focused on for a second round of hybridizations. One hundred and fifteen cDNAs of interest, along with thirteen possible constitutive clones were used for the secondary hybridizations. Each clone was spotted in quadruplet for statistical analysis. Secondary hybridizations were performed between control and water stressed samples for each of days 2, 8, 14, and 20 to show a timeline of gene expression that could be correlated with results from the physiological experiments. Differential hybridization was measured as described previously. Northern analysis with labeled probes from six genes of interest was also performed, using the previously described hybridization and washing techniques, to show changes in steady state mRNA levels of the two tolerant cultivars (Cavalier and Palisades) to confirm the results from the secondary hybridizations. Cavalier total RNA (5 ug) from days 2, 8, 14, and 20 along with Palisades total RNA (5 ug) from days 8 and 14 were electrophoresed through denaturing agarose gels and blotted onto HybondN (Pharmacia, Piscataway, NJ) positively charged nylon membranes and immobilized by UV crosslinking. RNA quantities were normalized using a Biodoc-it Imaging System (UVP, Upland CA) and a Fujifilm BAS-1800 II phosphoimager (Fujifilm Medican Systems USA, Inc., Stanford, CT)

All statistical analysis was done using standard error calculations and/or PROC MIXED in SAS version 8.2 (SAS Institute, Cary, NC) with cultivar (Cavalier, Diamond, Palisades, DALZ 8504), days (days 2, 8, 14, and 20), and treatment (control, water stressed, preconditioned) being main effects. Data that did not satisfy the tests for normality (normal quantile plot, tests for normality, and residual plots) were transformed by taking the square root or log of the raw data. If those transformations did not satisfy the tests for normality, and individual outliers were obvious, they were deleted as a last resort.

Two cDNAs of interest used in the secondary hybridizations that were identified as unigene "hypothetical", "expressed" or "unknown function" proteins in *Arabidopsis thaliana* var. *columbia* were targeted for study by knockout mutation. An *A. thaliana* mutant with a T-DNA insertion immediately upstream of the start site or in an exon of the genes of interest was obtained from the SALK Institute (http://signal.salk.edu/cgi-bin/tdnaexpress) and 20-30 seeds were germinated for each SALK line. DNA isolation and T-DNA insert characterization were performed as previously described (Koiwa et al., 2003) using wild type and T-DNA flanking sequence primer combinations. Four or five plants with homozygous T-DNA inserts in the gene of interest were grown out and regenerated. Twelve seedlings from a plant lacking the gene of interest, along with twelve seedlings from a wild type plant, were then transplanted into a single flat and irrigated until they were at the six to eight leaf stage (14 to 21 d after germination) after which no irrigation was supplied. Visual evaluations, measured in days until leaf wilting, were taken every day to determine if differences existed in the knockout population that negatively affected the plant's ability to deal with the water stress.

RESULTS

Water Relations

Soil moisture was unchanged throughout the growing season for the control tubs and the water stressed tubs prior to water stress treatment (Fig. 1). Greenhouse maximum and minimum temperatures remained constant throughout the growing season (Fig. 2). Water, osmotic, and turgor potential for each cultivar are shown in Fig. 3-6. Measurements from ten psychrometers for days two through 24 of the first repetition were omitted because of faulty input channels on the multiplexer and days two through 12 of the second repetition were omitted as the psychrometers malfunctioned (Fig. 7). Measurements from days 14 through 32 of the second repetition were taken with the same psychrometers used in the first repetition.

There was a continuum in water potential curves between the four cultivars and a significant difference between the repetitions ($P<0.0001$), with the onset of stress being sooner in repetition two (Fig. 8). As the plant responses were similar across both repetitions (Fig. 8), the two were pooled. The most obvious difference was among the two species with Palisades and DALZ 8504 maintaining turgor longer than either Cavalier or Diamond (Fig. 9). Diamond approached zero turgor by about day 24 while Cavalier did not approach turgor loss until about day 30. Palisades and DALZ 8504 were severely stressed towards the end of the experimental period but did not reach turgor loss (Fig. 9). There were also marked differences in how long the four cultivars maintained full turgor and the corresponding osmotic potentials. Diamond had lost full

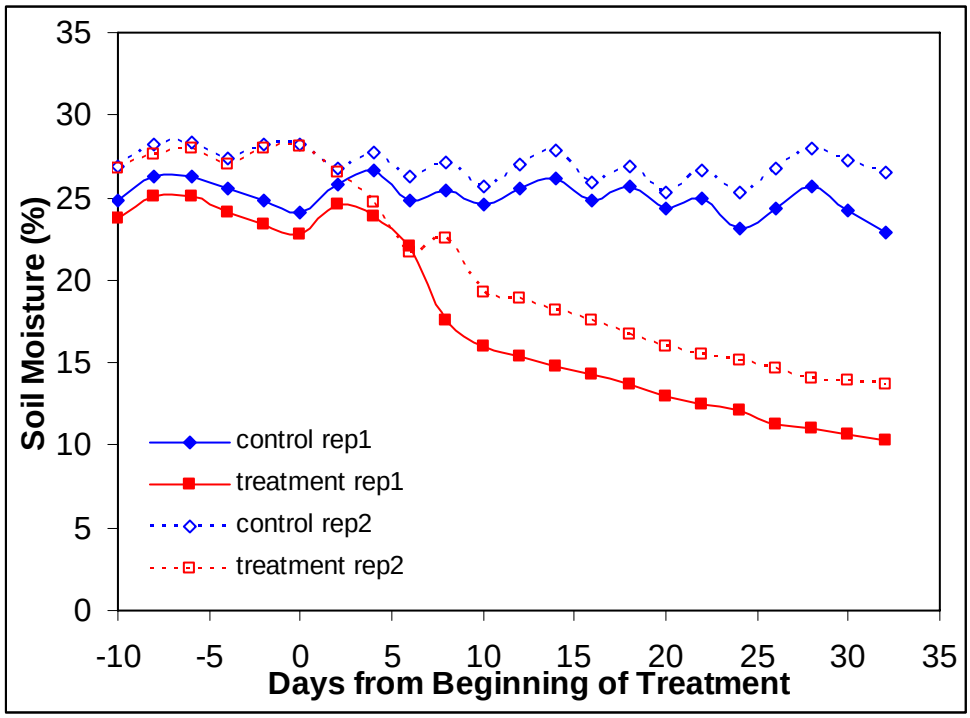


Fig. 1. Mean soil moisture of the water potential experiment. Soil moisture for treatments (squares) and controls (diamonds) of repetitions one (solid lines) and two (dashed lines) (July and August 2003).

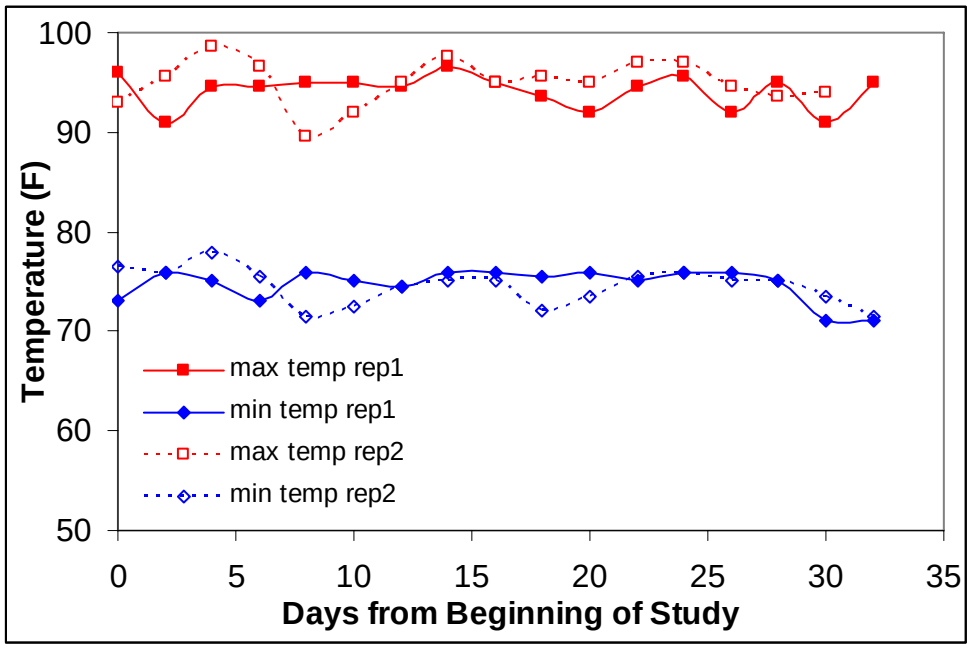


Fig. 2. Mean maximum and minimum greenhouse air temperatures of the water potential experiment. Maximum (squares) and minimum (diamonds) temperatures for repetitions one (solid lines) and two (dashed lines) (July and August 2003).

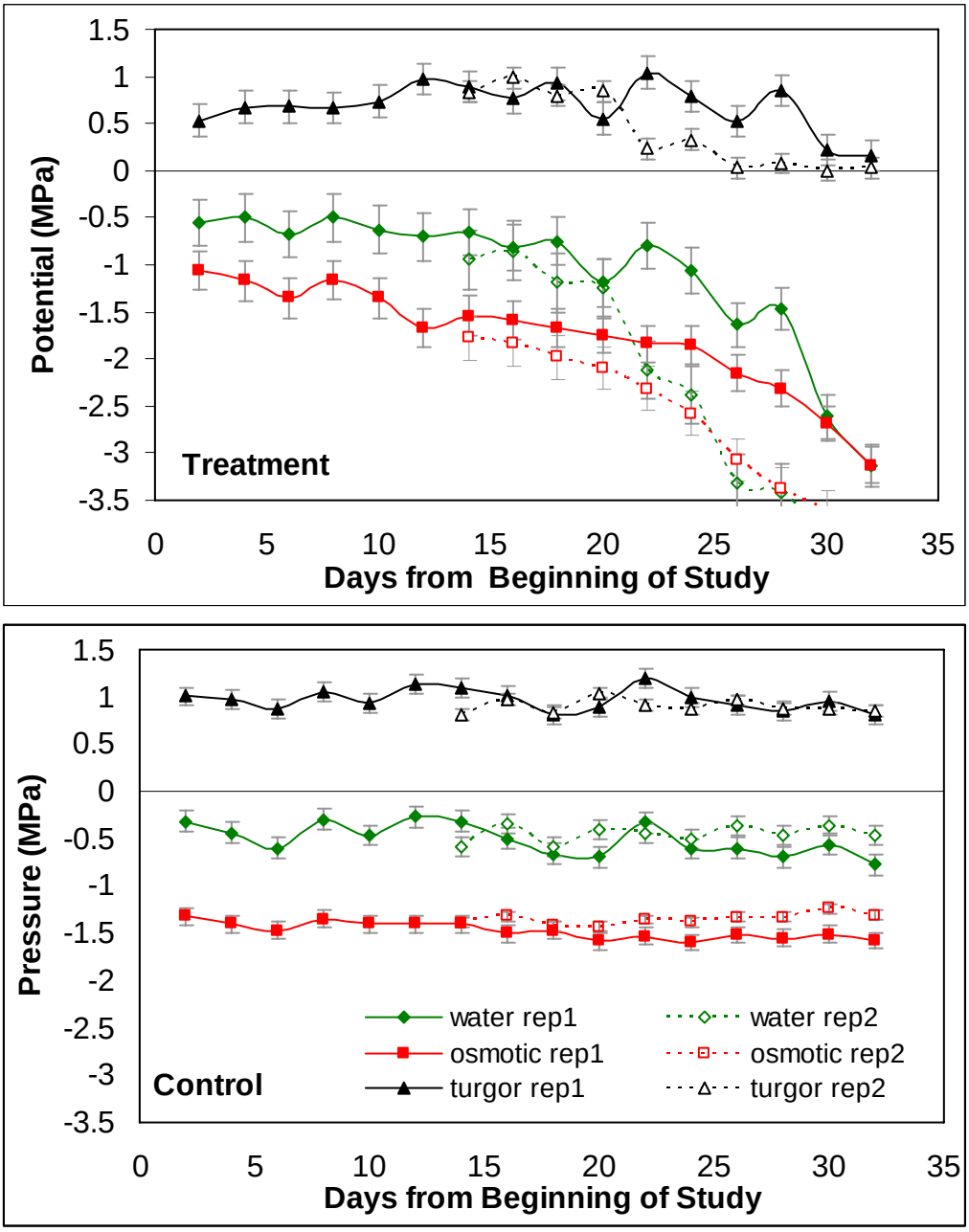


Fig. 3. Water potential curves of Cavalier. Water (diamonds), osmotic (squares), and turgor (triangles) potential curves for repetitions one (solid) and two (dashed) of Cavalier in response to water stressed and control treatments.

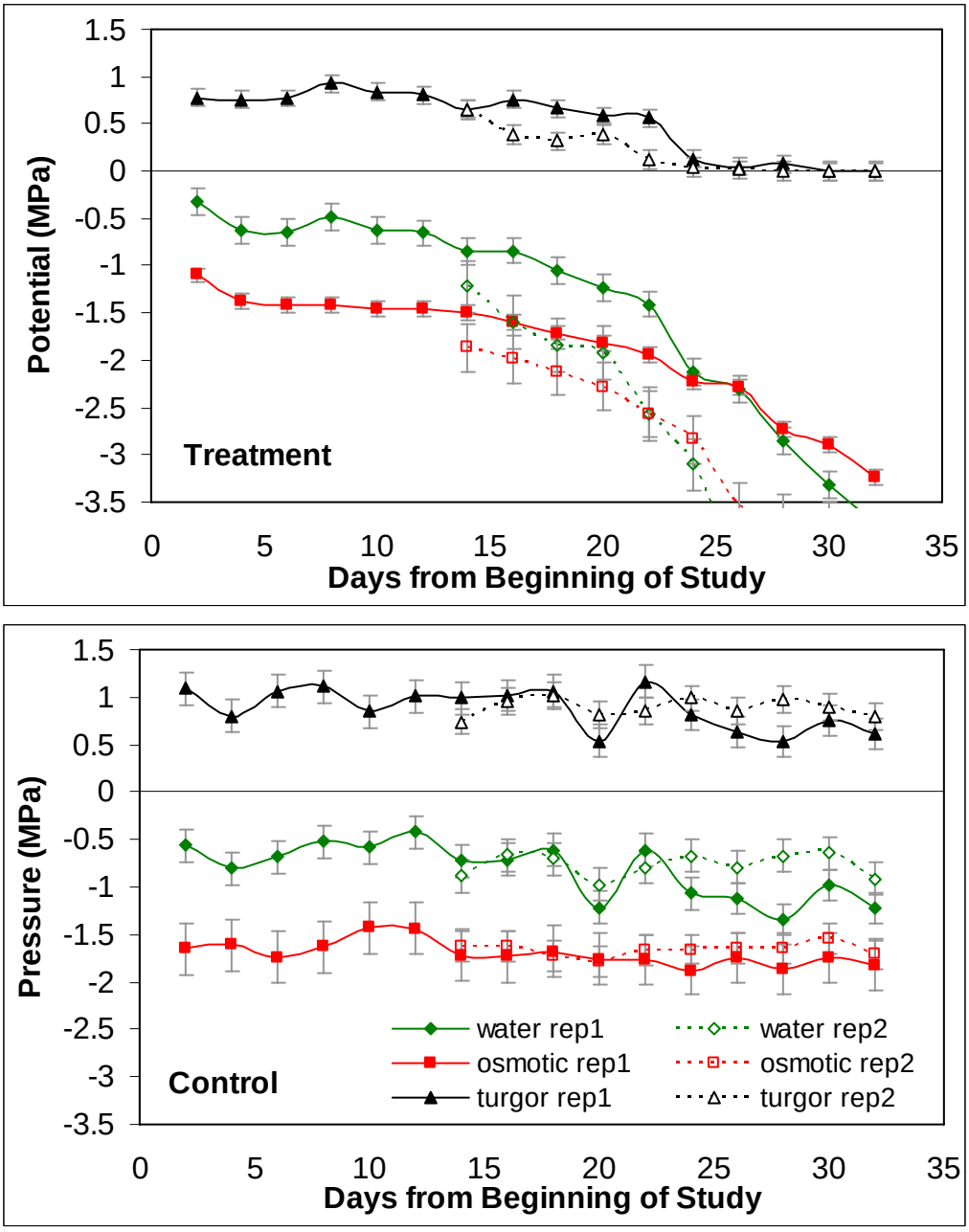


Fig. 4. Water potential curves of Diamond. Water (diamonds), osmotic (squares), and turgor (triangles) potential curves for repetitions one (solid) and two (dashed) of Diamond in response to water stressed and control treatments.

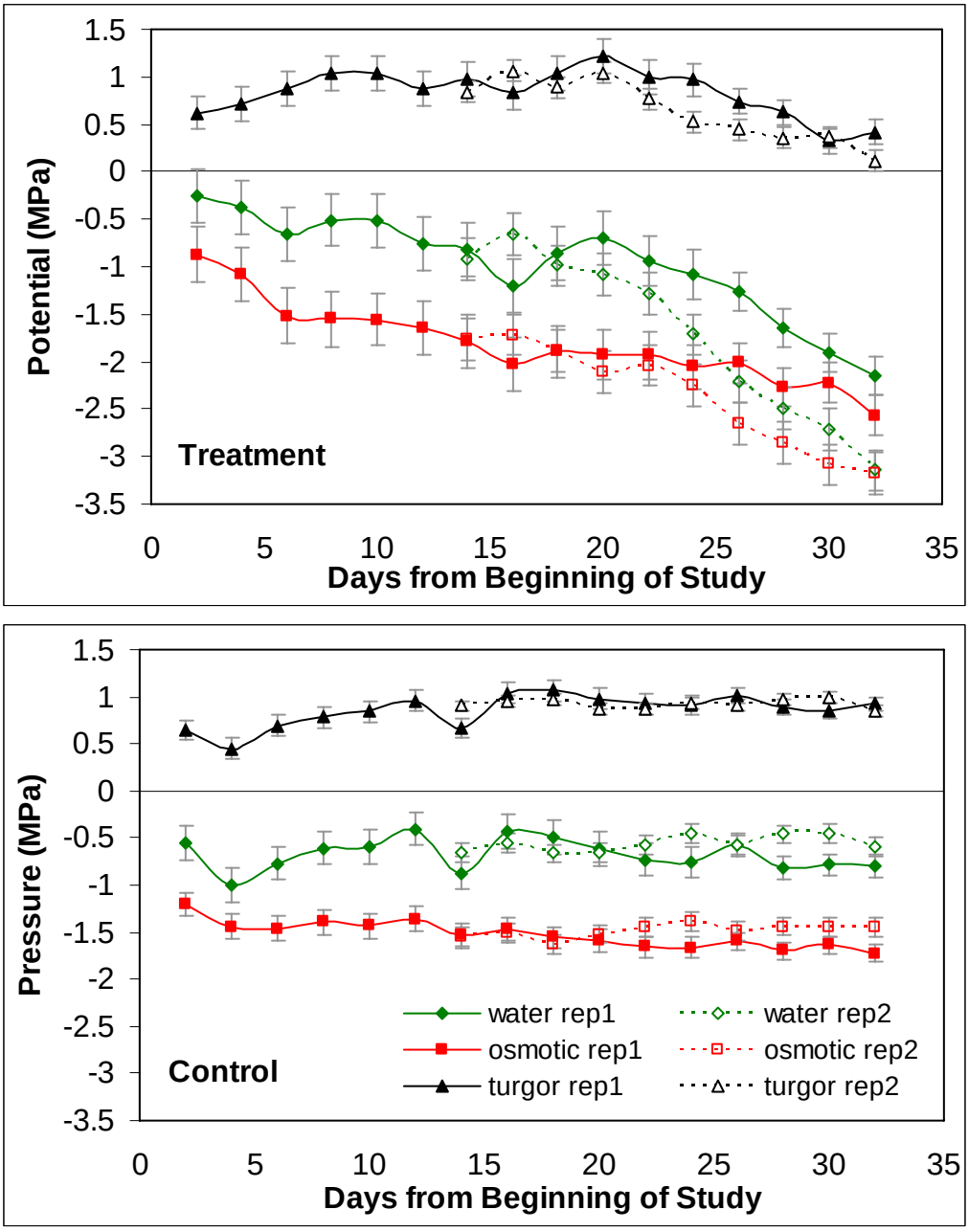


Fig. 5. Water potential curves of Palisades. Water (diamonds), osmotic (squares), and turgor (triangles) potential curves for repetitions one (solid) and two (dashed) of Palisades in response to water stressed and control treatments.

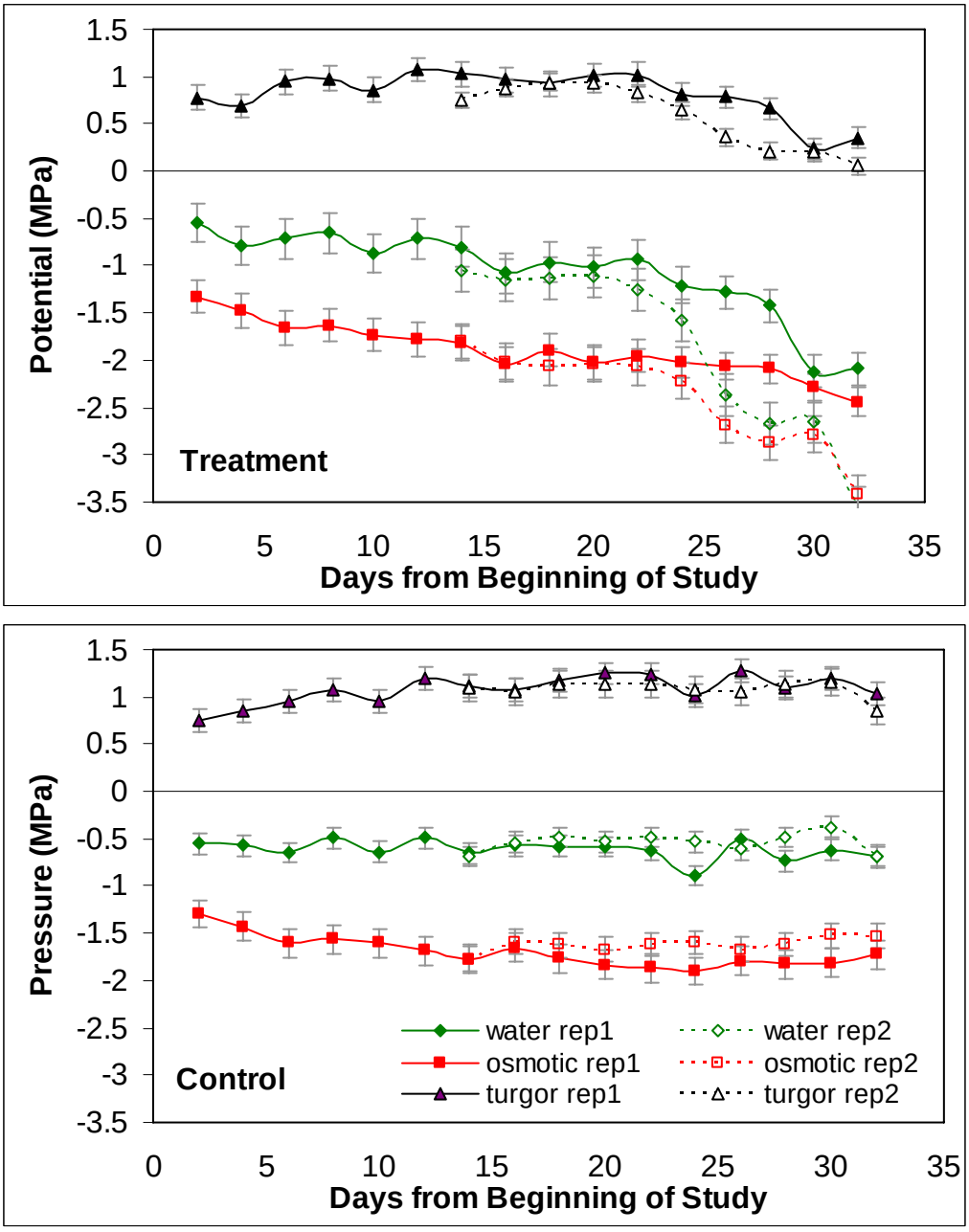


Fig. 6. Water potential curves of DALZ 8504. Water (diamonds), osmotic (squares), and turgor (triangles) potential curves for repetitions one (solid) and two (dashed) of DALZ 8504 in response to water stressed and control treatments.

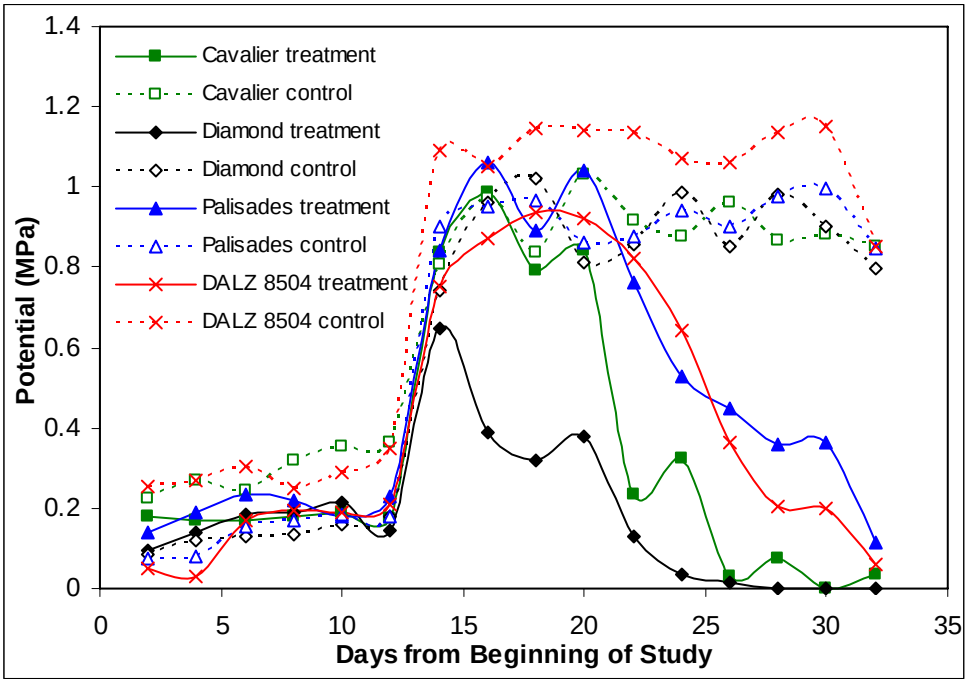


Fig. 7. Turgor potential of all four cultivars of repetition two. Malfunctioning psychrometers in second repetition (days two through 12). From days 14 through 32, psychrometers from the first repetition were used. Water stressed (solid lines) and controls (dashed lines) of Cavalier (squares), Diamond (diamonds), Palisades (triangles), and DALZ 8504 (Xs).

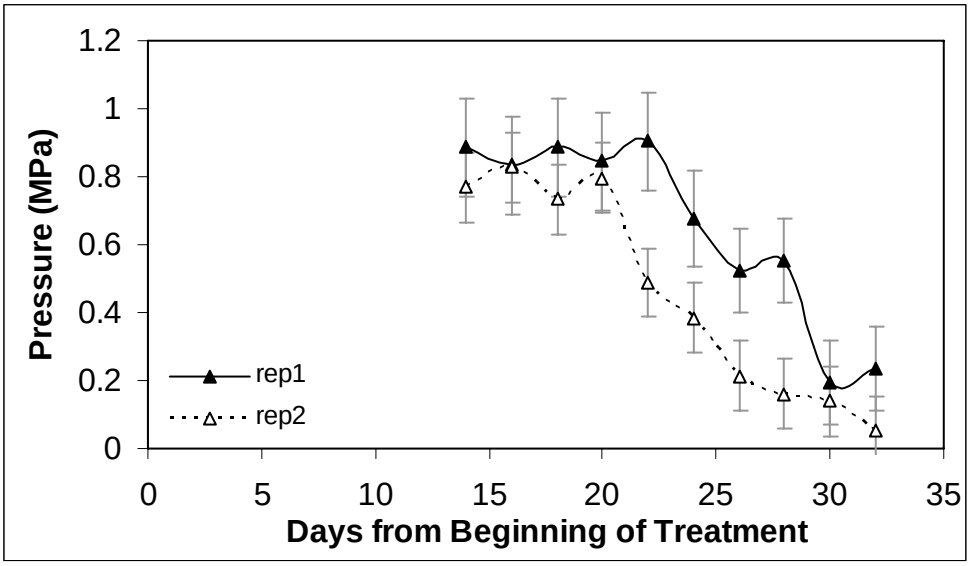


Fig. 8. Mean turgor potential across all four water stressed cultivars for repetitions one and two. Repetition two (dashed line) began drying sooner than repetition one (solid line), but the curves are relatively parallel between days twenty and thirty.

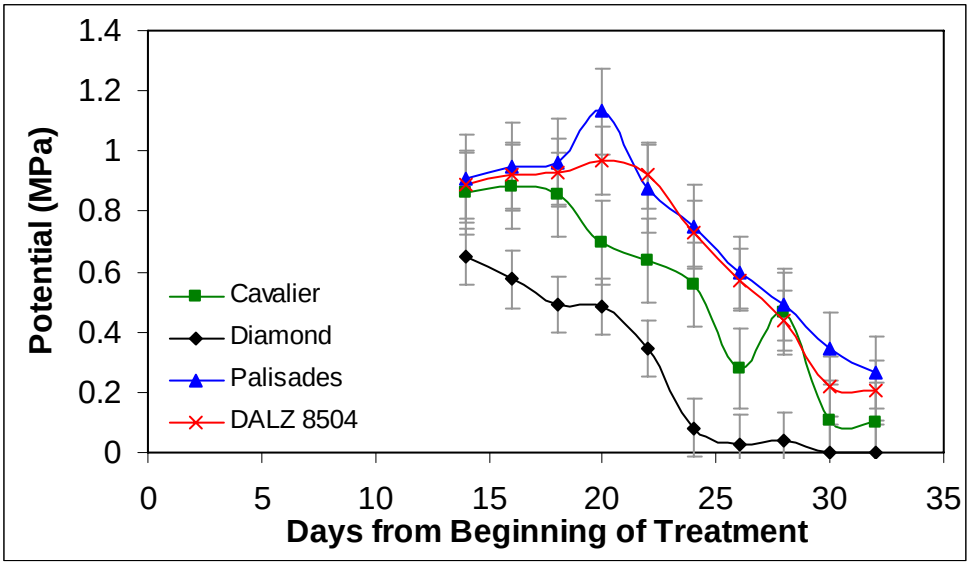


Fig. 9. Mean turgor potential of water stressed zoysiagrasses. Mean turgor pressure of Cavalier (squares), Diamond (diamonds), Palisades (triangles), and DALZ 8504 (Xs) across two repetitions.

turgor by day 14 (osmotic potential of -1.68 MPa) while Cavalier maintained full turgor until day 18 (osmotic potential of -1.82 MPa) and Palisades and DALZ 8504 maintained full turgidity until day 22 (osmotic potentials of -1.99 and -2.01 MPa, respectively). It appeared as though there was also a difference in rate of osmotic potential decrease once the four cultivars dropped below full turgor. Palisades and DALZ 8504 had a more gradual decline in turgor potential than either Cavalier or Diamond (Fig. 10).

There were also differences among the four cultivars in days until leaf rolling. Diamond and Cavalier began midday leaf rolling on day 15, but recovered through the night and were fully expanded by the following morning. However, midday leaf rolling was not observed until day 19 in DALZ 8504 and day 24 in Palisades (all data are means of two repetitions). Predawn recovery was not observed in Diamond and Cavalier by day 24 and by day 30 for DALZ 8504 and Palisades. Cultivar responses within species were statistically similar. Although no differences existed between Diamond and Cavalier in when they started leaf rolling or in failing to recover by morning, there was a difference in severity of rolling. Cavalier was much less severely rolled during the day and recovered more fully by the following morning than Diamond. It was also interesting that the differences in onset of leaf rolling between DALZ 8504 and Palisades were not reflected in their turgor potentials (Fig. 9).

Water Relations Characteristics

There was only one parameter ($\psi_{\pi 100}$) that differed among the two repetitions. Since there were no significant differences between repetitions of the other five

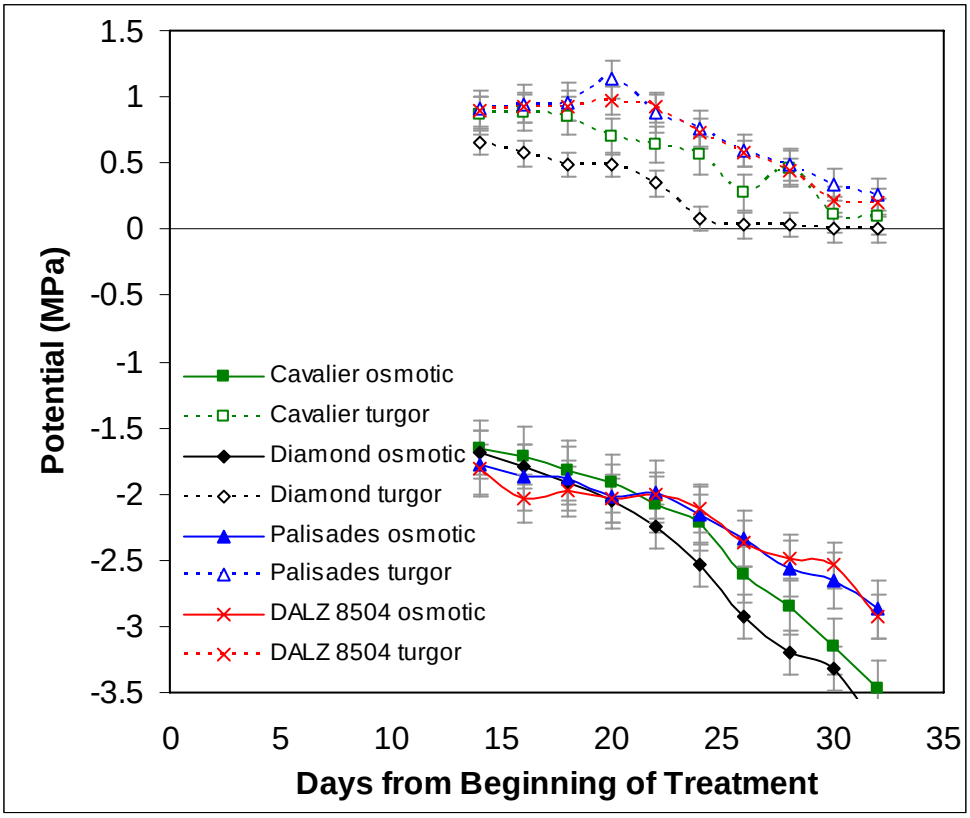


Fig. 10. Mean osmotic and turgor potentials of water stressed zoysiagrasses. Mean osmotic (solid lines) and turgor (dashed lines) potentials of Cavalier (squares), Diamond (diamonds), Palisades (triangles), and DALZ 8504 (Xs) across two repetitions.

parameters, and the difference between the two repetitions of $\psi_{\pi 100}$ was barely significant (P=0.04), the repetitions were pooled. There were two significant interactions. Both were two-way interactions and involved elasticity and TWDW. The elasticity interaction arose because plants from the water stress treatments had lower elasticity values than control plants prior to the water stress treatment (water stressed mean was 0.39 MPa lower than controls) but had higher values than non-stressed plants after the water stress treatment (water stressed mean was 0.74 MPa higher than controls). The TWDW interaction arose because water stress treatments had higher TWDW than control plants prior to the water stress treatment but had lower TWDW than control plants after the water stress treatment. These interactions are not surprising as water stress tends to decrease cell wall elasticity and TWDW, so the main effects of cultivar, days, and treatment will be the focus of further discussion.

The mean values of cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor ($\psi_{\pi 100}$), bulk modulus of elasticity (ε), and turgid weight/dry weight ratios (TWDW) of pre- and post-treatment plants are shown in Table 1.

Of the main effects, the only parameter that exhibited non-significant differences between cultivars was TWDW (P=0.74). Significance in the other four parameters was due to Diamond exhibiting significantly less water stress tolerance (lower β, RWC_0, and ε, with less negative ψ_{L0} and $\psi_{\pi 100}$) than the other three cultivars.

When cultivars and treatments were pooled to isolate the effects of days, the only parameter that exhibited similarity among days (before and after stress) was $\psi_{\pi 100}$

Table 1. Water release curve measurements of well watered and water stressed zoysiagrasses before and after water stress. Mean cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor ($\psi_{\pi 100}$), elasticity (ε), and turgid weight/dry weight ratios (TWDW) of the four cultivars; Cavalier, Diamond, Palisades, and DALZ 8504. Measurements were taken from control and water stressed plants before (BWS) and after (AWS) water stress (28 days).

		β		RWC_0		ψ_{L0}		$\psi_{\pi 100}$		ε		TWDW	
Cultivar	Treatment	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS
		g g^{-1}		g g^{-1}		MPa		MPa		MPa			
Cavalier	Control	0.18^{ab}	0.22^{a}	0.63^{ab}	0.66^{a}	-2.36^{a}	-2.20^{a}	-1.31^{a}	-1.25^{ab}	5.04^{ab}	5.05^{a}	3.20^{a}	$2.75^{**\,a}$
Cavalier	Stressed	0.19	0.21	0.63	0.65	-2.15	-2.13	-1.18	-1.21	4.47	4.99	3.25	2.71^{**}
Diamond	Control	0.11^{a}	0.17^{a}	0.59^{a}	0.65^{a}	-2.24^{a}	-2.03^{a}	-1.19^{a}	-1.17^{a}	4.23^{a}	4.34^{a}	3.36^{a}	2.91^{a}
Diamond	Stressed	0.13	0.29^{*}	0.59	0.70^{*}	-2.05	-2.13	-1.09	-1.24^{**}	3.91	5.68^{*}	3.22	2.70^{**}
Palisades	Control	0.24^{ab}	0.29^{a}	0.68^{b}	0.71^{a}	-2.25^{a}	-2.28^{a}	-1.34^{a}	-1.36^{ab}	5.45^{b}	5.84^{a}	3.30^{a}	$2.79^{**\,a}$
Palisades	Stressed	0.23	0.28	0.67	0.69	-2.20	-2.16	-1.25	-1.22	5.15	5.99	3.52	2.62^{**}
DALZ8504	Control	0.26^{b}	0.23^{a}	0.67^{b}	0.67^{a}	-2.34^{a}	-2.15^{a}	-1.28^{a}	-1.25^{b}	5.92^{b}	5.14^{a}	3.08^{a}	3.02^{a}
DALZ8504	Stressed	0.26	0.34	0.66	0.73	-2.31	-2.27	-1.27	-1.35	5.54	6.67	3.43	2.66^{**}

*, ** Significant differences between before and after stress at $P<0.05$ and 0.01 levels, respectively.
Different letters indicate cultivar differences within a water stress column.

(P=0.43). Leaf water potential at zero turgor was barely significant (P=0.04) and actually increased during water stress rather than decrease as expected. The other four parameters showed significant differences between days and the direction of change was consistent with water stress responses.

The most interesting aspect of this experiment was the similarity among water stressed and non-stressed plants. Control and water stressed plants were similar for all six of the water relations characteristics measured. These data indicated that both the control and treatment plants were responding to the water stress although the water stressed plants were the only plants that were actually water stressed (Fig. 1).

Except for TWDW, Diamond was the only species that demonstrated a significant response to the water stress for any of the parameters measured (it showed significant differences in every parameter measured except ψ_{L0}). Most of the response to water stress, and the cultivar differences presented in Table 1 were the result of significantly lower β, RWC_0, and ε values of Diamond prior to the treatment period with values being similar to the other three cultivars after the water stress treatment. The only other cultivar difference observed was for $\psi_{\pi 100}$, with Diamond being the only cultivar with active osmotic adjustment (a decrease in $\psi_{\pi 100}$).

The TWDW was the only parameter measured that showed a consistent response to the water stress treatment across all four cultivars with significant reductions of TWDW in response to water stress. Although not significant, it is interesting to note that both *Z. japonica* cultivars (Palisades and DALZ 8504) decreased their TWDW (0.9 and 0.77 reductions respectively) more than either of the *Z. matrella* cultivars (Cavalier and Diamond; reductions of 0.54 and 0.52 respectively).

When the water stress treatment plants were evaluated separately, all of the parameters, except ψ_{L0}, were different when the before and after treatment values were compared. Mean values increased for β (from 0.20 to 0.28 g g^{-1}), RWC_0 (from 0.57 to 0.69 g g^{-1}), and ε (from 4.77 to 5.83 MPa), while mean values decreased for $\psi_{\pi 100}$ (from –1.20 to –1.26 MPa) and TWDW (from 3.36 to 2.67). These responses are consistent with changes that occur in water stressed plants.

When the after stress plants were analyzed separately, significant differences existed between the control and treatment plants. Stressed plants had significantly higher β (0.28 compared to 0.23 g g^{-1} for the controls) and ε values (5.83 compared to 5.09 MPa), and significantly lower TWDW (2.67 compared to 2.87). However, there were no differences between the other three parameters.

One of the most interesting outcomes of the water relations characteristic estimates is the apparent response of control plants. When control plants were analyzed separately, TWDW decreased significant ($P<0.0001$) among days (3.24 before and 2.87 after). The only other significant response by control plants was in ψ_{L0} (-2.30 MPa before and –2.17 MPa after stress). As there were no changes in soil water content of the controls (Fig. 1) or greenhouse air temperatures (Fig. 2) during the treatment period, the plants did not experience water stress and most likely received a signal from the water stressed plants.

Water Stress Preconditioning

The six water relations characteristics were statistically similar across repetitions, so the repetitions were pooled for analysis. However, significant species by day interaction effects were observed for RWC_0, $\psi_{\pi 100}$, and TWDW. Looking at the

interaction plots and P values (data not presented) of these three interactions revealed that they were due to differences in trends of one cultivar. The RWC_0 interaction arose because DALZ 8504 responded more to the water stress than the other three cultivars. The $\psi_{\pi 100}$ interaction arose because Cavalier did not respond to the water stress while the other three cultivars did respond. The TWDW interaction arose because Palisades had a much higher TWDW before water stress than the other three cultivars with all four cultivars having very similar TWDW after stress. These interactions will be addressed with the discussion of cultivar differences, but the main effects of cultivar, days, and treatment will be the major focus of discussion. Mean values of cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor ($\psi_{\pi 100}$), elasticity (ε), and turgid weight/dry weight ratios (TWDW) of pre- and post-treatment plants are shown in Table 2.

Of the main effects, the only parameter that exhibited non-significant differences between cultivars was TWDW (P=0.07). Significance in the other four parameters was generally divided between the species with the *Z. matrella* cultivars (Cavalier and Diamond) showing significantly less water stress tolerance (lower β, RWC_0, and ε, with less negative ψ_{L0} and $\psi_{\pi 100}$) than the *Z. japonica* cultivars (Palisades and DALZ 8504).

The ψ_{L0} and ε were similar among days (before and after stress; P=0.23 and 0.08 respectively). The other four parameters were different among days and the direction of change was consistent with typical water stress responses. The 2 and 4 wk values for β, RWC_0, ψ_{L0}, $\psi_{\pi 100}$, or ε were similar and so the 2 wk values were not included in Table 2. The mean TWDW value from the 2 wk measurement (2.58) was significantly less than the before water stress measurement (3.11; $P<0.0001$) and significantly greater than the 4

Table 2. Water release curve measurements of well watered, pre-conditioned, and water stressed zoysiagrasses before and after water stress. Mean cell wall bound water (β), relative water content at zero turgor (RWC_0), leaf water potential at zero turgor (ψ_{L0}), osmotic potential at full turgor($\psi_{\pi 100}$), elasticity (ε), and turgid weight/dry weight ratios (TW:DW) of the four cultivars; Cavalier, Diamond, Palisades, and DALZ 8504. Measurements were taken from control, water stressed, and pre-conditioned (Precond.) plants before (BWS) and after (AWS) water stress (28 days). Measurements were taken at two weeks but were no different than the four week (AWS) measurements.

		β		RWC_0		ψ_{L0}		$\psi_{\pi 100}$		ε		TW:DW	
Cultivar	Treatment	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS	BWS	AWS
		g g^{-1}		g g^{-1}		MPa		MPa		MPa			
Cavalier	Control	0.28^{ab}	0.29^{a}	0.65^{a}	0.68^{a}	-2.01^{ab}	-2.08^{ab}	-1.06^{ab}	-1.13^{a}	5.26^{a}	5.26^{a}	3.14^{a}	$2.43^{** ab}$
Cavalier	Stressed	0.24	0.28	0.66	0.67	-2.01	-1.85	-1.10	-1.01	5.00	5.06	3.14	2.46^{**}
Cavalier	Precond.	0.23	0.25	0.65	0.64	-1.97	-1.97	-1.08	-1.04	4.76	4.85	2.95	2.44^{**}
Diamond	Control	0.21^{a}	0.18^{a}	0.64^{a}	0.64^{a}	-1.82^{a}	-1.99^{a}	-1.01^{a}	-1.13^{a}	4.40^{a}	4.25^{a}	2.99^{a}	$2.55^{** ab}$
Diamond	Stressed	0.23	0.27	0.65	0.68	-1.92	-1.94	-1.06	-1.10	4.37	4.93	3.28	2.49^{**}
Diamond	Precond.	0.26	0.26	0.65	0.67	-1.84	-1.92	-0.97	-1.07	4.96	4.93	3.01	2.47^{**}
Palisades	Control	0.34^{c}	0.35^{b}	0.70^{b}	0.73^{b}	-2.17^{b}	-2.25^{c}	-1.19^{b}	-1.33^{b}	6.29^{b}	6.43^{b}	3.28^{a}	$2.41^{** b}$
Palisades	Stressed	0.28	0.39	0.68	0.74	-2.15	-2.15	-1.19	-1.25	6.47	7.27	3.29	2.37^{***}
Palisades	Precond.	0.33	0.37	0.72	0.76	-2.05	-2.20	-1.21	-1.37	6.30	6.88	3.08	2.38^{***}
DALZ 8504	Control	0.34^{bc}	0.37^{b}	0.69^{b}	$0.75^{* b}$	-2.00^{ab}	-2.14^{bc}	-1.08^{ab}	$-1.33^{* b}$	6.27^{b}	6.81^{b}	3.02^{a}	$2.56^{** a}$
DALZ 8504	Stressed	0.26	0.44	0.69	0.77	-2.07	-2.05	-1.21	-1.21	5.81	7.79	3.13	2.52^{**}
DALZ 8504	Precond.	0.32	0.38	0.71	0.75	-2.02	-2.09	-1.18	-1.26	6.38	6.85	3.12	2.47^{***}

*, **, *** Significant differences between before and after stress at $P<0.05$, 0.01, and 0.0001 levels, respectively.
Different letters indicate cultivar differences within a water stress column.

wk measurement (2.46; P=0.0002), but was consistent across species and therefore was omitted from Table 2 as well. All further discussion of the main effect of days will be comparing before and after the water stress treatment.

The most interesting aspect of this experiment was that the water relations characteristics of the control and both water stress treatments were similar, which indicated that control plants were responding in a manner typical of plants exposed to water stress.

As with the initial water relations characteristic experiment, TWDW was the only consistently significant response across all four cultivars in response to water stress. The only other significant differences observed were in the RWC_0 and $\psi_{\pi 100}$ measurements of DALZ 8504 control plants. Similar trends were seen in the water stressed and preconditioned treatment plants but were not significant.

There were significant cultivar differences in the extent of TWDW decrease after water stress. No significant differences existed before stress, but Palisades had significantly lower TWDW (2.39) than DALZ 8504 (2.52) after water stress. Significant species differences were observed for β, RWC_0, ψ_{L0}, $\psi_{\pi 100}$, and ε both before and after water stress but cultivars within species were similar for these characteristics.

When the preconditioned and stressed treatment plants were evaluated separately, β and TWDW were the only characteristics that showed significant differences when the before and after water stress measurements were compared. Cell wall bound water increased in the stressed plants from 0.25 g g^{-1} before water stress to 0.35 g g^{-1} after water stress, and TWDW decreased in both preconditioned (from 3.21 to 2.46) and stressed plants (from 3.04 to 2.44) in response to the water stress.

When the after stress plants were analyzed separately, only one significant difference was found between any of the treatments. Stressed plants had significantly higher ψ_{L0} values (-2.00 MPa) than control plants (-2.12 MPa), which is contrary to a typical water stress response.

Similar to the initial water relations characteristic experiment, some of the water relations characteristics of the control plants in the preconditioning experiment changed in a manner that was similar to the water stressed plants. When control plants were analyzed separately, the control plants of all cultivars exhibited an average decrease in TWDW of 0.62. Although not significant, the ψ_{L0} and $\psi_{\pi 100}$ of all control plants decreased by 0.12 and 0.14 MPa, respectively, and the ε of Palisades and DALZ 8504 control plants increased by 0.34 MPa when the initial and final measurements were compared. Since soil water content of the controls (Fig. 11) and greenhouse air temperatures (Fig. 12) were relatively constant during the experiment, the plants were not directly exposed to water stress.

Water Stress Signaling

In this experiment, as with the zoysiagrass experiments, the well watered Kentucky bluegrass responded to adjacent water stressed plants. There were no differences in TWDW between any of three distances (0, 0.5, and 1.0 m from water stressed plants). The TWDW decreased in all well watered plants after water stress occurred on adjacent water stressed plants. The TWDW then increased once the water stressed plants were removed. As the change in TWDW of non-stressed plants placed 0, 0.5, and 1.0 m from the water stressed plants was similar, the data were pooled across distance for analysis. The TWDW of non-stressed plants decreased from 5.6 ± 0.14 to 4.1

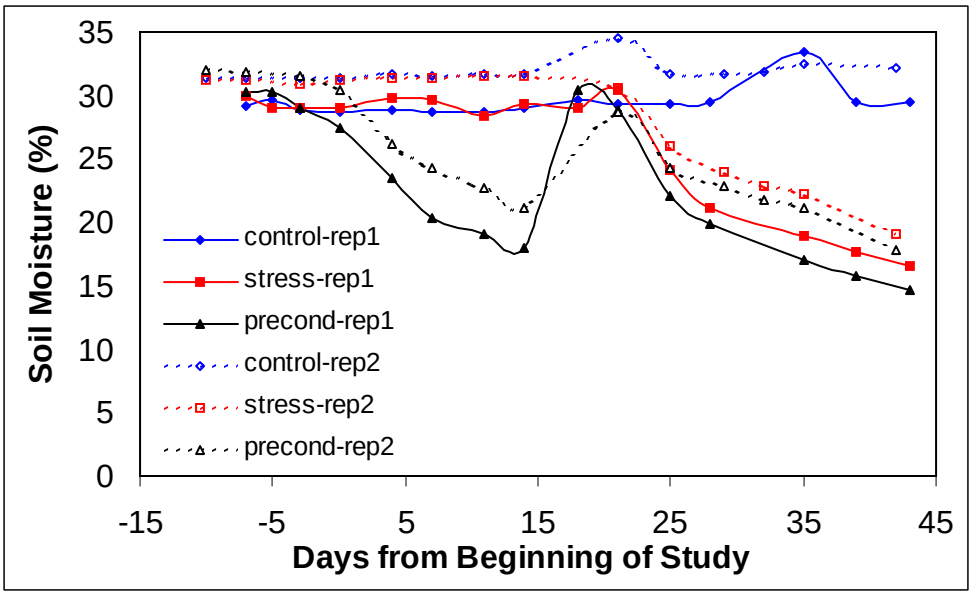


Fig. 11. Mean soil moisture of the preconditioning experiment. Mean soil moisture for preconditioned (triangle), non-preconditioned (squares), and control (diamonds) treatments of repetitions one (solid lines) and two (dashed lines) of the preconditioning experiment (June and July 2004).

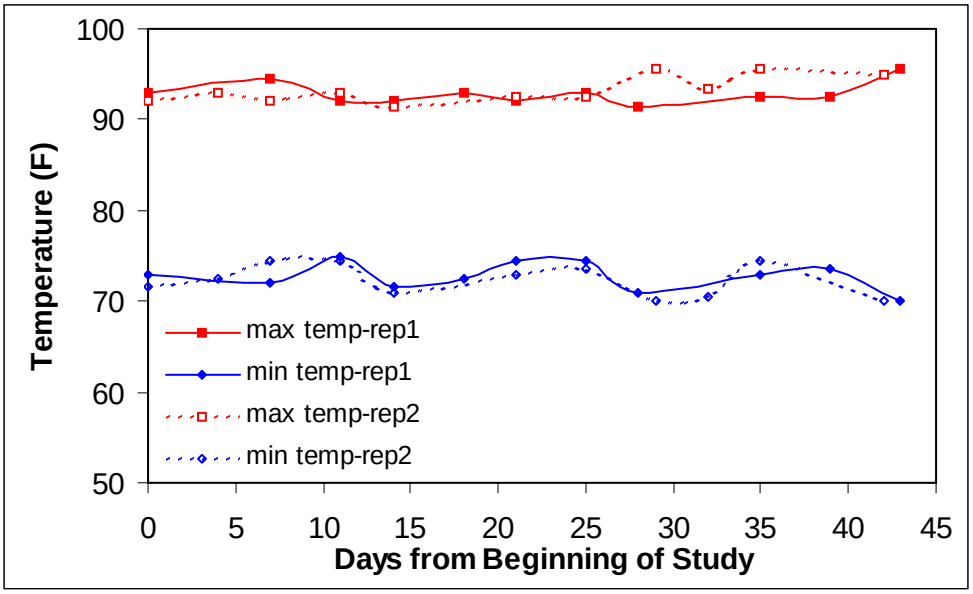


Fig. 12. Mean maximum and minimum greenhouse air temperatures of the preconditioning experiment. Maximum (squares) and minimum (diamonds) greenhouse air temperatures for repetitions one (solid lines) and two (dashed lines) of the preconditioning experiment (June and July 2004).

± 0.30 after treatment of adjacent stressed plants, and then increased to 5.0 ± 0.15 after water stressed plants were removed. These data support the results from the zoysiagrass study and indicated that water stress signaling may be a widespread response in grasses, since Kentucky bluegrass and zoysiagrass are not closely related.

Gene Expression

Sequencing and sequence identification from the two subtracted cDNA libraries (created from the two water stress tolerant cultivars (Cavalier and Palisades)) showed that the library construction appeared successful (i.e. high quality sequences identified as coming from plants; Tables 3 and 4). The first round of macroarrays, conducted from the same plant tissue as the cDNA libraries were constructed, demonstrated that the cDNA library subtraction appeared to work as well, with the treatment membranes having higher gene expression than the control membranes of all four cultivars (Fig. 13-16; Table 5). Two hundred and seventy six Cavalier and 403 Palisades clones of interest (unusually bright spots or spots whose image analysis revealed more than a five-fold difference between spots on the control and treatment membranes) were sequenced and Blastx searches revealed the putative identities of the sequenced clones (Table 6). Of the 302 identified clones, only ten were present in both Cavalier and Palisades. However, those ten accounted for 75 percent of the repeats found in Table 6 (Table 7).

GenBank searches revealed that 35 of the 302 identified clones have been previously associated with stress responses in other species. These 35 clones, along with all clones that were identified as clones of interest by both the visual and image analysis methods, as well as some clones that were repeatedly identified (Table 7), were used in the second round of macroarrays. From the first round of macroarrays, 37 clones from

Table 3. Putative sequence identities of Cavalier plate 1. Columns for species, accession and E value refer to the most homologous DNA sequence from the Blastx database. Fourteen sequences (A3, A5, A7, C2, D6, D12, E1, F3, F10, G7, G11, H4, H7, and H10) resulted in no Blastx hits and are not shown in this table.

Well	Putative Sequence Identification	Species	Accession	E value
A01	LacZ cloning vector	vector pTA6		2.00E-45
A02	Chloroplast hypothetical protein	Maize	AAR91119	3.00E-42
A04	Hypothetical protein	Rice	BAD07869	3.00E-12
A06	adenalate cyclase	Cow		1.50E+00
A08	Gag-pol polyprotein	Rice	NP_918356	4.00E-41
A09	Beta galactosidase	phage		2.00E-13
A10	Phosphatidylinositol transfer protein	Rice		2.00E-29
A11	ABC transporter	Rice	NP_915325	2.00E-72
A12	Hypothetical protein	Rice	BAD07869	1.00E-17
B01	Hypothetical protein	Rice	BAD07869	1.00E-18
B02	Chloroplast hypothetical protein	Maize	AAR91119	7.00E-30
B03	Chloroplast mutational hotspot ORF85	Rice	NP_039397	5.00E-05
B04	Transcript antisense to ribosomal RNA	Yeast		9.00E-10
B05	Non-ribosomal peptide synthetase	Bacteria	NP_794272	6.30E+00
B06	Similar to mitogen-activated protein kinase	Rice	CAD40821	5.00E-45
B07	Hypothetical protein	Evening primrose	NP_084748	4.00E-09
B08	Photosystem I assembly protein Ycf4	Corn	NP_043035	2.00E-18
B09	Chloroplast hypothetical protein	Corn	AAR91119	7.00E-30
B10	Chloroplast hypothetical protein	Corn	AAR91119	9.00E-17
B11	LacZ cloning vector	vector pTA6		1.00E-40
B12	Chloroplast hypothetical protein	Corn	AAR91119	4.00E-30
C01	Ribosomal protein L28-like	Rice	NP_916542	1.00E-29
C03	Hypothetical protein	Rice	BAD07869	1.00E-18
C04	Protein F21D18.22	*A. thaliana*	A96521	2.00E-05
C05	Heat shock protein 70	*A. thaliana*	NP_567510	8.00E-64
C06	Vesicle-associated membrane protein	Rice	BAC99503	4.00E-36
C07	Senescence-associated protein	Pea	BAB33421	5.00E-39
C08	Chloroplast hypothetical protein	Corn	AAR91119	4.00E-30
C09	Unknown	Rice	AAO72659	4.00E-43
C10	Polyprotein	Virus	AAQ76546	8.80E+00
C11	Unknown	Bacteria	NP_759470	2.00E-03
C12	Probable histidine kinase	*A. thaliana*	T00842	6.40E+00
D01	Conserved protein	Bacteria	NP_633235	9.80E+00
D02	Expressed protein	*A. thaliana*	NP_196392	1.00E-03
D03	Myb protein	Frog	Q08759	3.40E+00
D04	Senescence-associated protein	Pea	BAB33421	7.00E-51
D05	Chloroplast hypothetical protein	Corn	AAR91119	1.00E-29
D07	Iron deficiency protein IDS3	Rice	BAC84567	6.00E-06
D08	Hypothetical protein	Bacteria	NP_780783	7.00E-21
D09	Senescence-associated protein	Pea	BAB33421	2.00E-01
D10	OSJNBa0028I23.20	Rice	CAE04638	9.00E-06
D11	ATP-binding region	*A. thaliana*	NP_564908	1.00E-22
E02	LacZ cloning vector	vector pTG8	AAF61634	5.00E-13

Table 3 (cont.)

Well	Putative Sequence Identification	Species	Accession	E value
E03	Chloroplast hypothetical protein	Corn	AAR91119	6.00E-29
E04	Unknown	*A. thaliana*	AAG51531	6.00E-05
E05	Senescence-associated protein	Pea	BAB33421	3.00E-40
E05	Senescence-associated protein	Pea	BAB33421	3.00E-40
E06	Hypothetical protein	Rice	BAD07869	4.00E-18
E07	Lac Z cloning vector	vector pTA6	AAR19394	5.00E-21
E08	Conserved protein	Bacteria	NP_633235	9.80E+00
E09	Myosin heavy chain-like protein	Rice	NP_917326	1.00E-07
E10	Unknown	Rice	AAO72659	8.00E-46
E11	Casein kinase	Rice	NP_916323	6.00E-06
E12	Unknown	Zebrafish	AAH65983	9.00E-03
F01	ORF46c	Pine	NP_817268	9.00E-06
F02	Chloroplast hypothetical protein	Corn	AAR91119	6.00E-25
F04	Pyruvate dehydrogenase kinase	Corn	AAC63961	1.00E-05
F05	Hypothetical protein	Rice	BAD07869	1.00E-18
F06	Casein kinase I	Rice	CAD32377	5.00E-14
F07	Proline-rich family protein	*A. thaliana*	NP_171713	2.00E-28
F08	Hypothetical protein	Nematode	CAE57658	6.90E-01
F09	Hypothetical protein	Evening primrose	NP_084748	1.00E-13
F11	Hypothetical protein	Rice	BAD07869	1.00E-18
F12	Hypothetical protein	Bacteria	NP_603248	5.00E+00
G01	Hypothetical protein	Bacteria	NP_759470	2.00E-03
G02	Kinase	Rice	NP_915832	3.00E-03
G03	Hypothetical protein	Bacteria	D75542	8.00E-27
G04	Acorbate peroxidase	Rice	BAC79363	2.00E-21
G05	Zinc finger family protein	*A. thaliana*	NP_177528	1.00E-11
G06	Beta-galactosidase alpha peptide	vector	AAC53709	1.00E-32
G08	Expressed protein	*A. thaliana*	NP_196392	2.00E-03
G09	Gamma-adaptin 1	Rice	AAK98709	2.00E-11
G10	Splicing factor 3b	Rice	BAD10377	1.00E-20
G12	Chloroplast hypothetical protein	Corn	AAR91119	6.00E-29
H01	Cytochrome P450 monooxygenase	Corn	T02955	6.00E-20
H02	OSJNBa0086O06.17	Rice	CAE04869	1.00E-15
H03	Hypothetical protein	Virus	A56644	9.90E+00
H05	Hypothetical protein	Rice	AAS01952	2.00E-14
H06	Leucine zipper protein	Rice	NP_922012	7.00E-18
H08	Glycine-rich protein	Tobacco	S34666	7.00E-12
H09	Unknown	Zebrafish	AAH65983	9.00E-03
H11	Sucrose-phosphate synthase	Barley	AAF75266	4.00E-20
H12	Hypothetical protein	Rice	BAD07869	3.00E-14

Table 4. Putative sequence identities of Palisades plate 1. Columns for species, accession and E value refer to the most homologous DNA sequence from the Blastx database. Nineteen sequences (A6, B1, B2, B3, C6, D1, D3, D4, D12, E1, E3, E4, E7, E8, E9, F3, F8, G2, and H4) resulted in no Blastx hits and are not shown in this table.

Well	Putative Sequence Identification	Species	Accession	E value
A01	60S ribosomal protein	Corn	P45633	2.00E-09
A02	Antisense to Ribosomal RNA	Yeast	NP_690845	4.00E-11
A03	Ring box-1 protein	Rice	AAL87158	2.00E-19
A04	Photosystem I subunit 9	Corn	NP_043044	1.70E-01
A05	Antisense to Ribosomal RNA	Yeast	NP_690845	3.00E-12
A07	LacZ alpha peptide	vector	AAA56741	2.00E-21
A08	Hypothetical protein	Rice	AAL84286	7.00E-21
A09	Cytochrome oxidase subunit I	Lice	AAO11988	6.10E+00
A10	Elongation factor	Soybean	BAC22127	7.00E-10
A11	Hypothetical protein	Rice	BAD07869	4.00E-20
A12	Cytochrome P450 monooxygenase	Corn	T02955	3.00E-36
B04	GRounDhog, hedgehog-like (grd-11)	Nematode	NP_507923	3.00E+00
B05	Methyl-accepting chemotaxis protein	Bacteria	ZP_00054748	1.60E+01
B06	Hypothetical protein	Rice	BAD07869	3.00E-18
B07	Pyrroline-5-carboxylate reductase	Rice	BAC15792	5.00E-26
B08	Antisense to Ribosomal RNA	Yeast	NP_690845	4.00E-11
B09	ATP synthase CF0 C chain	Rice	NP_039378	8.00E-08
B10	Hypothetical protein	Rice	BAD07869	4.00E-20
B11	Oxidoreductase	Bacteria	NP_828460	9.40E+00
B12	spermidine/putrescine ABC transporter	Bacteria	NP_814947	4.30E+00
C01	Polyketide synthase modules	Bacteria	ZP_00108808	7.50E+00
C02	Unnamed protein product	Mouse	BAB27270	6.00E-01
C03	Calcium-binding EF hand family protein	*A. thaliana*	NP_194377	1.00E-27
C04	Expressed protein	*A. thaliana*	NP_568492	2.00E-17
C05	Photosystem I subunit 9	Corn	NP_043044	2.40E+00
C07	Bundle sheath cell specific protein 1	Corn	BAB20906	3.00E-10
C08	Hypothetical protein	Nematode	CAE73759	3.40E+00
C09	Cyclophilin	Rice	AAP73848	4.00E-08
C10	Ribonuclease III, 5'-partial	Rice	AAS07189	9.00E-32
C11	OSJNBb0065J09.5	Rice	CAE05709	8.00E-50
C12	Hypothetical protein	Rice	BAD07869	6.00E-11
D02	Phosphoinositide-specific phospholipase C	Rice	AAK01711	4.00E-06
D05	hypothetical protein	Evening primrose	NP_084741	8.00E-07
D06	Grg1 protein	Fungi	CAC24571	5.00E-11
D07	S-adenosylmethionine synthetase 1	Rice	P46611	4.00E-56
D08	Antisense to Ribosomal RNA	Yeast	NP_690845	2.00E-11
D09	Ankyrin-kinase	Alfalfa	AAL78675	8.00E-18
D10	S-adenosylmethionine synthetase 1	Rice	P46611	9.00E-64
D11	Similar to FLJ40243 protein	Mouse	XP_355221	7.50E+00
E02	Glycoside hydrolase	*A. thaliana*	NP_850987	3.00E+00
E05	Expressed protein	*A. thaliana*	NP_200323	2.00E-43
E06	RNA helicase	*A. thaliana*	CAA66825	3.00E-04
E10	C2 domain-containing protein	*A. thaliana*	NP_196671	7.5
E11	Hypothetical protein	Rice	NP_916222	2.00E-40

Table 4 (cont.)

Well	Putative Sequence Identification	Species	Accession	E value
E12	Similar to Gcn5 INdependent; Gin1p	Yeast	AAO51238	2.9
F01	Zinc finger protein	Rice	AAP85546	1.00E-28
F02	Sphingosine kinase	Rice	NP_922341	8.00E-41
F04	Malate dehydrogenase	Rice	BAC83246	2.00E-43
F05	Small GTP-binding protein (RAB5A)	Rice	AAK38149	1.00E-34
F06	hypothetical protein	Tobacco	T02948	1.00E-06
F07	ribosomal protein S3	Rice	NP_039424	3.00E-28
F09	pentatricopeptide (PPR) repeat	*A. thaliana*	NP_180698	1.20E-02
F10	Proline-rich protein	Human	NP_005030	7.30E-01
F11	Hypothetical protein	Bacteria	G72580	7.60E-01
F12	Flagellar hook-associated protein	Bacteria	NP_244487	4.10E-01
G01	LacZ cloning vector	vector pTA6	AAR19394	1.00E-18
G03	Plastidic ATP/ADP transporter	Citrus	AAM29152	9.00E-35
G04	Mitochondrial protein of unknown function	Yeast	NP_690845	5.00E-13
G05	Xylose isomerase	Rice	BAC83596	5.00E-51
G06	Cloning vector	vector pZeRO-2T	CAA71575	8.00E-05
G07	Chaperone/heat shock protein	Fungi	AAB69701	2.00E-05
G08	Hypothetical protein	Rice	BAD07869	1.00E-10
G09	Tetracycline transporter protein	*A. thaliana*	F84546	3.00E-04
G10	Olfactory receptor-like protein	Rat	AAC17222	8.90E+00
G11	P0498B01.20	Rice	NP_913129	5.10E-01
G12	ATP synthase CF0 C chain	Rice	NP_039378	8.00E-08
H01	Hypothetical protein	Rice	BAD07869	1.60E-02
H02	Mitochondrial protein of unknown function	Yeast	NP_690845	4.00E-11
H03	Xylose isomerase	Rice	BAC83596	5.00E-51
H05	Formin homology 2 domain	*A. thaliana*	NP_566311	1.90E+00
H06	Voltage-dependent anion channel	Rice	CAB82853	4.00E-42
H07	Hydrolase	Rice	NP_909997	1.00E-25
H08	rRNA promoter binding protein	Rat	NP_671477	1.00E-06
H09	Ribosomal protein S3	Rice	NP_039424	8.00E-27
H10	Cloning vector	vector pZeRO-2T	CAA71575	4.00E-09
H11	Hypothetical protein	Rice	BAD07869	4.00E-20
H12	Hypothetical protein	Rice	BAD07869	3.00E-02

Table 5. Quantified gene expression for the first round of macroarrays. Values are the mean of 768 data points.

Cultivar	Control (PSL)	Treatment (PSL)
Cavalier	57	214
Diamond	61	223
Palisades	42	153
DALZ 8504	82	532

Table 6. Identity of clones of interest from the first round of macroarrays.

Clone Identity	Cavalier	Palisades
Identified Clones	101	201
Repeats	110	91
Unidentified (possibly unique)	28	49
Unidentified (poor quality)	37	62
Total Clones of Interest	276	403

Table 7. Putative identity of repeated clones and the number of repeats from each cDNA library. Columns for species and accession refer to the most homologous DNA sequence from the Blastx database.

			Number of Repeats	
Putative Clone Identity	Species	Accession	Cavalier	Palisades
Chloroplast hypothetical protein	Corn	AAR91119	41	3
Photosystem I apoprotein A2	Rice	AAS46120	0	0
Senescence-associated protein	Pea	BAB33421	7	0
Hypothetical protein	Rice	BAD07869	7	6
Unknown function (mitochondrial)	Yeast	NP_690845	11	43
Unknown	Bacteria	NP_759470	9	1
Hypothetical protein	Rice	NP_910689	0	0
Myosin heavy chain protein	Rice	NP_917326	0	0
Monooxygenase	Corn	T02955	4	2
Hypothetical protein	Bacteria	ZP_00203429	12	4

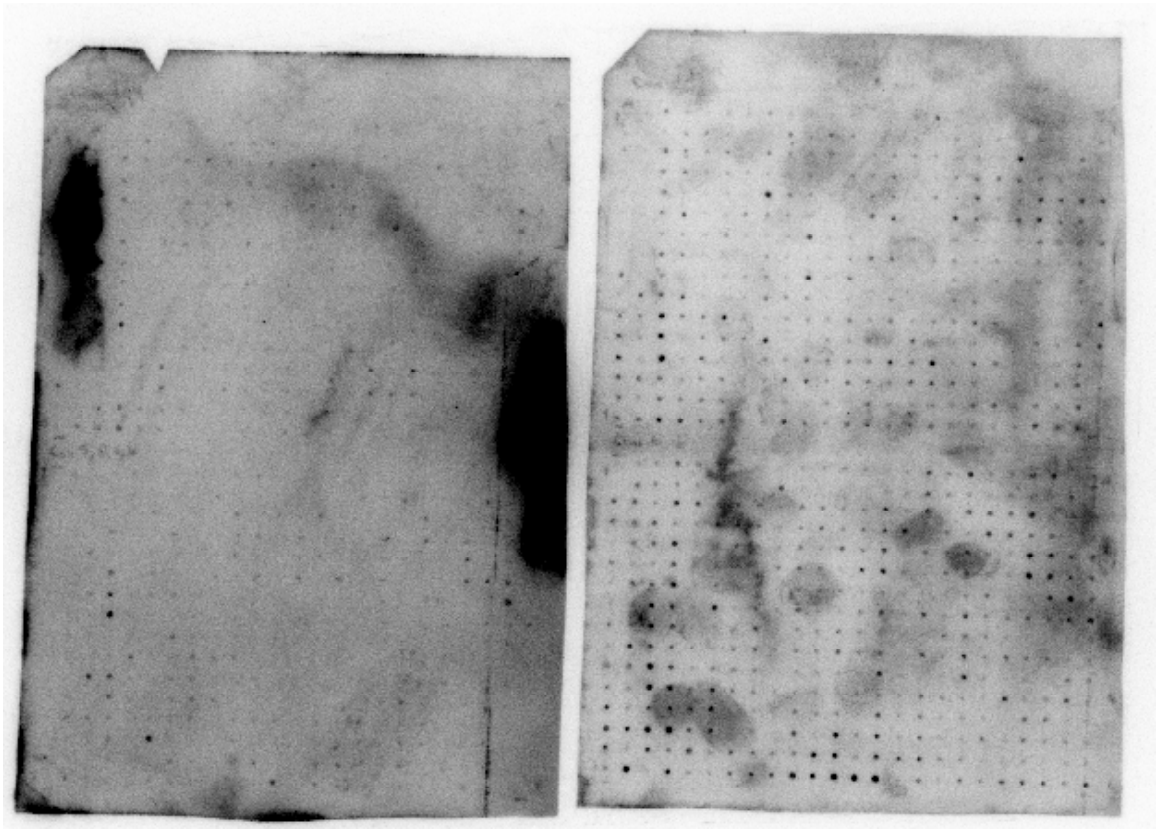


Fig. 13. First round macroarrays of Cavalier. RNA was pooled from days 8, 14, and 20. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane.

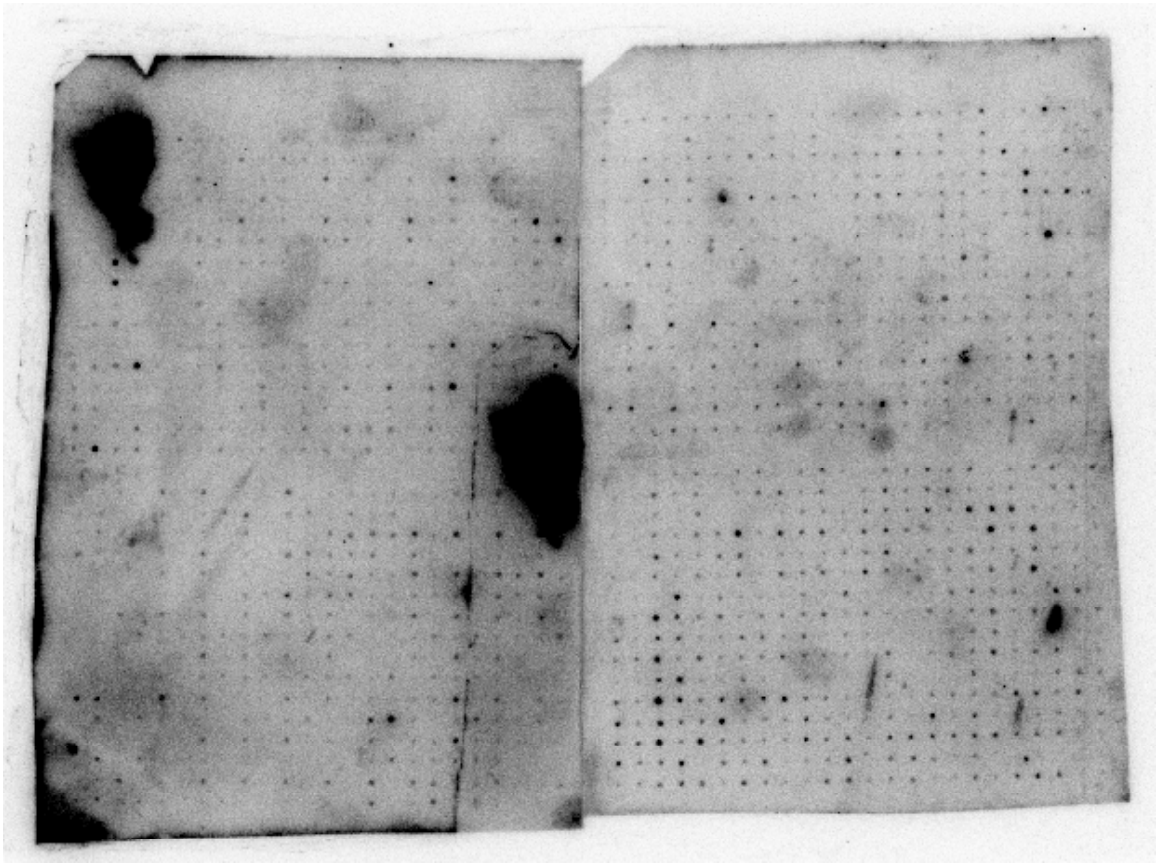


Fig. 14. First round macroarrays of Diamond. RNA was pooled from days 8, 14, and 20. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane.

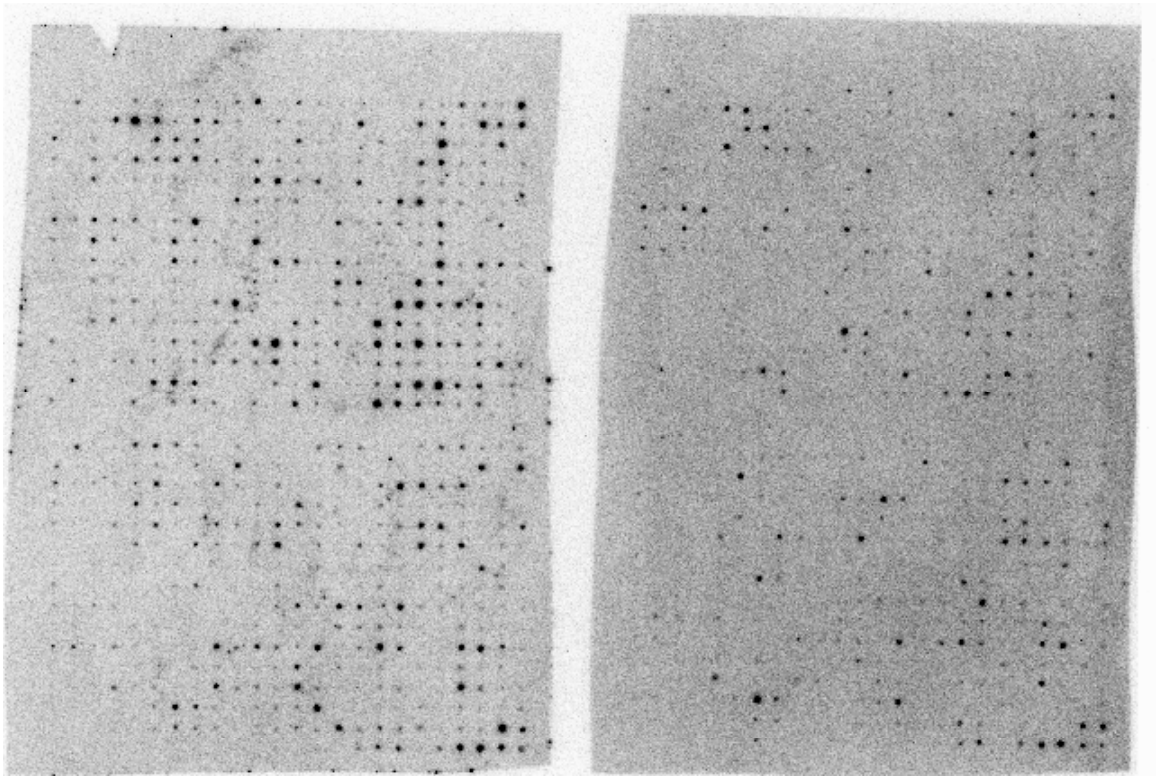


Fig. 15. First round macroarrays of Palisades. RNA was pooled from days 8, 14, and 20. Treatment RNA was hybridized to the left membrane and control RNA to the right membrane.

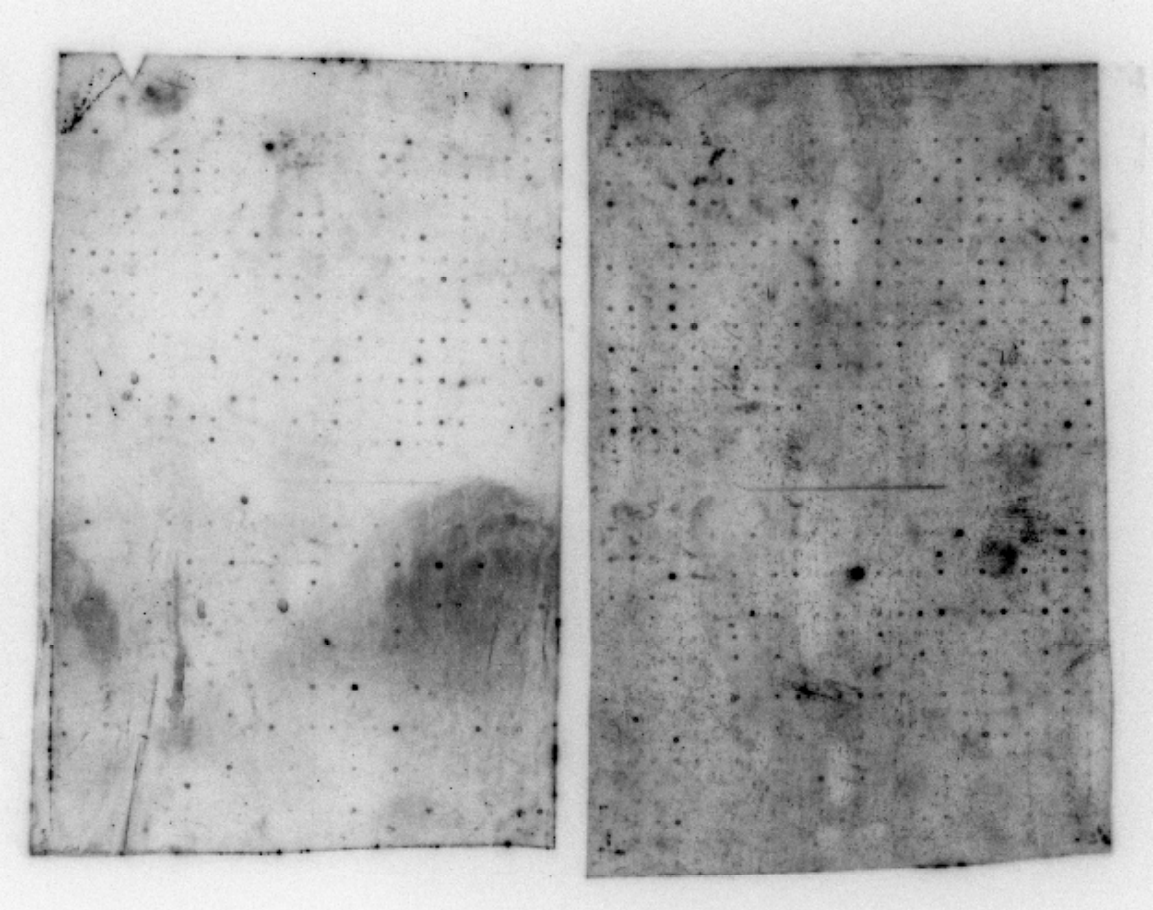


Fig. 16. First round macroarrays of DALZ 8504. RNA was pooled from days 8, 14, and 20. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane.

the Cavalier cDNA library and 78 clones from the Palisades cDNA library were chosen for the second round of macroarrays (Table 8). The 115 genes were divided into functional groups (Fig. 17). Eleven clones from Tables 3 and 4 that were similar among non-stressed and water stressed treatment membranes of each species, along with an actin clone and a histone H3 clone obtained from Dr. Scott Finlayson at Texas A&M University, were used as constitutive genes in the second round of macroarrays (Table 9).

Gene expression from the second round of macroarrays showed much less difference between non-stressed and water stressed treatment membranes than did the first round (Table 10). The plant tissue used in the first round had been exhausted, so tissue that was collected from a second set of tubs was used for the second round. Soil moistures were no different between the two sets of tubs (Fig. 18), and no visual differences in plant response between the tubs were observed (unpublished data). Mean gene expression for days 8, 14, and 20 of the 13 "constitutive" genes is shown in Table 11. Of the 13 genes, only six were similar among the three days. These six constitutive genes were used to normalize the data. Data was also normalized with scintillation counts from each probe (Table 12).

Normalized gene expressions of the 115 genes from the second round of arrays are shown in Tables 13-16 and Fig. 19-34. Median gene expression of all 115 genes for all cultivars are shown in Fig. 35-38. Of the 115 genes, some did not show significant quantitative differences between days or treatment ($P>0.05$). Forty-five, 39, 9, and 43 genes showed no significant quantitative differences between days or treatments for Cavalier, Diamond, Palisades, and DALZ 8504, respectively (Tables 13-16).

Table 8. Putative identity of clones used in the second round of macroarrays. Columns for species, accession and E value refer to the most homologous DNA sequence from the Blastx database. Genes 1 through 37 were from the Cavalier cDNA library and genes 38 through 115 were from the Palisades cDNA library.

Gene	Putative Sequence Identification	Function	Species	Accession	E value
1	Histidine kinase	transferase activity	*A. thaliana*	T00842	6.40E+00
2	Unknown	unknown	Rice	AAO72659	8.00E-46
3	Splicing factor 3b	spliceosome	Rice	BAD10377	1.00E-20
4	None	none			
5	Nuclear antigen EBNA1	miscellaneous	Human	NP_039875	8.00E-04
6	Hypothetical protein At2g05580	hypothetical	*A. thaliana*	C84470	1.00E-04
7	N-acetylglucosaminyltransferase	transferase activity	Bacteria	CAD90583	3.6
8	None	none			
9	Hypothetical protein	hypothetical	Bacteria	ZP_00327143	4.00E-11
10	None	none			
11	Photosystem I assembly protein Ycf4	photosynthesis	Corn	NP_043035	2.00E-49
12	Hypothetical protein At2g24100	hypothetical	*A. thaliana*	F84632	1.00E-14
13	Extensin	cell wall	Chickpea	CAA07235	5.00E-03
14	Manganese superoxide dismutase	oxidoreductase activity	Fungi	CAD42938	2.80E-02
15	Senescence-associated protein	senescence associated	Pea	BAB33421	3.00E-55
16	Unknown	unknown	Bacteria	NP_759470	6.00E-04
17	Allatotropin neuropeptide precursor	miscellaneous	Fall armyworm	CAD98809	3.90E-02
18	Systemin degrading enzyme	hydrolase activity	Tomato	CAC67408	6.00E-26
19	None	none			
20	Aspartate transaminase	transferase activity	Millet	S53303	6.00E-36
21	RuBP carboxylase; At1g67090	Calvin cycle	*A. thaliana*	AAG40356	8.00E-28
22	Homeotic gene regulator	DNA binding	*A. thaliana*	NP_187252	4.00E-32
23	Expressed protein; At2g42670	unknown	*A. thaliana*	NP_565980	4.00E-26
24	d-TDP-glucose dehydratase	lyase activity	Reed	CAC14890	2.00E-54
25	Hypothetical protein	hypothetical	Primrose	NP_084748	3.00E-13
26	Lyncein	miscellaneous	Rice	AAN64504	6.00E-57
27	ORF42f	unknown	Pine	NP_042478	1.00E-07
28	Hypothetical protein	hypothetical	Rice	BAD07869	2.00E-18

Table 8 (cont.)

Gene	Putative Sequence Identification	Function	Species	Accession	E value
29	Expressed protein; At1g54650	unknown	*A. thaliana*	NP_175866	3.00E-16
30	Chloroplast hypothetical protein	hypothetical	Corn	AAR91119	5.00E-30
31	Hydroxypyruvate reductase	oxidoreductase activity	Mangrove	BAB44155	2.00E-17
32	Expressed protein; At3g62580	hypothetical	*A. thaliana*	NP_567130	3.00E-42
33	Senescence-associated protein	senescence associated	Pear	AAR25995	8.00E-26
34	None	none			
35	Cytochrome P450 monooxygenase	oxidoreductase activity	Corn	T02955	4.00E-16
36	Integral membrane protein-like	miscellaneous	Rice	BAC20797	7.00E-08
37	Hypothetical protein Avar020175	hypothetical	Bacteria	ZP_00203429	2.00E-18
38	None	none			
39	GRounDhog, hedgehog-like (grd-11)	miscellaneous	Nematode	NP_507923	3.00E+00
40	Pyrroline-5-carboxylate reductase	oxidoreductase activity	Soybean	P17817	7.00E-23
41	phosphoinositide-specific phospholipase C	hydrolase activity	Rice	AAK01711	4.00E-06
42	S-adenosylmethionine synthetase 2	ATP binding	Tomato	P43281	1.00E-60
43	RING-H2 zinc finger protein	transcription factor	Rice	AAP85546	1.00E-28
44	Hypothetical protein APE1926	hypothetical	Bacteria	G72580	7.60E-01
45	Chaperone/heat shock protein	chaperone	Fungi	AAB69701	2.00E-05
46	Tetracycline transporter protein	transporter	*A. thaliana*	F84546	3.00E-04
47	Voltage-dependent anion channel	transporter	Rice	CAB82853	4.00E-42
48	Hypothetical protein	hypothetical	Rice	BAD07869	4.00E-20
49	None	none			
50	Protein kinase	transferase activity	Rice	AAO72572	2.00E-82
51	Lac z	none	vector	AAD31805	5.00E-32
52	Cellobiohydrolase II	hydrolase activity	Fungi	AAM76664	1.00E-10
53	Disease-resistant-related protein	miscellaneous	Rice	AAL78367	2.00E-38
54	Hyperosmotic protein 21	water stress	Salmon	AAK29182	4.00E-18
55	None	none			
56	Pyrroline-5-carboxylate reductase	oxidoreductase activity	*A. thaliana*	NP_196984	7.00E-22
57	Lac z	none	vector	AAR19394	4.00E-29
58	ADP-ribosylation factor	protein binding	Corn	P49076	5.00E-25

Table 8 (cont.)

Gene	Putative Sequence Identification	Function	Species	Accession	E value
59	None	none			
60	None	none			
61	Chloroplast hypothetical protein	hypothetical	Corn	AAR91119	5.00E-30
62	None	none			
63	None	none			
64	Phosphoenolpyruvate carboxykinase	lyase activity	Tomato	AAL37428	3.00E-17
65	Delta-1-pyrroline-5-carboxylate synthetase	oxidoreductase activity	Rice	O04226	7.00E-16
66	OSJNBa0027H09.17	unknown	Rice	CAE03817	1.00E-30
67	Lac z	none	vector	AAS78488	4.00E-11
68	20 kDa chaperonin	chaperone	Rice	BAD19442	8.00E-38
69	None	none			
70	Polyubiquitin	metabolism	Corn	1604470A	1.00E-79
71	bZIP protein HY5	transcription factor	Rice	BAD15505	2.00E-24
72	Unnamed protein product	unknown	Pufferfish	CAF96048	1.10E+00
73	Ubiquitin / ribosomal protein CEP52	metabolism	Tobacco	S28420	4.00E-18
74	GAP dehydrogenase	Calvin cycle	Barley	P26517	5.00E-06
75	phospholipase D1	hydrolase activity	*S. lepidophylla*	CAB43063	2.00E-21
76	Extensin precursor	cell wall	Tobacco	P13983	5.00E-07
77	Nidogen 2 protein	miscellaneous	Rat	XP_346115	7.00E+00
78	None	none			
79	Glycine-rich cell wall precursor	cell wall	Rice	BAC84049	2.00E-05
80	hydratase/isomerase family protein	lyase activity	*A. thaliana*	NP_193072	1.00E-52
81	None	none			
82	Unnamed protein product	unknown	Pufferfish	CAF99908	9.80E-02
83	Aquaporin TIP-type 1	aquaporin	Rice	P50156	2.00E-11
84	Lac z	none	vector	AAR19394	3.00E-43
85	Mitochondrial protein of unknown function	unknown	Yeast	NP_690845	7.00E-13
86	Enolase (2-phosphoglycerate dehydratase)	lyase activity	Rice	AAP94211	4.00E-09
87	None	none			
88	Oxidase	oxidoreductase activity	Rice	AAC35554	3.00E-14

Table 8 (cont.)

Gene	Putative Sequence Identification	Function	Species	Accession	E value
89	Inositol-3-phosphate synthase	isomerase activity	Iceplant	Q40271	4.00E-10
90	Coronatine-insensitive 1	stress signaling	Tomato	AAR82925	2.00E-15
91	None	none			
92	Ribosomal protein L17	hypothetical	Rice	BAD23438	4.00E-46
93	Senescence-associated protein	senescence	Pea	BAB33421	1.00E-25
94	Lac z	none	vector	AAA72534	9.50E-01
95	None	none			
96	GAMYB-binding protein	transcription factor	Barley	AAO25543	6.00E-84
97	OJ1414_E05.5	unknown	Rice	NP_916175	6.00E-47
98	Leucine-rich repeat resistance protein	resistance	Cotton	AAK70805	2.00E-15
99	Protein F20B24.8	unknown	*A. thaliana*	H86239	3.20E+00
100	Senescence/dehydration-associated protein	senescence	*A. thaliana*	NP_567995	4.40E-02
101	Phosphoenolpyruvate carboxylase	Calvin cycle	Rhodes grass	AAG42288	4.00E-46
102	Unknown	unknown	Bacteria	NP_759470	2.60E-02
103	COG0038: Chloride channel protein EriC	channel	Bacteria	ZP_00286186	2.10E+00
104	None	none			
105	Hypothetical protein	hypothetical	Primrose	NP_084748	1.50E-02
106	Photosystem I P700 apoprotein A2	Photosynthesis	Rice	AAS46120	4.00E-27
107	None	none			
108	Hypothetical protein AN9258.2	hypothetical	Fungi	XP_413395	8.20E-01
109	Cytochrome P450 monooxygenase	oxidoreductase activity	Corn	AAL66766	6.00E-42
110	Protein phosphatase 2C	hydrolase activity	Beech	CAB90634	7.00E-19
111	Elongation factor 1 beta	protein synthesis	Barley	CAB90214	1.00E-19
112	None	none			
113	Heat shock protein 12p	chaperone	Yeast	NP_116640	4.00E-08
114	Bet v I allergen family protein	miscellaneous	*A. thaliana*	NP_173813	5.00E-09
115	Cysteine protease	hydrolase	Barley	CAD66657	3.00E-09

Table 9. Putatively identified clones that appeared to be constitutively expressed in the first round of macroarrays. The actin and histone H3 clones (genes 127 and 128) were not used until the second round of macroarrays.

Gene	Putative Sequence Identity
116	vector
117	ribosomal protein L28-like
118	conserved protein
119	OSJNBa0028I23.20
120	vector
121	hypothetical protein
122	NONE
123	NONE
124	NONE
125	vector
126	NONE

Table 10. Quantified gene expression for the first and second rounds of macroarrays. Values for the first round are from the mean of 768 clones. Due to a small number of astronomically high numbers that drastically altered the means, the values for the second round are the median of four time-points of 115 clones.

	First Round		Second Round	
Cultivar	Control (PSL)	Treatment (PSL)	Control (PSL)	Treatment (PSL)
Cavalier	57	214	122	137
Diamond	61	223	87	85
Palisades	42	153	74	121
DALZ 8504	82	532	111	110

Table 11. Gene expression (PSL) of 13 theoretically constitutive genes. Only six genes showed non-significant differences between days (bolded genes). The six non-significant genes were used for normalization.

		Gene Expression		
Gene	P value	Day 8	Day 14	Day 20
116	0.0119	238	215	128
117	0.0006	424	224	191
118	**0.2226**	**76**	**63**	**50**
119	**0.1953**	**79**	**59**	**42**
120	0.0067	352	293	800
121	**0.9206**	**81**	**74**	**73**
122	0.0064	485	355	203
123	0.0077	474	461	2085
124	**0.1089**	**189**	**198**	**116**
125	0.0056	484	716	2032
126	0.0121	443	332	1175
127	**0.1073**	**106**	**58**	**50**
128	**0.1415**	**525**	**462**	**292**

Table 12. Scintillation counts for each of the ^{32}P incorporated mRNA probes from the second round of macroarrays.

	Day 8		Day 14		Day 20	
Cultivar	Control	Treatment	Control	Treatment	Control	Treatment
Cavalier	447472	550821	460481	523377	426675	277286
Diamond	788371	774675	577163	679439	500000*	460703
Palisades	669572	566528	769337	848980	446419	365534
DALZ 8504	660349	523966	532758	1138715	459594	485870

* Actual scintillation count was 198,289, but hand held scintillation counts and the membrane itself indicated that the control and treatment membranes contained similar amounts of radioactivity.

Table 13. Gene expression of 115 genes from control and treatment tissue of Cavalier on days 2, 8, 14, and 20. P values are for treatment (Trtmt), Day, and their interaction (Int). The Data column indicates data was normal (norm) or normalized by square root (srt) or log (log) transformations. The standard error is also shown (SE) with * indicating that the SE of one or more values varied from the value shown due to deleted outliers.

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
1	norm	none	0.535	0.0734	0.3902	383	246	321	132	381	328	231	260	66
2	norm	none	0.3344	0.5351	0.3129	79	60	116	109	69	89	95	41	25
3	srt	none	0.3908	0.8108	0.6963	7.2	7.8	9.1	7.4	7.7	11.2	7.9	10	2.1
4	log	none	0.2241	0.1365	0.1763	2.19	2.46	2.44	2.3	2.48	2.75	2.2	2.43	0.13
5	log	none	0.0239	0.0123	0.0056	1.48	2.46	2.31	2.22	2.45	2.5	2.38	2.13	0.15
6	log	none	0.0481	0.0008	0.0033	1.77	2.11	2.09	2.36	2.03	2.64	2.17	2.06	0.1
7	norm	1	0.0021	<.0001	0.0027	117	95	51	51	131	306	59	78	26 *
8	norm	none	0.1887	0.0004	<.0001	72	201	142	97	408	121	71	45	35
9	norm	2	0.0003	0.0007	0.0314	65	186	118	221	158	516	246	235	44 *
10	norm	none	0.2618	<.0001	0.0009	723	561	425	356	1446	515	325	135	110
11	norm	none	0.1789	0.0179	0.3112	985	863	602	647	822	695	754	366	117
12	log	none	0.912	0.0007	0.2193	1.08	1.76	1.14	1.9	0.56	2.24	1.48	1.68	0.26
13	log	none	0.9011	0.0139	0.0029	2.04	0.96	1.89	0.85	2.28	1.63	0.06	1.64	0.35
14	srt	none	0.0106	0.0022	0.0056	10.9	12.5	12.7	13.7	9.9	25.4	16.4	13.3	2
15	norm	none	0.8836	0.5706	0.8997	231	305	183	333	261	287	212	258	81
16	norm	none	0.6693	0.0445	0.085	26	96	170	262	70	186	128	113	47
17	norm	none	0.0828	0.4459	0.0485	98	175	229	154	437	263	172	148	71
18	srt	none	0.6639	0.0033	0.0845	5.6	10.4	13.4	14.9	6	18.3	10.9	12	2.2
19	log	none	0.1922	0.1884	0.8067	2.66	2.75	2.51	2.66	2.74	2.97	2.68	2.66	0.12
20	srt	none	0.3608	0.7954	0.9949	18.8	21.1	19.7	21	17	19.6	18.6	18.6	2.6
21	norm	none	0.1231	0.4856	0.1274	1098	1247	654	777	723	696	870	733	167
22	log	none	0.0213	0.4042	0.4517	0.48	1.04	1.2	0.24	1.14	1.19	1.74	1.67	0.4
23	norm	none	0.0248	0.2724	0.01	55	52	47	139	182	79	141	76	27
24	srt	none	0.6548	0.1104	0.0583	11.9	9.3	7	13.8	5.3	10.1	14.6	15.3	2.4
25	log	none	0.9748	0.014	0.2075	2.11	2.18	1.58	0.41	2.56	1.33	1.17	1.25	0.43
26	srt	none	0.9598	0.0421	0.0993	8.8	17.9	14.3	22.7	11.8	18.7	20	13.5	3

Table 13 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
27	srt	none	0.0031	0.8029	0.6723	8.1	7.6	6.8	10.2	14.7	19.6	14.3	14.2	3.2
28	srt	none	0.0003	0.153	0.0101	8.5	6.3	7.1	12.1	13.9	16.5	10.1	11.5	1.5
29	norm	2	0.0075	<.0001	0.0627	62	156	108	176	23	292	188	268	31 *
30	srt	none	0.2029	0.4184	0.2456	8.1	13.7	14	15.5	15.2	14.7	14.5	14.5	2.1
31	norm	2	<.0001	<.0001	<.0001	35	68	85	109	42	515	115	114	17 *
32	srt	none	0.802	0.3497	0.2342	12.5	6.8	7.3	10.3	7.5	7.7	11.3	12.2	2.2
33	norm	none	0.07	0.5509	0.682	18	14	24	44	35	60	38	54	16
34	srt	none	0.0246	0.5219	0.0445	9.5	11.7	11.7	11.2	17.9	11	13.1	12.9	1.6
35	norm	none	0.029	0.4995	0.1731	59	78	141	101	227	101	135	179	40
36	log	none	0.2189	0.0018	0.9488	0.5	1.47	1.42	1.87	0.72	1.92	1.77	1.98	0.32
37	srt	none	0.0043	0.0201	0.0198	11.7	15.1	4.8	8.2	16.8	14.2	16.7	9.7	2
38	norm	none	0.0217	0.4248	0.0123	236	371	407	184	170	157	181	309	55
39	srt	none	0.013	0.0004	0.656	24.7	28.4	29.3	18.1	23	23.9	22.3	15.1	2.1
40	srt	none	0.7411	0.0065	0.0547	3.6	12.2	11.7	4.5	5.4	10	6.2	8.7	1.8
41	norm	none	0.0562	0.1494	0.0449	149	286	280	111	36	147	102	233	54
42	norm	none	0.3674	0.0017	0.0484	205	152	207	74	269	127	64	85	35
43	srt	none	0.1312	0.0022	0.1529	2.8	8.7	9.5	6.8	4.8	14.6	7	9.2	1.8
44	norm	none	0.0824	0.0471	0.3527	52	144	199	109	45	101	87	103	33
45	norm	none	0.0051	0.7855	0.7882	5	17	15	21	50	43	32	55	14
46	srt	1	0.0491	0.0296	0.8027	11.9	14.3	8.8	16.5	13.1	17.7	14.4	19.3	2.2 *
47	norm	none	0.9952	0.0356	0.6812	18	39	68	48	1	66	62	44	19
48	norm	4	<.0001	0.0006	0.0001	39	88	41	95	201	250	101	92	15 *
49	log	none	0.4089	0.8251	0.2332	1.75	0.46	1.56	0.84	1.35	1.92	1.17	1.38	0.51
50	srt	none	0.4382	0.1224	0.7394	3	9.5	6	7.4	3.5	6	5.7	6.5	1.9
51	srt	none	0.5545	<.0001	0.1608	10.4	13.1	14.2	18.2	8.2	11.5	17.6	21.3	1.6
52	norm	none	0.9411	0.2463	0.5388	23	62	26	28	7	42	28	58	19
53	log	2	0.003	0.0363	0.0091	2.9	2.56	2.95	2.6	2.67	2.59	2.41	2.59	0.07 *
54	norm	none	0.5149	0.5996	0.2104	25	59	97	57	50	70	31	45	22
55	norm	2	0.0027	<.0001	<.0001	2547	1159	1442	533	4093	211	219	135	101 *
56	log	none	0.7377	<.0001	0.017	0.54	1.46	1.87	1.11	0.17	2.09	1.14	1.82	0.25

Table 13 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
57	norm	none	0.2508	0.4623	0.0079	62	131	159	83	87	58	36	159	29
58	srt	3	0.0776	0.3601	0.11	8.6	8.8	16.3	10.3	10.7	8.1	7.9	7.7	1.6 *
59	norm	none	0.0147	0.0302	0.0002	78	117	281	81	88	71	52	136	28
60	norm	none	0.2153	0.0018	0.3182	123	94	140	326	76	265	183	364	57
61	norm	none	0.723	0.0553	0.1273	270	402	458	121	580	184	407	190	108
62	log	none	0.4706	0.4759	0.6947	2.49	2.69	2.62	2.59	2.55	2.6	2.49	2.59	0.08
63	srt	none	0.4207	0.0039	0.0713	11.5	15.1	14.6	14	11.1	22.7	14	11.7	1.9
64	srt	none	0.0252	0.0003	0.0149	32.8	38.6	42.5	24.1	35.5	30.5	30.2	25.1	2.5
65	log	none	0.5353	0.0646	0.9512	0.98	1.92	1.48	1.39	0.63	2.01	1.18	1.23	0.41
66	srt	none	0.4946	0.0965	0.1003	1.7	6.7	9.9	4.4	4.9	9.1	4.8	7.6	1.9
67	srt	none	0.9888	0.0166	0.0289	8	4.9	10	2.7	10.3	4.7	4.3	6.2	1.5
68	srt	none	0.0237	0.0094	0.0128	2.6	7.8	7.9	4.4	2.1	3.3	3.6	6.5	1.1
69	srt	none	0.0102	0.0571	0.2073	14.8	14.7	15.9	10	14.7	10	7.1	7.6	2
70	norm	none	0.0351	<.0001	0.0341	419	204	382	228	459	166	157	181	43
71	srt	none	0.0485	0.0034	0.9026	1.2	7.4	4	5.8	3.8	9.8	6.7	6.6	1.5
72	srt	none	0.0436	0.9477	0.6411	8.7	9.6	10.6	9.6	8	5.5	4.6	7.5	2.2
73	norm	none	0.72	0.4724	0.1554	287	646	670	423	482	440	428	557	116
74	norm	none	0.0015	0.8585	0.3431	492	717	645	506	404	286	208	310	114
75	srt	none	0.2223	0.118	0.0418	13.8	18.1	18.9	17.3	15.1	13.3	12.3	20.7	1.9
76	srt	none	0.4652	0.2788	0.445	20.5	19.4	22	20.8	21.2	14.9	18	23.3	2.6
77	srt	none	0.1884	0.0591	0.812	2.5	7.4	7.7	5.4	5.1	9.7	7.3	7.4	1.7
78	norm	none	0.877	0.0492	0.0537	25	39	101	115	39	102	60	69	21
79	srt	none	0.0054	0.8903	0.1395	5.8	7.2	8.1	8	12.6	11.4	8.8	8.9	1.5
80	srt	none	0.5786	0.1433	0.6786	6.2	7.2	9.3	6.8	4.3	8.5	7.8	6.7	1.4
81	srt	none	<.0001	0.0003	0.004	6.7	6.7	8.9	6	6.7	1	5.4	4.1	0.7
82	norm	none	0.4809	0.6558	0.4417	44	61	59	46	47	57	53	85	16
83	srt	2	0.2643	0.0042	0.0983	4.8	10.5	13.3	9.6	7.1	8.5	8.9	9.6	1.2 *
84	srt	none	0.7857	0.019	0.3516	1.6	7.5	6.9	10.2	5.2	6.3	4.7	8.7	1.8
85	srt	3	0.7952	<.0001	<.0001	10.8	5.8	10	7	20	4.3	0.7	9.6	1.3 *
86	log	none	0.0388	0.4615	0.1973	2.28	2.22	2.3	2	2.19	2.4	2.49	2.45	0.12

Table 13 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
87	srt	none	0.2409	0.0049	0.8834	6.7	13.8	10.4	11.5	8.6	13.6	12.2	13.3	1.6
88	norm	2	0.142	0.0375	0.7017	169	230	165	223	179	334	181	269	38 *
89	srt	1	0.5884	0.0113	0.0093	4.2	8.2	6.2	9.4	8.5	0.7	9.6	11.7	1.7 *
90	norm	2	0.0002	<.0001	0.001	4	87	98	114	24	301	105	155	21 *
91	norm	none	0.6983	0.0438	0.0542	224	444	497	331	241	361	338	653	87
92	norm	none	0.9112	0.123	0.6509	321	215	263	292	274	191	235	372	55
93	srt	none	0.043	0.0029	0.2912	3.3	14.2	12.4	13.4	11	17.3	11.1	17.6	2.3
94	norm	none	0.67	0.0003	0.6771	58	441	302	380	141	379	289	455	69
95	srt	none	0.6256	0.0004	0.0118	5	13	13.9	11.9	9.5	13.7	9.1	13.3	1.3
96	srt	none	0.8121	0.0417	0.1771	3.9	7.7	9	11.6	9	7.4	5.1	12.1	1.9
97	srt	2	0.3609	<.0001	0.0011	17.3	8.9	6.8	8.5	14.7	4.9	17.1	8.9	1.5 *
98	srt	none	0.1672	0.2816	0.2267	4.2	4.6	4.8	9.5	5.6	8.1	9	7.1	1.7
99	norm	none	0.6268	0.4058	0.4111	87	97	82	147	46	125	110	88	32
100	srt	none	0.2779	<.0001	0.0914	19	9	6.9	10.2	13.7	12.3	5.4	8.7	1.6
101	srt	none	0.7424	0.0236	0.8274	37.3	25	19.1	22.1	34.1	30.7	21.9	21.7	5
102	norm	none	0.012	0.0001	<.0001	107	83	107	159	353	182	86	40	27
103	norm	none	0.0471	0.0422	0.0124	200	128	142	183	178	295	121	236	30
104	norm	4	0.9317	0.0746	0.2142	16	60	31	47	32	-	32	27	10
105	srt	none	0.1383	0.0002	0.001	10.9	7	6.6	11.7	16.5	14.8	4.5	7.1	1.5
106	log	none	<.0001	<.0001	<.0001	3.25	3.17	3.1	3.02	3.76	2.89	2.42	2.15	0.08
107	norm	none	0.0039	0.002	0.4467	643	765	503	629	412	725	159	406	93
108	srt	none	0.49	0.3506	0.0181	4.5	4.2	8.1	8.4	7.2	7.2	0.7	6.7	1.7
109	srt	none	0.0728	0.0077	0.7113	8.4	2.6	4	0.9	9.4	3.6	6.9	5.3	1.7
110	srt	none	0.2125	0.9698	0.5167	8.7	7	8.3	7.5	8.1	10	8.4	10.5	1.5
111	norm	none	0.9364	0.0507	0.3518	261	75	99	175	178	131	140	151	42
112	norm	none	0.1137	0.032	0.2144	13	101	53	64	102	147	50	53	26
113	srt	2	0.1353	<.0001	0.1506	16.1	10.4	0.8	9.9	17.7	13.8	6.8	7.2	1.8 *
114	srt	none	0.223	0.0762	0.5491	9.1	6.6	4.4	9.8	12.6	7.2	8	8.8	1.9
115	norm	none	0.2559	0.9257	0.7416	231	354	327	375	475	435	337	416	114

Table 14. Gene expression of 115 genes from control and treatment tissue of Diamond on days 2, 8, 14, and 20. P values are for treatment (Trtmt), Day, and their interaction (Int). The Data column indicates data was normal (norm) or normalized by square root (srt) or log (log) transformations. The standard error is also shown (SE) with * indicating that the SE of one or more values varied from the value shown due to deleted outliers.

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
1	srt	4	0.0708	<.0001	0.0009	26.4	13.2	11	8.3	21.9	10.3	11.3	11	0.7 *
2	log	none	0.0711	0.0128	0.3406	1.8	1.4	1.15	1.12	1.79	1.41	1.52	1.53	0.15
3	norm	1	0.0276	<.0001	0.0036	122	31	28	28	57	35	29	26	9 *
4	log	none	0.167	<.0001	<.0001	2.36	2.28	2.02	2.25	2.59	1.96	2.09	2.08	0.05
5	norm	none	0.0023	0.0004	<.0001	145	264	221	126	370	218	229	167	24
6	norm	none	0.4208	0.4581	0.1588	74	88	86	84	116	42	68	60	19
7	srt	none	<.0001	<.0001	<.0001	15.2	8.1	8.2	7.1	7.2	6.1	6	7.6	0.7
8	norm	none	0.0458	<.0001	<.0001	170	188	63	223	346	53	87	47	18
9	norm	none	0.9083	0.0176	0.0055	86	109	46	121	189	41	66	58	25
10	norm	none	0.0073	<.0001	<.0001	756	1298	387	759	2623	445	1080	199	138
11	norm	none	0.7112	0.4368	0.0271	1176	1039	533	628	772	612	1055	758	170
12	srt	none	0.5849	0.034	0.2119	4.8	5.2	3.3	3.8	8.1	4	3	3.6	1.1
13	log	none	0.4954	<.0001	0.0348	2.37	1.73	1.28	1.61	2.19	1.46	1.54	1.63	0.09
14	srt	none	0.1738	0.0874	0.7963	11.8	8.7	9.9	7.7	10	8.2	7.4	7.5	1.3
15	norm	6	0.0031	<.0001	0.0115	263	90	77	139	205	58	97	101	10 *
16	norm	none	0.5533	0.8551	0.0152	61	92	45	117	78	63	95	49	18
17	norm	none	0.9244	0.0012	0.0038	195	202	81	201	385	98	129	78	43
18	norm	none	0.498	0.0321	0.2097	39	73	51	54	13	50	76	52	13
19	norm	none	0.9832	0.2626	0.0395	459	507	233	227	401	264	326	440	78
20	norm	none	0.3107	0.2734	0.3306	333	417	237	249	326	359	320	383	55
21	norm	none	0.4262	0.1689	0.0804	779	956	564	421	777	427	684	522	136
22	log	none	0.8033	0.058	0.0063	1.29	1.56	1.25	1.22	1.95	0.73	0.91	1.58	0.21
23	norm	none	0.9491	0.0101	0.0218	130	83	61	128	181	49	123	44	25
24	log	none	0.5299	0.0772	0.0302	2.24	2.2	2.09	2.14	2.25	1.88	2.23	2.17	0.08
25	srt	none	0.0139	<.0001	0.0022	18.2	13.8	8.9	15.4	17.9	9.2	11.7	8.5	1.2
26	norm	none	0.3115	0.5578	0.8433	155	120	88	146	114	86	100	112	33

Table 14 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
27	norm	none	0.7831	0.3461	0.002	93	96	103	56	57	73	95	134	14
28	srt	none	0.0996	<.0001	0.5972	11.8	4.8	6.1	7.9	9.3	5	4.6	7	1
29	log	none	0.6516	0.055	0.0693	1.86	1.96	2.11	1.93	1.75	1.82	1.89	2.26	0.11
30	srt	none	0.5457	0.1571	0.0003	9.5	13.1	9.5	12.8	14.4	8.6	10.1	10.1	1
31	log	none	0.0217	0.2193	0.014	1.28	1.76	1.6	1.28	1.7	1.47	1.84	1.75	0.12
32	log	none	0.3639	0.1208	0.5735	1.83	2.09	2.01	1.98	1.91	2.05	2.25	1.98	0.11
33	srt	none	0.1244	0.0974	0.0144	2.7	4.2	3.9	6.4	7.5	2.7	5.8	5.7	1
34	norm	none	0.3939	<.0001	<.0001	126	136	73	180	318	65	101	80	20
35	log	1	0.4139	0.0005	0.0331	2.15	1.77	1.67	1.87	2.21	1.5	2.12	1.89	0.11 *
36	norm	none	0.0144	0.4735	0.0558	13	40	28	29	58	27	72	42	12
37	log	none	0.0424	<.0001	0.2117	2.31	1.94	1.64	2.07	2.07	1.69	1.71	1.97	0.08
38	log	none	0.4954	0.0173	0.0355	2.58	2.28	2.18	2.18	2.31	2.16	2.14	2.44	0.09
39	norm	none	0.3708	<.0001	<.0001	1600	559	453	155	704	522	604	733	80
40	norm	1	0.1676	0.0706	0.0032	51	38	40	31	19	34	58	97	12 *
41	norm	none	0.0634	0.1193	0.0005	201	191	311	140	83	171	130	276	33
42	norm	none	0.9067	0.1026	0.2378	205	141	176	97	212	94	122	179	36
43	log	none	0.1815	0.3306	0.6737	0.78	0.7	0.74	1.16	0.65	1.09	1.49	1.56	0.36
44	srt	none	0.3443	0.0011	0.0237	8.4	8.5	10.2	7.4	5.3	7.7	15.1	10.1	1.3
45	norm	none	0.0605	0.4077	0.7836	16	19	31	26	10	14	14	18	7
46	norm	none	0.9053	0.4891	0.0078	207	200	186	138	188	124	171	239	24
47	srt	none	0.355	0.3482	0.9573	7.3	4.8	6.5	5.1	8	5.1	7.7	7.1	1.6
48	log	none	0.9196	0.0001	0.864	2.19	1.13	1	1.82	2.28	1.15	1.08	1.55	0.24
49	srt	none	0.0432	0.067	0.0038	9.9	6.6	3.6	5.5	3.9	5.5	5	5.5	0.9
50	log	none	0.0018	0.2451	0.0106	1.67	0.9	0.95	1.02	0.27	0.81	0.28	1.04	0.21
51	srt	none	0.2826	0.012	0.0009	12.8	12	12.1	11.5	7	11.2	10.5	16.4	1.1
52	log	none	0.191	0.4458	0.6663	0.97	0.58	0.36	0.79	0.34	0.39	0.32	0.68	0.26
53	srt	none	0.0414	0.0238	0.0053	32.3	21	25	20.4	21.2	19.1	20.8	25.3	2
54	norm	none	0.8935	<.0001	0.2704	72	26	26	17	97	22	9	18	10
55	log	none	0.1608	<.0001	<.0001	3.45	3.33	2.95	3.06	3.7	3.06	3.53	2.72	0.06
56	log	none	0.723	0.0193	0.4265	0.32	1.11	1.51	1.16	0.74	0.54	1.6	1.58	0.34

Table 14 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
57	norm	none	0.7804	0.0333	0.0268	90	111	101	95	61	86	92	173	19
58	norm	none	0.0653	0.9726	0.3251	82	86	67	105	119	123	152	90	26
59	srt	none	0.8731	0.3191	0.0634	11.3	8.5	10.4	8.2	9.2	7.8	8.4	12.4	1.3
60	log	none	0.4541	0.3421	0.4482	2.17	1.93	2.19	2.11	2.07	2.15	2.24	2.15	0.1
61	log	none	0.0154	<.0001	0.2833	2.69	2.24	1.96	2.38	2.66	1.96	1.91	2.11	0.08
62	norm	none	0.5592	0.0002	0.0101	450	134	66	144	213	146	71	279	51
63	norm	4	0.235	0.0213	0.0243	144	55	-	59	74	52	71	83	16
64	srt	none	0.0272	<.0001	0.0061	47.4	28	28.2	18.5	43.3	30	31.5	37.3	3
65	log	none	0.2028	0.0273	0.2901	1.49	1.46	1.99	1.49	0.77	1.5	1.76	1.56	0.23
66	log	none	0.3808	0.3299	0.0158	1.48	1.03	1.29	1.15	0.34	1.26	1.22	1.53	0.24
67	srt	3	0.0104	0.0025	0.0419	9.6	4.9	6.1	5.9	8.8	5.2	12	9.8	1 *
68	norm	5	0.4236	<.0001	<.0001	53	28	27	28	48	21	69	10	5 *
69	norm	none	0.6832	0.0609	<.0001	238	432	141	254	322	170	421	95	48
70	norm	none	0.4096	<.0001	0.0159	411	162	146	189	457	71	293	170	35
71	srt	none	0.7869	0.0354	0.0847	2.9	4.5	6.8	6.4	4	5.7	3.5	6.7	1
72	norm	none	0.0271	<.0001	0.1555	161	35	42	48	170	49	125	63	18
73	norm	none	0.0031	<.0001	<.0001	740	250	234	153	379	203	209	242	37
74	log	none	0.8866	0.0002	0.024	2.86	2.44	2.41	2.27	2.74	2.46	2.18	2.64	0.1
75	log	none	0.8176	0.0635	0.0179	2.44	2.27	2.4	2.18	2.42	2.15	2.18	2.48	0.08
76	srt	none	0.0129	<.0001	<.0001	28.8	13.8	14.8	10.4	19.8	12.5	11.6	15.7	1.1
77	log	none	0.7812	0.2563	0.1476	1.54	0.89	0.62	1.24	0.88	1.23	0.91	1.08	0.23
78	norm	none	0.0098	0.0025	0.0191	25	17	16	17	86	11	29	29	10
79	srt	2	0.0237	0.0093	0.3808	7.4	5.1	5.8	5.9	10.8	5.6	8.9	6.5	1 *
80	srt	none	0.0019	<.0001	<.0001	6.5	6.3	6	5.7	7.9	6.1	19.2	4	1.3
81	norm	none	0.044	0.0026	0.6787	89	35	35	36	98	64	69	44	13
82	srt	none	0.0243	0.0412	0.5269	8	6.3	5.3	4	8.6	7	7.4	7	0.9
83	norm	none	0.9775	0.113	0.1522	29	79	69	88	65	62	70	69	13
84	srt	none	0.1395	0.2495	0.4952	4.3	2.4	4.7	4.2	7.9	4.6	5.4	3.7	1.4
85	srt	none	0.0367	0.0002	0.0245	16.9	4.2	5.6	8.6	8.9	6.4	5.1	4.7	1.6
86	norm	none	0.2804	0.0453	0.3818	142	87	111	86	189	107	77	136	26

Table 14 (cont.)

			P value			Control				Treatment				
Gene	Data	Outlier	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
87	norm	none	0.5371	<.0001	0.0322	86	29	48	14	63	31	33	36	7
88	log	none	0.2176	<.0001	0.0003	2.39	1.67	1.65	1.27	2.3	1.56	1.6	1.77	0.07
89	norm	none	0.7692	0.0009	0.2305	78	24	70	27	75	23	43	50	12
90	log	none	0.5053	0.8175	0.4156	1.33	1.14	1.21	0.94	0.84	1.34	0.82	1.11	0.26
91	log	none	0.0485	0.0385	0.0184	2.41	2.23	2.25	2.15	2.48	2.09	2.36	2.69	0.1
92	log	none	0.0048	<.0001	0.0083	2.54	2.14	2.26	1.87	2.52	2.18	2.41	2.37	0.08
93	log	none	0.1801	0.963	0.6517	1.87	1.82	1.93	1.87	2	2.09	1.86	2.05	0.13
94	log	none	0.439	0.4026	0.0022	2.17	2.24	2.41	2.13	2.25	2.21	2.18	2.46	0.07
95	norm	none	0.0016	0.1111	0.0696	66	93	94	64	97	87	134	131	13
96	norm	3	<.0001	0.0001	0.0004	32	25	49	35	171	31	73	54	10 *
97	srt	none	0.2496	<.0001	0.1027	15	4.7	3.4	5.4	12	6.8	7.7	6.7	1.4
98	norm	9	0.0392	0.0002	0.001	36	21	3	21	-	21	24	16	3 *
99	norm	none	0.156	0.7025	0.3118	81	93	67	60	40	62	68	68	15
100	log	none	0.6932	<.0001	0.009	2.06	1.63	1.95	1.92	2.37	1.6	1.83	1.67	0.08
101	srt	none	0.0002	<.0001	0.0202	30.7	30.2	26.6	21.4	45.8	30.4	31.9	27.9	2.2
102	norm	none	0.8749	<.0001	<.0001	97	162	63	265	371	79	110	37	19
103	log	none	0.1463	0.0009	0.3267	2.36	2.23	1.89	2.01	2.45	2.13	2.04	2.26	0.09
104	log	none	0.6088	0.0001	<.0001	0.04	1.33	0.67	1.62	1.97	0.72	0.28	0.89	0.14
105	log	none	0.1005	<.0001	0.0206	2.35	1.87	1.8	2.28	2.5	1.85	1.7	1.69	0.11
106	log	none	0.2153	<.0001	<.0001	3.45	3.46	2.95	3.25	3.71	3.23	3.26	2.62	0.08
107	srt	none	0.4036	0.0079	0.0803	21.8	23.1	18.9	20.2	26.8	20.4	17.7	22.7	1.6
108	srt	none	0.8354	0.0401	0.473	4.1	3	4.1	5.6	5.2	3.1	2	7.3	1.3
109	srt	none	0.4835	0.1189	0.447	6.8	4.9	5.9	7.8	4	5.9	5.1	7.9	1.2
110	norm	3	0.4879	<.0001	<.0001	57	140	29	69	73	77	72	56	7 *
111	srt	none	0.7119	0.0312	0.2685	11.4	9.9	10.3	9.9	13	8.3	9.6	11.6	1
112	norm	none	0.9464	0.1031	0.5412	29	46	75	75	48	52	78	50	15
113	norm	none	0.0197	0.0005	0.0026	157	185	58	136	346	138	159	113	31
114	norm	none	0.4631	0.1799	0.1093	97	137	109	134	107	123	166	115	16
115	norm	none	0.0053	0.2137	0.2115	190	199	210	238	466	223	288	383	60

Table 15. Gene expression of 115 genes from control and treatment tissue of Palisades on days 2, 8, 14, and 20. P values are for treatment (Trtmt), Day, and their interaction (Int). The Data column indicates data was normal (norm), normalized by square root (srt) or log (log) transformations, or non-normal (non). The standard error is also shown (SE) with * indicating that the SE of one or more values varied from the value shown due to deleted outliers.

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
1	srt	none	<.0001	<.0001	0.019	18.7	12.8	6.8	18.5	37.1	37	20.6	27.9	2.2
2	srt	none	<.0001	<.0001	0.0001	5.4	4.8	4.2	7.3	16.7	25.6	3.7	13.1	2
3	log	none	0.1963	0.0057	0.1393	1.2	1.68	1.31	0.77	1.33	2.86	0.79	1.33	0.36
4	norm	1	<.0001	0.0026	<.0001	196	77	83	330	511	832	221	192	72 *
5	norm	none	0.0014	<.0001	<.0001	72	150	287	199	106	851	229	26	49
6	srt	none	<.0001	<.0001	<.0001	7.1	5.8	7.1	6.9	11.1	26.7	6.4	3.9	1.4
7	srt	none	0.0001	0.0087	0.0005	6.3	4.4	5.3	9.3	18.3	22	6.4	7.2	2.2
8	srt	2	0.0232	<.0001	<.0001	13.5	7.7	6	2.9	2.3	25.9	8.7	2.1	1.2 *
9	norm	3	<.0001	<.0001	<.0001	43	82	62	1	114	552	70	431	36 *
10	srt	none	0.0363	0.0001	0.0194	18.5	23.8	15.4	13.1	7.9	21.2	21.4	3.1	2.8
11	norm	none	0.1612	<.0001	0.1206	373	660	837	366	417	981	1089	191	108
12	srt	none	<.0001	<.0001	<.0001	3.4	4.5	6.7	1.8	2.8	34.3	3	1.7	1.3
13	srt	none	<.0001	0.0004	<.0001	10.3	6.6	5	1.6	6.7	27.2	7	18.4	2.2
14	srt	none	0.0109	0.0017	0.0029	9.4	7.6	9.9	2.7	11.6	21.3	5.5	7.8	2.1
15	log	none	0.0003	0.155	0.0294	2.1	2.18	2.29	2.37	2.73	2.86	2.23	2.7	0.13
16	srt	none	0.0026	0.0118	0.0049	4.5	5.8	9.7	4.6	6.5	23.7	7.5	11.8	2.6
17	srt	none	0.4358	<.0001	<.0001	11.1	10.5	14	6.4	4.2	25	12.1	5.1	2
18	log	none	0.4398	0.0001	0.0037	0.34	1.59	1.88	0.74	0.79	2.84	0.61	0.98	0.31
19	norm	none	0.0226	<.0001	<.0001	179	372	572	471	539	1019	450	107	76
20	norm	none	0.0493	<.0001	<.0001	382	493	612	523	649	942	695	126	68
21	srt	none	<.0001	0.0004	<.0001	22.4	27.2	27.8	26.5	50	39.2	24.7	28.3	2.3
22	srt	1	0.0175	<.0001	<.0001	1.7	5.8	10.1	4.2	3.8	22.2	1.7	2.3	1.1
23	log	none	0.1689	0.079	0.7887	1.94	1.84	1.87	1.11	1.16	1.55	1.88	0.52	0.41
24	srt	2	0.0001	<.0001	0.0003	10	12.2	11.3	9	19.9	36.5	9.9	9	2.4
25	norm	none	<.0001	<.0001	<.0001	350	133	62	29	173	1048	189	421	80
26	srt	none	0.9197	0.0373	0.0017	8.4	8.7	14.1	14.4	7.3	25	8.5	5.7	3

Table 15 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
27	srt	none	<.0001	<.0001	<.0001	5	11.5	10.9	6.8	20.8	24.9	5.7	23.5	1.7
28	norm	2	<.0001	<.0001	<.0001	80	17	67	60	161	514	60	299	32 *
29	norm	2	<.0001	0.0045	0.0003	18	131	285	156	448	694	211	922	77 *
30	srt	none	0.0027	<.0001	<.0001	8.5	9.8	11.3	9.8	14.7	24.9	8.3	1.7	1.1
31	log	none	0.1942	0.0018	0.0015	1.33	1.47	1.59	1.55	0.66	2.75	0.71	0.77	0.28
32	norm	none	0.0006	<.0001	<.0001	27	152	99	308	16	742	199	35	37
33	srt	none	0.0002	<.0001	<.0001	1.5	3.9	7.1	9	5.8	29.9	1.7	4.2	1.7
34	srt	2	0.0022	<.0001	<.0001	12.7	8.6	8.7	10.9	13.8	25.8	10.3	1.7	1 *
35	srt	none	<.0001	<.0001	<.0001	5.6	6.2	7.5	3.6	12.9	40.6	6	9.6	3
36	srt	2	0.0002	<.0001	<.0001	2.8	6.6	4.4	6.9	8.6	32.7	1.1	1.7	1.7 *
37	srt	none	0.0003	0.0001	<.0001	14.9	10.2	10.6	4.1	14.6	30.8	5.6	17.2	2.4
38	srt	none	<.0001	0.0001	<.0001	11.4	15.3	20.8	17.9	46	31.4	14.7	21.4	2.1
39	norm	none	<.0001	<.0001	<.0001	1133	744	1083	924	4004	1693	1091	1415	223
40	srt	none	0.0004	<.0001	0.0009	3.5	6.7	7	11.7	6.6	25.1	5.6	17.1	2.2
41	srt	none	<.0001	0.0006	<.0001	7	12.4	15.9	19.1	32.4	25.6	10.6	24.9	1.8
42	norm	none	0.0643	<.0001	<.0001	131	145	77	156	24	595	106	3	40
43	log	none	0.714	0.0025	0.0004	0.3	0.94	0.165	1.63	0.5	2.67	0.04	0.94	0.34
44	srt	none	<.0001	<.0001	<.0001	4.4	6.6	9.1	7.3	15.2	27.7	4.9	6.7	1.9
45	log	none	0.0245	0.0007	0.0006	0	0.93	1.5	0.93	0.85	2.96	0.04	1.59	0.37
46	srt	3	<.0001	0.0003	<.0001	7.5	12.9	16.8	17.2	33.7	36.4	14	10.2	2 *
47	srt	none	0.0004	<.0001	<.0001	3.2	6.6	11.3	5.2	6.4	31.2	1.1	11.45	2.1
48	srt	none	0.0096	<.0001	<.0001	10.6	9	3.5	5.3	8.8	32.4	3.3	1.7	2.3
49	log	none	0.1539	0.0222	0.12	1.62	1.65	1.48	1.28	1.64	2.94	1.39	1.37	0.31
50	log	none	0.0887	0.0054	0.0206	1.38	1.12	0.37	1.51	1.69	2.68	0.87	0.8	0.33
51	norm	none	<.0001	0.0143	<.0001	48	214	368	292	760	864	215	609	70
52	log	1	0.0176	0.1343	0.0048	0	0.28	0.8	1.09	1.3	2.73	0.47	0.64	0.41 *
53	norm	2	0.0013	0.2385	<.0001	429	557	1052	567	907	1060	620	943	84 *
54	srt	2	<.0001	<.0001	<.0001	4.8	6.1	5.1	1	7.7	22.5	4.1	1.7	1.3 *
55	norm	2	<.0001	<.0001	<.0001	3227	1296	681	392	508	942	931	281	95 *
56	srt	none	0.0003	<.0001	<.0001	3.7	4.3	7.1	5.6	5.1	24.9	4.9	3.9	1.5

Table 15 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
57	srt	5	<.0001	<.0001	<.0001	5.4	8.6	11.3	15.8	25.3	33.4	10.3	1.7	0.8 *
58	norm	2	<.0001	<.0001	<.0001	55	80	119	120	14	1061	68	3	36 *
59	srt	none	<.0001	<.0001	<.0001	5.9	9.2	9.6	6.3	21.2	37.7	5.9	13.9	2.5
60	srt	none	<.0001	<.0001	<.0001	8.1	13.8	11.1	3.5	26.6	41.5	15	10.8	2.1
61	norm	2	0.0063	<.0001	0.1364	498	157	112	187	609	503	129	267	61 *
62	norm	2	<.0001	0.0052	<.0001	262	267	365	294	662	696	272	614	44 *
63	norm	none	0.0043	<.0001	<.0001	132	224	163	28	82	569	118	107	37
64	norm	none	<.0001	<.0001	<.0001	1666	1159	2017	1292	5144	1682	2296	1308	239
65	log	none	0.0174	0.0007	0.2113	0.14	1.43	0.88	1.46	0.98	2.94	0.81	1.89	0.37
66	log	none	0.4549	0.0097	0.0077	0.95	1.04	1.01	1.63	0.99	2.81	0.41	1.16	0.35
67	srt	2	0.0193	0.0917	0.0293	7.7	7.1	9.3	6	11.9	19.1	6.2	8.7	2.1 *
68	srt	none	0.0251	0.087	0.1274	3.4	4.3	4.2	6.7	4.1	18.1	6.5	9.6	2.9
69	srt	5	0.1518	<.0001	<.0001	26.8	14.8	9	6.5	3	27.9	13.5	-	1.7 *
70	srt	none	0.6139	<.0001	<.0001	14.7	12.2	17.1	7.1	4.7	31.2	16.3	1.7	2
71	log	none	0.0015	<.0001	0.018	0.74	1.29	1.58	0.34	1.51	3.18	1.48	0.76	0.3
72	srt	1	0.0009	<.0001	0.002	8	7.8	5.5	1	14.7	29.6	3.1	4.4	2.7 *
73	srt	none	0.0004	0.0008	0.0003	17	15.6	14.3	20	41.6	38.4	15.6	13.3	3.6
74	norm	2	<.0001	0.0005	<.0001	586	476	329	896	1470	1762	682	507	128 *
75	norm	none	<.0001	0.0003	<.0001	95	315	236	408	1448	956	404	502	91
76	norm	none	<.0001	<.0001	<.0001	221	130	213	517	3126	854	273	744	68
77	log	none	0.0946	<.0001	0.0073	0.64	1.3	0.95	2.31	1.2	2.79	0.42	2.17	0.28
78	log	none	0.0265	0.0056	0.0064	0.85	0.93	0.81	2.15	1.1	3.13	1.17	1.69	0.35
79	log	none	0.4632	0.0136	0.0081	1.23	1.56	1.8	1.49	1.48	3.25	0.79	1.31	0.36
80	log	none	0.8427	0.8664	0.1677	1.42	1.27	1.21	1.81	1.21	1.7	1.91	0.65	0.43
81	srt	none	0.2086	0.8194	0.3062	8.4	4.3	6.8	4.8	6.8	13.1	4.8	13.6	3.9
82	srt	none	0.0009	0.0008	<.0001	6.3	4.5	7.4	6.1	3.5	30.5	8.2	11.4	2.7
83	non	10+	<.0001	<.0001	<.0001	14	54	39	47	88	1195	13	3	80
84	non	10+	0.0005	0.0002	0.0002	10	26	61	1	152	847	1	100	88
85	log	none	0.6555	0.2545	0.1938	2.32	0.74	2.15	1.1	2.18	2.31	1.34	1.18	0.55
86	log	none	0.0274	0.0025	0.0106	1.89	1.77	0.9	1.8	2.55	3.25	1.83	0.92	0.33

Table 15 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
87	srt	none	<.0001	<.0001	<.0001	6.1	6.4	9.4	10	18.2	26.5	3.1	6.1	1.5
88	norm	2	<.0001	<.0001	<.0001	139	49	123	44	734	354	8	71	26 *
89	srt	none	0.3958	0.0002	<.0001	5.4	5.2	8.2	10.2	4	22.9	2	4.8	1.9
90	log	none	0.0038	0.0004	<.0001	0.88	0.7	1.15	1.32	0.82	3.22	0.36	2.36	0.3
91	log	none	<.0001	0.0059	<.0001	1.85	1.96	2.31	2.1	3.05	3.36	1.9	2.38	0.15
92	srt	none	<.0001	<.0001	<.0001	14.4	10.4	13	14.7	22	69.9	14.1	15.3	3.4
93	log	none	0.0018	0.0087	0.005	0.98	1.05	1.28	0.02	1.67	3.39	0.56	1.71	0.4
94	srt	3	<.0001	0.004	<.0001	5.1	12.9	13.2	11.8	31.7	35.1	6.5	23.8	2.8 *
95	non	10+	0.1163	0.4488	0.0939	38	51	60	204	311	522	125	3	132
96	srt	3	0.0006	<.0001	<.0001	6.9	7	9	1	6.2	30.6	3.6	7.3	1.9 *
97	srt	none	0.0888	<.0001	0.0006	11.7	6.4	1.4	16.2	21.6	1.1	11.1	12.3	2.1
98	log	none	0.0813	0.0084	0.0468	1.11	1.02	1.88	1.67	1.48	0.09	0.67	1.82	0.31
99	srt	none	0.0021	0.0018	0.1564	8	10	12.2	11.7	2.5	6.3	6.3	12.2	1.5
100	srt	3	0.0005	<.0001	0.0029	11.4	8.1	5.9	14.4	11.7	25.5	7.7	16.1	1.6 *
101	norm	none	0.7863	0.0641	0.7946	1106	1051	1269	589	951	746	1498	604	278
102	srt	none	0.0012	0.0114	0.0002	11.2	7.9	7.8	8.2	4.8	16.7	11.9	19.8	1.7
103	srt	none	<.0001	0.0012	<.0001	11.5	9.2	15.4	13.3	33.1	22.2	7.9	23.8	2.4
104	log	none	0.6906	0.0778	0.0263	1.32	1.04	0.81	0.73	0.57	1.51	0.04	2.24	0.41
105	srt	none	0.0013	<.0001	0.0013	7.6	6.5	9.5	10.4	14.3	8.2	6.7	25.4	2
106	log	none	0.0455	0.0007	0.0231	3.36	3.18	2.97	2.58	2.77	2.86	3.18	2.54	0.13
107	srt	2	0.8951	<.0001	<.0001	12	22.2	21.2	19.9	34.5	1.1	22.6	17.6	1.4 *
108	non	10+	0.4883	0.5353	0.29	7	34	25	516	178	1	40	3	182
109	norm	5	<.0001	0.0004	0.0005	0	63	1	-	3	1	9	3	6 *
110	norm	none	0.0066	0.0641	0.0094	61	71	83	118	102	10	35	45	17
111	norm	none	0.8985	0.001	0.0015	175	214	151	225	345	82	96	255	35
112	norm	2	0.5513	<.0001	<.0001	17	51	56	162	143	1	26	86	16
113	srt	none	0.2429	0.646	0.6682	11.7	11.7	14.1	10.1	12.5	10	8.7	7.9	2.5
114	srt	none	0.0236	0.0033	0.1636	4.9	8.2	6.1	9.5	5.7	17.5	7	12.7	2.1
115	log	none	0.0173	0.9607	0.2415	2.01	2.3	2.38	2.2	2.63	2.45	2.37	2.62	0.16

Table 16. Gene expression of 115 genes from control and treatment tissue of DALZ 8504 on days 2, 8, 14, and 20. P values are for treatment (Trtmt), Day, and their interaction (Int). The Data column indicates data was normal (norm), normalized by square root (srt) or log (log) transformations, or non-normal (non). The standard error is also shown (SE) with * indicating that the SE of one or more values varied from the value shown due to deleted outliers.

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
1	srt	none	0.0887	<.0001	0.037	32.5	6.8	18	31		20.9	19.4	28.9	3.1
2	srt	none	0.3602	0.1293	0.4157	11.9	4	7.1	12.5		10.7	6.7	12.7	2.9
3	norm	4	<.0001	0.0009	0.0286	80	12	52	83		2	14	15	9 *
4	log	none	0.0181	<.0001	<.0001	2.74	1.18	2.12	4.08		2.24	2.12	3.69	0.11
5	srt	1	0.5135	0.5219	0.0015	8.9	7	18	9.1		18.2	8.5	11.4	2.4 *
6	srt	none	0.0059	0.3204	0.9939	6.7	3.3	3.2	2		8.4	8.1	6.7	1.9
7	log	none	0.2752	0.0007	0.7879	2.21	0.98	1.94	2.06		0.73	1.89	1.65	0.26
8	log	4	0.9635	0.0007	0.0514	1.96	1.3	0.52	0.8		0.8	0.62	1.17	0.16 *
9	log	none	0.0534	0.223	0.429	2.04	0.62	1.04	1.39		1.75	2.04	1.46	0.44
10	srt	none	0.0799	0.0002	<.0001	11.7	20.4	34.6	13.4		33	8.6	13.4	3
11	srt	none	0.0071	0.0264	<.0001	23.1	14.5	29.1	43.7		25.6	23.8	14.7	3.2
12	log	none	0.2293	0.0972	0.0148	0.75	2.22	1.46	0.62		0.72	1.41	1.17	0.33
13	log	none	0.8785	0.0745	0.3224	1.91	1.26	1.84	1.62		0.85	1.74	2.27	0.35
14	norm	4	0.9353	<.0001	0.2407	114	39	156	34		2	160	64	16 *
15	log	none	0.6388	0.048	0.0605	2.46	1.7	2.15	1.65		0.78	1.85	2.46	0.35
16	log	5	0.0002	0.007	<.0001	0.75	1.21	1.79	0.84		0.31	0.4	1.37	0.13 *
17	log	none	0.8347	0.0059	0.1503	2	0.96	1.8	1.83		0.79	1.28	2.7	0.36
18	log	none	0.1336	0.8918	0.1928	1.16	0.7	1.16	0.93		1.68	0.94	1.4	0.32
19	srt	none	0.5417	0.0105	0.0116	27.2	11.8	28.4	25.1		23.9	26.6	19.1	2.8
20	norm	3	0.0075	0.0003	0.0835	521	107	217	268		315	208	569	63 *
21	srt	none	0.0941	<.0001	0.0208	46.1	17.1	27.6	77.6		21.8	25.6	54.4	4.8
22	log	none	0.0995	0.3628	0.0287	0.3	0.37	0.9	1.09		1.85	0.66	1.17	0.31
23	srt	none	0.2162	0.2037	0.4612	7.4	4.8	7.8	4.7		9.9	11	3.9	2.4
24	srt	2	0.0023	<.0001	<.0001	15.7	3.4	17.7	66.2		14.3	7	38.8	2.9 *
25	norm	none	0.2089	0.6856	0.3643	121	30	59	129		113	184	98	55
26	srt	none	0.04	0.0061	0.1593	7.8	7.2	15.8	5.4		3.8	8.5	5.8	1.9

Table 16 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
27	srt	5	<.0001	<.0001	<.0001	20.9	5.4	12.5	92.1		12.2	6.4	46.5	2.5 *
28	norm	none	0.8773	0.4092	0.2541	108	7	46	89		76	37	42	34
29	log	none	<.0001	<.0001	0.0071	2.46	1.46	2.56	3.78		0.31	2.35	3.19	0.13
30	srt	1	0.0102	<.0001	0.0324	12.9	4.7	14.1	46.8		6.4	7.7	33.7	2.5
31	log	none	0.1227	0.4477	0.0487	1.06	1.01	1.76	1.63		1.37	0.44	1.33	0.32
32	srt	1	0.0127	0.004	<.0001	4.5	4.8	14.5	3.2		7.8	2	4.5	1.2 *
33	log	none	0.0127	0.2564	0.1144	0.9	0.1	1.13	1.21		1.95	1.02	2.56	0.46
34	log	none	0.3958	0.0209	0.0748	1.51	1.21	1.84	2.56		1.81	1.06	2.1	0.3
35	norm	none	0.4625	0.4504	0.0103	44	5	102	226		247	85	103	55
36	srt	4	0.0013	<.0001	<.0001	1.4	18.1	3.2	3.5		2.2	7.6	3.9	1 *
37	log	none	0.6073	0.3324	0.0328	2.24	1.42	1.91	2.29		2.09	1.51	1.72	0.24
38	srt	none	0.0291	<.0001	0.0045	35.9	7.7	23.3	131.8		10.5	23.1	100.1	5.1
39	log	none	0.035	<.0001	0.0002	3.5	2.28	2.97	3.2		2.85	2.89	3.12	0.07
40	srt	none	0.0444	0.6192	0.0454	5.9	3.5	7.9	7.3		5.1	3.5	3.9	1.2
41	log	none	0.0433	<.0001	<.0001	2.84	1.44	2.5	3.57		2.2	2.52	3.29	0.09
42	log	none	0.3698	0.3407	0.161	1.48	1.11	2.44	1.7		1.45	1.38	1.61	0.36
43	log	none	0.8502	0.0162	0.3093	1.15	0.58	1.5	1.89		0.79	0.71	2.28	0.4
44	log	none	0.021	0.0186	0.6448	2.06	1.01	1.96	2.03		0.68	1.42	1.17	0.28
45	log	none	0.9243	0.0812	0.0416	0.51	0.45	1.19	2.01		1.82	0.36	1.57	0.43
46	srt	none	0.0199	<.0001	<.0001	27.9	5.3	17.3	99.5		14.6	17.1	69.2	3.4
47	non	10+	0.7273	0.2035	0.2899	5	167	48	159		467	16	15	143
48	srt	none	0.0611	0.3098	0.0442	2.8	11.5	7.7	4.6		1.4	4.3	6.9	2.3
49	srt	4	<.0001	<.0001	0.0002	8	5	8.7	13.4		1.4	3.4	3.9	0.6 *
50	srt	none	0.3021	0.0597	<.0001	6.9	2.4	5.6	13		17.6	4.2	3.9	1.8
51	srt	none	<.0001	<.0001	0.0001	26.1	8.3	20.6	73.1		7.4	11.3	41.5	3
52	log	none	0.0553	0.0022	0.2142	0.59	0.05	0.52	1.14		0.31	1.35	1.17	0.23
53	norm	3	0.0006	<.0001	0.3998	568	130	470	382		25	254	134	52 *
54	srt	none	0.3706	0.1043	0.0314	7.2	1.5	7.1	3.3		4	3.1	8.4	1.6
55	norm	1	<.0001	<.0001	<.0001	434	596	1546	448		2718	662	798	123 *
56	log	none	0.3293	0.0103	0.859	1.78	0.37	0.91	1.79		0.86	0.99	2.26	0.42

Table 16 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
57	srt	4	<.0001	<.0001	<.0001	17	2.9	11.7	18.6		37.3	9.3	4.6	1 *
58	srt	none	0.8378	0.29	0.0124	2.9	6.3	7.6	4.1		5.3	2.8	10.6	1.8
59	norm	none	0.2065	<.0001	0.3449	3009	38	136	4		12	95	15	18
60	norm	none	0.8848	0.0072	0.3395	523	296	310	128		455	229	15	99
61	norm	none	0.1335	0.1007	0.0553	364	47	177	187		367	176	156	75
62	norm	1	0.0623	<.0001	<.0001	649	73	345	401		350	243	15	42 *
63	srt	2	0.5318	0.0163	<.0001	7.6	5	10.3	6.3		13.8	5.7	3.9	1.1 *
64	log	none	0.2124	<.0001	<.0001	3.58	2.8	3.26	3.45		3.13	3.01	3.23	0.04
65	log	none	0.7641	0.3889	0.002	1.14	0.46	1.67	1.71		2.15	0.36	1.61	0.37
66	log	none	0.8222	0.9319	0.0917	1.31	0.59	1.66	1.46		1.67	1.07	1.17	0.39
67	log	none	0.2581	0.2387	0.0103	1.92	0.85	1.93	2.38		1.84	0.8	1.55	0.34
68	srt	none	0.107	0.0005	0.0022	3.5	6.1	6.6	8.7		6.7	1.5	23.5	2.5
69	log	3	0.0054	0.097	0.0023	0.92	2.19	2	1.16		0.31	0.95	1.8	0.27 *
70	log	none	0.0062	0.4304	0.6486	1.34	2.08	2	1.88		0.8	1.32	1.17	0.36
71	non	10+	0.0721	0.077	0.0372	28	92	13	690		50	68	15	143
72	srt	none	0.7406	0.1462	0.8136	4.6	10.2	6.5	5.2		11.8	8.6	3.8	2.8
73	srt	none	0.6055	<.0001	0.3526	38	7.8	22.6	25.6		11.5	18.6	22.2	2.9
74	srt	none	0.7435	0.0545	<.0001	30.7	9.7	26.6	33		31.9	19.1	20.8	3
75	log	none	0.0499	<.0001	<.0001	2.73	1.81	2.81	3.97		2.65	2.53	3.86	0.09
76	norm	3	<.0001	<.0001	<.0001	1579	60	512	13377		418	414	8927	139 *
77	non	10+	0.5319	0.2145	0.0239	3	18	36	102		179	3	36	41
78	log	none	0.1141	0.9475	0.4249	1.04	1.14	1.37	1.04		1.89	1.31	2.04	0.42
79	log	none	0.0858	0.0147	0.0199	0.62	1.17	0.98	2.46		2.41	1.72	1.85	0.31
80	srt	none	0.0003	<.0001	<.0001	6.7	5.2	7.6	4.6		26.2	1.5	9.1	1.8
81	log	none	0.1141	0.3934	0.274	2.07	1.57	1.4	1.18		2.1	1.31	2.67	0.48
82	srt	none	0.7842	0.1321	0.4046	3.1	7.1	4.6	16.3		10.2	8.3	11.8	3.3
83	non	10+	0.6663	0.3408	0.1158	12	41	86	21		97	2	15	32
84	log	none	0.1293	0.2359	0.7435	0.74	1.65	1.15	1.98		1.01	0.98	1.17	0.42
85	srt	none	<.0001	0.0221	0.0074	9.2	1.1	5.1	11		22	9.7	19.3	2.4
86	log	none	0.157	0.0927	0.5996	2.41	1.42	1.93	2.54		1.35	1.57	1.81	0.33

Table 16 (cont.)

			P value			Control				Treatment				
Gene	Data	Outliers	Trtmt	Day	Int	Day 2	Day 8	Day 14	Day 20	Day 2	Day 8	Day 14	Day 20	SE
87	srt	3	0.5218	0.0233	0.2436	8.8	4.2	8.7	6.6		1.4	9.7	12	2.1 *
88	norm	2	0.0021	<.0001	0.883	437	13	81	504		155	183	631	40 *
89	srt	none	0.8658	0.1687	0.2433	2.6	3.6	9.3	12.2		9.6	7.3	9.5	2.8
90	log	none	0.0255	0.0648	0.0578	0.3	0.81	1.27	1.42		1.98	2.03	1.17	0.29
91	srt	none	0.0427	<.0001	0.0015	26.2	4.1	20.6	45.5		14	9.1	30.2	3.2
92	norm	1	0.0745	0.0008	0.0002	332	18	188	788		239	185	214	75 *
93	srt	none	0.2831	0.5591	0.0523	5	2.8	8.4	14.8		21.7	6.1	11.2	4.8
94	srt	4	<.0001	<.0001	<.0001	25.4	6.9	16.4	155		9.7	14.2	91	3.1 *
95	srt	none	0.0049	0.4965	0.0085	11	5.8	16.3	19.7		9.9	4.8	3.9	3
96	log	none	0.9533	0.2043	0.1328	0.43	0.98	1.37	1.43		1.86	0.77	1.22	0.36
97	srt	none	0.0015	0.0021	0.0029	20.4	9.4	10.5	23.5		10.5	5.9	8.2	2.1
98	log	1	0.0222	0.0022	0.3456	1.02	0.6	1.33	2.19		0.31	0.98	1.17	0.27 *
99	srt	none	0.9328	0.1192	<.0001	6.4	3.6	10.8	17.3		16.9	6.3	8.1	2.2
100	srt	none	0.4103	<.0001	0.073	12.3	3.5	5.4	38.8		9.4	18.1	29.7	4.6
101	srt	none	0.2883	0.2243	0.0008	34.9	23.2	35.2	23.8		30.6	16.6	26.8	3.1
102	srt	none	0.807	0.0004	0.5907	5.4	4.7	6.8	22.1		3.9	11.5	20.2	3.4
103	srt	none	0.5171	0.0036	0.0557	30.5	4.7	21.4	23		13.4	9.5	19.7	4
104	log	3	0.3556	0.1163	0.199	0.3	0.54	1.16	1.04		0.56	0.36	1.17	0.26 *
105	log	none	0.3526	0.6374	0.3307	0.93	0.38	1.26	1.87		1.63	1.68	1.47	0.54
106	srt	none	0.0367	<.0001	<.0001	22.4	31.9	41.9	20.7		51.8	24.4	6	2.2
107	srt	none	0.1475	<.0001	0.0223	37.6	12.9	26.2	87.3		21.5	12.6	77.9	3.9
108	log	none	0.5914	0.9168	0.9696	1.14	0.92	1.11	1.1		1.04	1.25	1.41	0.42
109	log	none	0.304	0.1829	0.0527	1.86	1.82	1.2	2.51		1.15	1.85	1.72	0.31
110	srt	none	0.0247	0.2042	0.0004	10.6	7.2	8.2	20.6		12.2	6.3	3.9	2.3
111	srt	3	0.1406	0.0044	0.0004	15.2	8.2	15.5	21.8		15.2	7.8	15.8	1.6 *
112	srt	none	0.0228	0.4153	0.0062	9.6	5	7.3	10.1		18.4	13.3	5.6	2.5
113	srt	9	0.8968	<.0001	<.0001	12	4.7	7.1			10.5	1.5	21.1	0.8 *
114	srt	none	0.0004	0.0125	0.2554	2.7	4.7	10.5	2		8.7	18	13.9	2.3
115	srt	none	0.8981	0.0312	0.0323	24.6	6	19	25.1		18	11.7	19.2	3.8

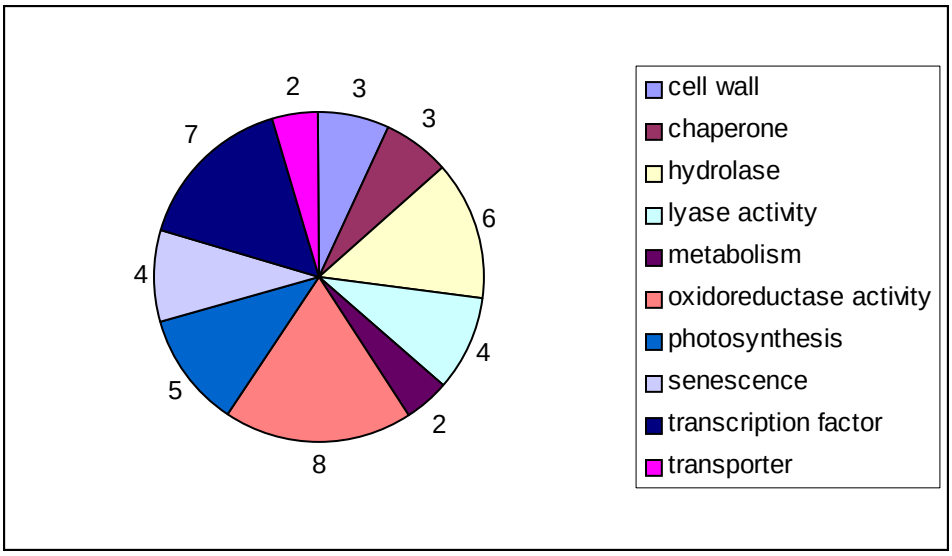


Fig. 17. Major functional groups found in second round of macroarrays. Seventy one genes were not shown as their identities were unknown, hypothetical, or were only present once.

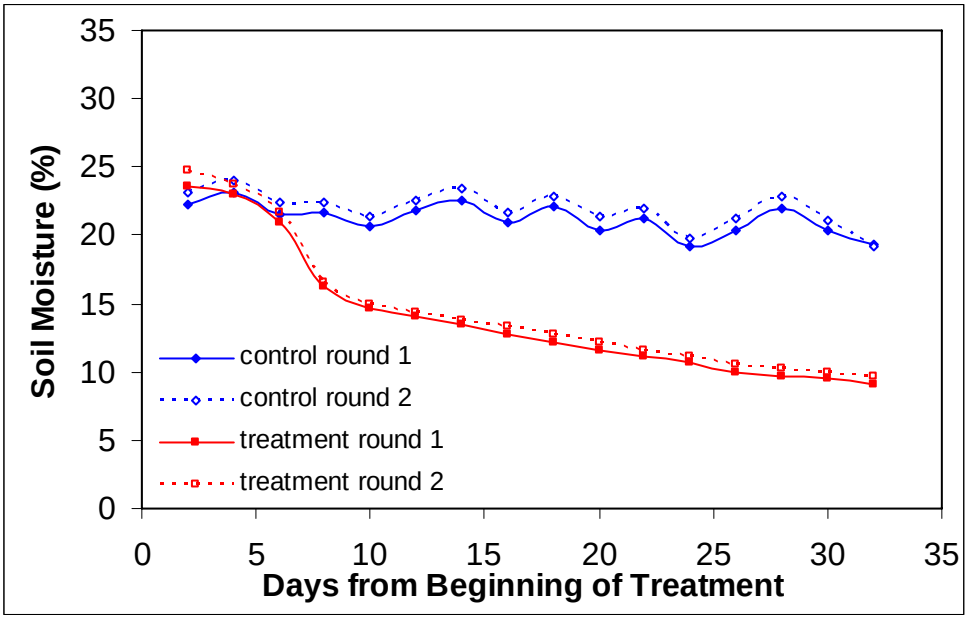


Fig. 18. Soil moistures from which tissues used in the first and second round of macroarrays were taken. Soil moisture for treatments (squares) and controls (diamonds) of tubs used for the cDNA library syntheses and first round of macroarrays (solid lines) and the tubs used for the second round of macroarrays (dashed lines).

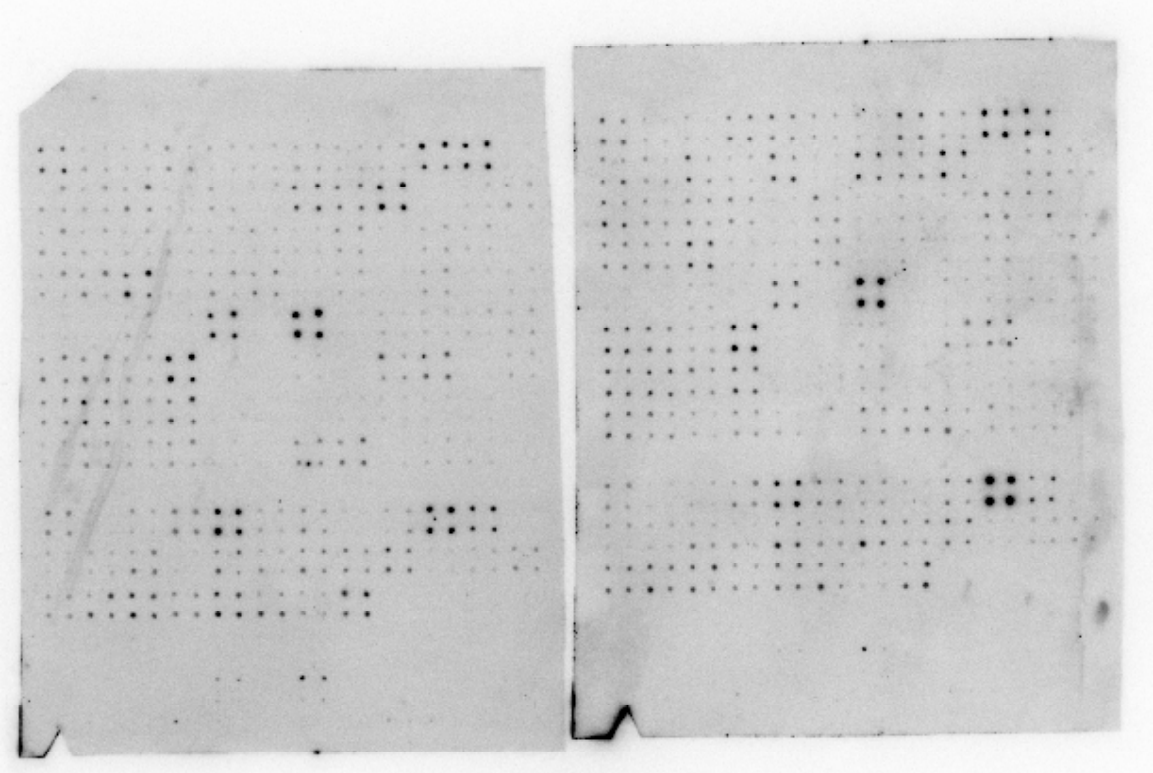


Fig. 19. Second round macroarrays from Cavalier tissue taken on day two. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

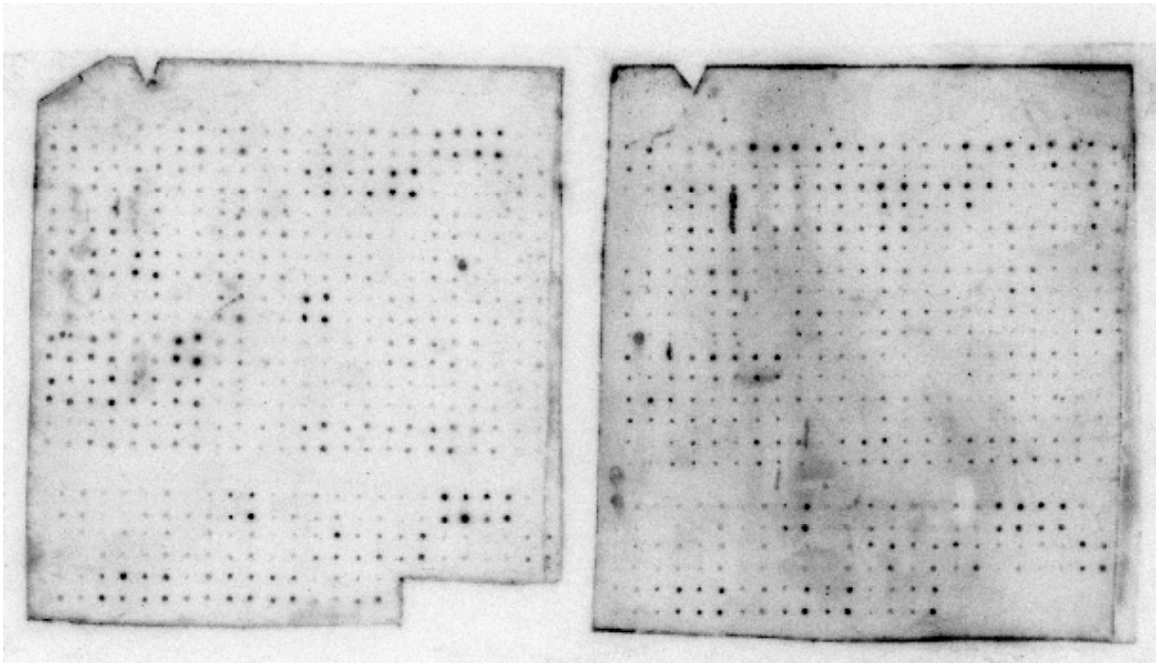


Fig. 20. Second round macroarrays from Cavalier tissue taken on day eight. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

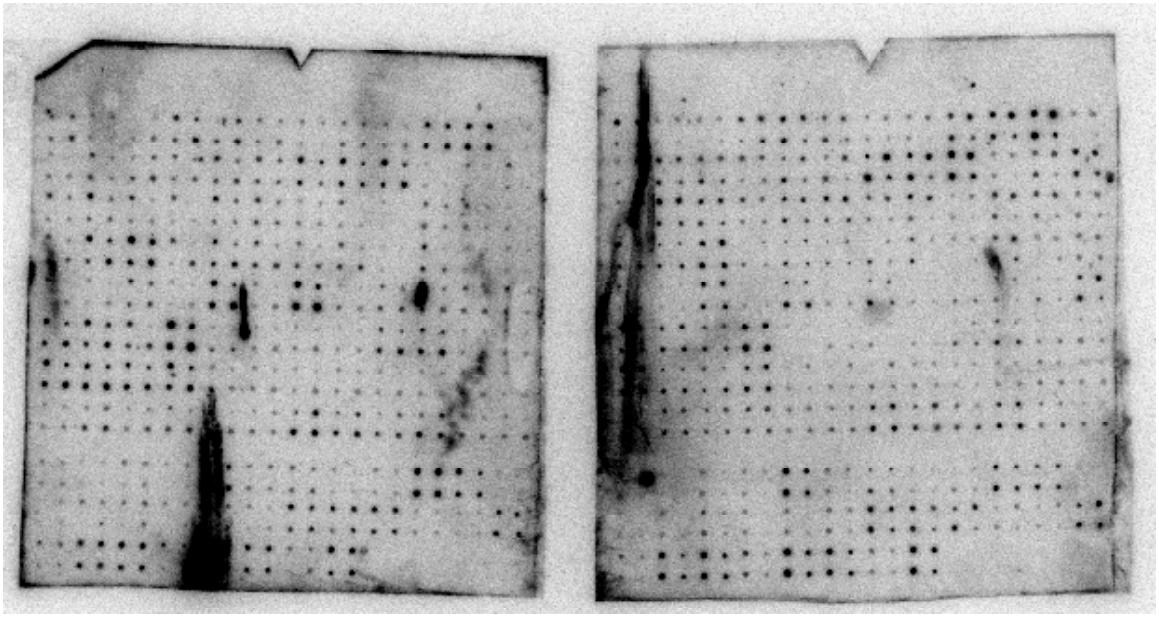


Fig. 21. Second round macroarrays from Cavalier tissue taken on day fourteen. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

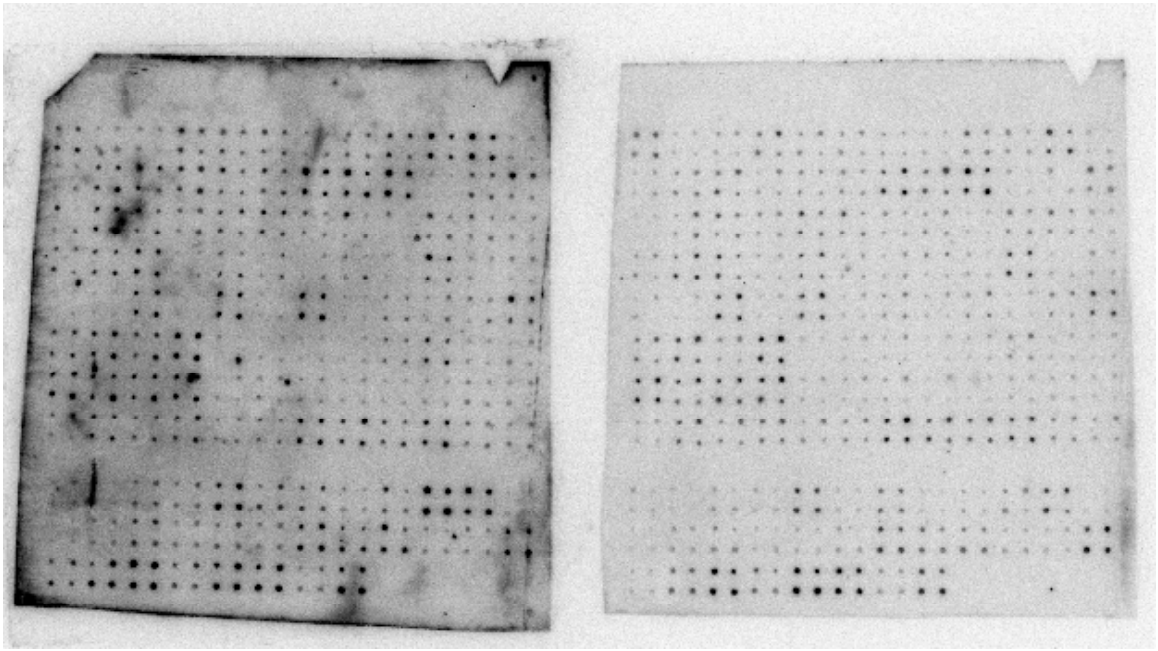


Fig. 22. Second round macroarrays from Cavalier tissue taken on day twenty. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

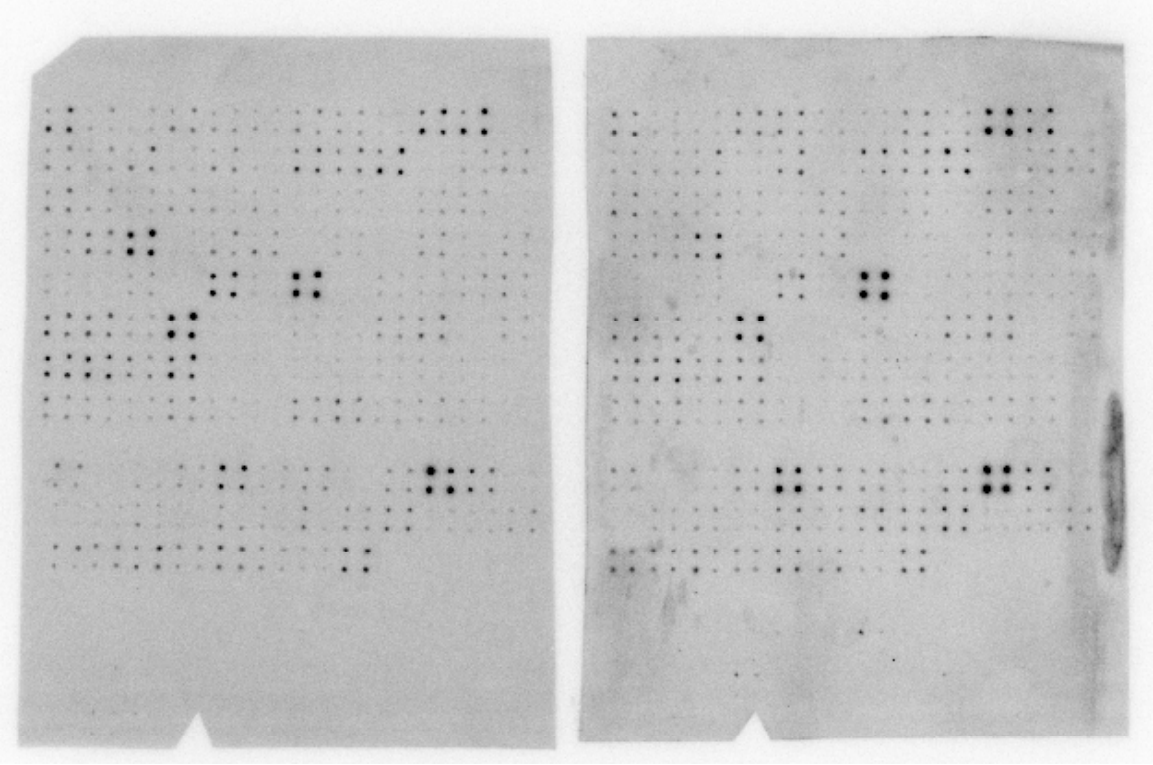


Fig. 23. Second round macroarrays from Diamond tissue taken on day two. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

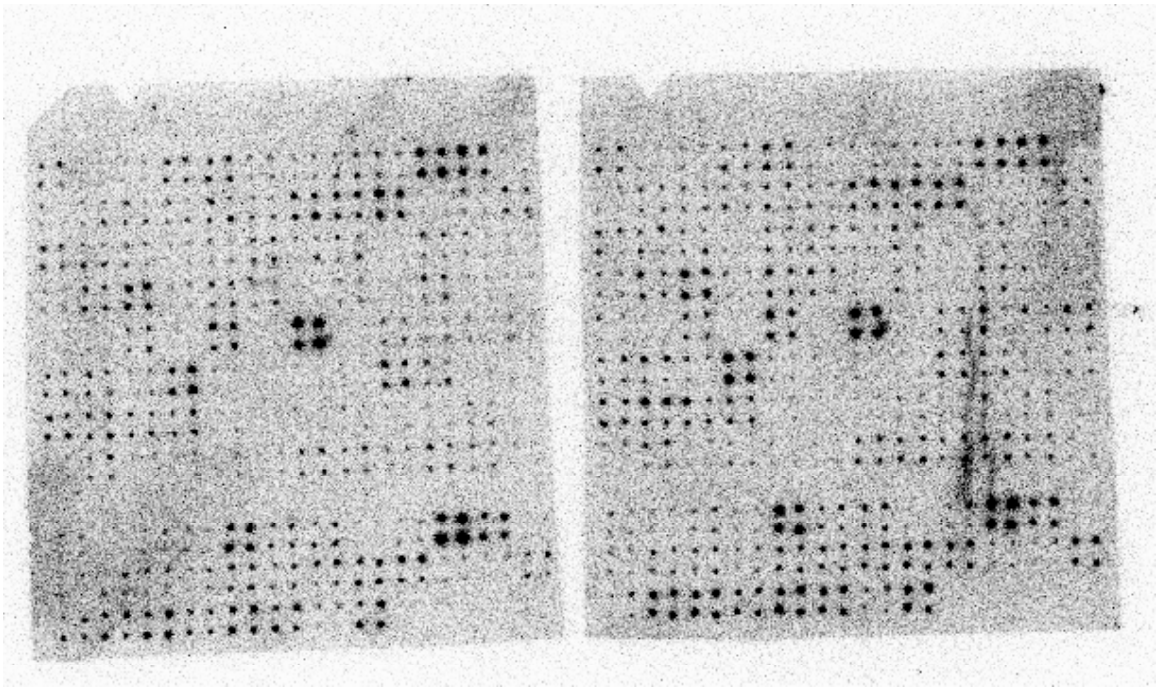


Fig. 24. Second round macroarrays from Diamond tissue taken on day eight. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

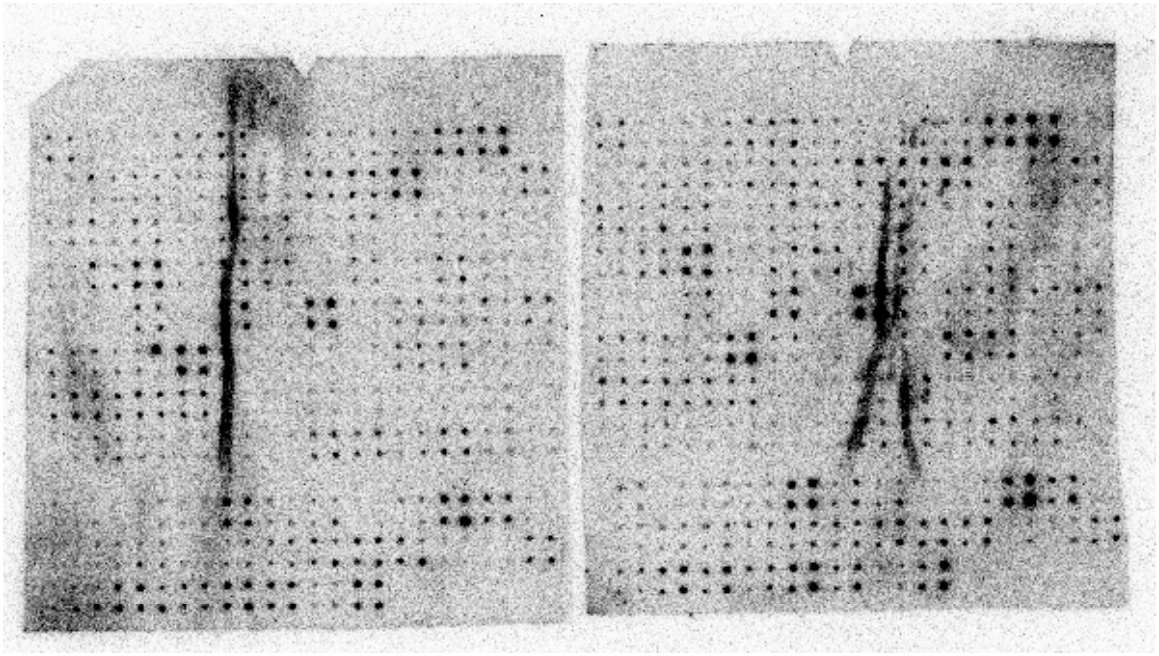


Fig. 25. Second round macroarrays from Diamond tissue taken on day fourteen. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

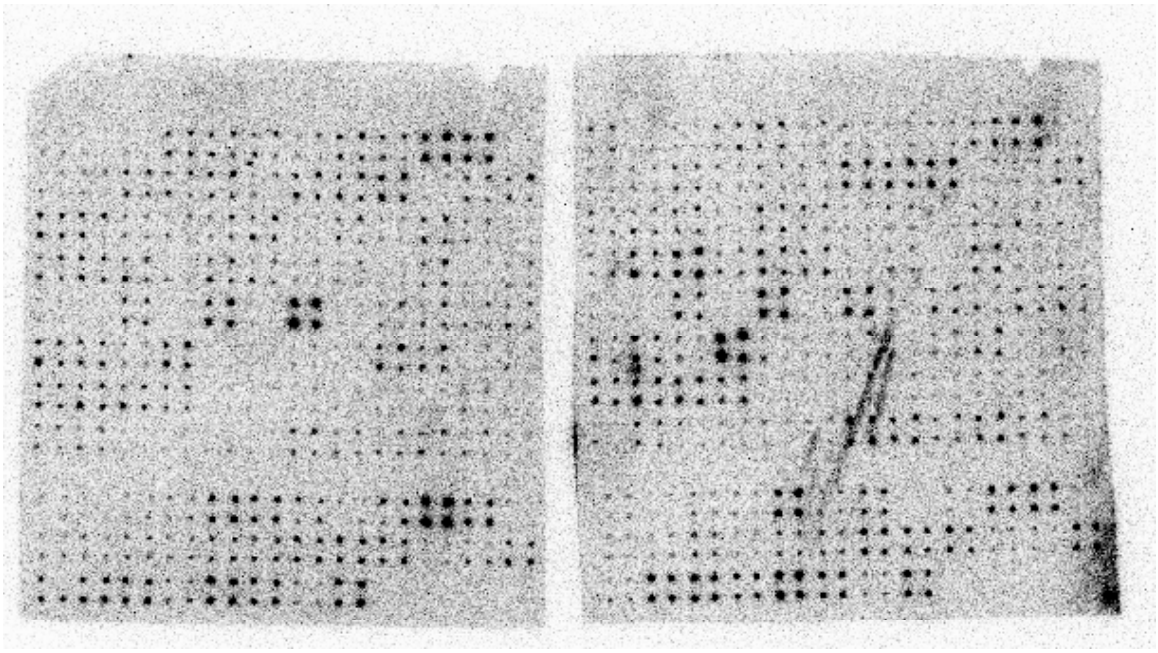


Fig. 26. Second round macroarrays from Diamond tissue taken on day twenty. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

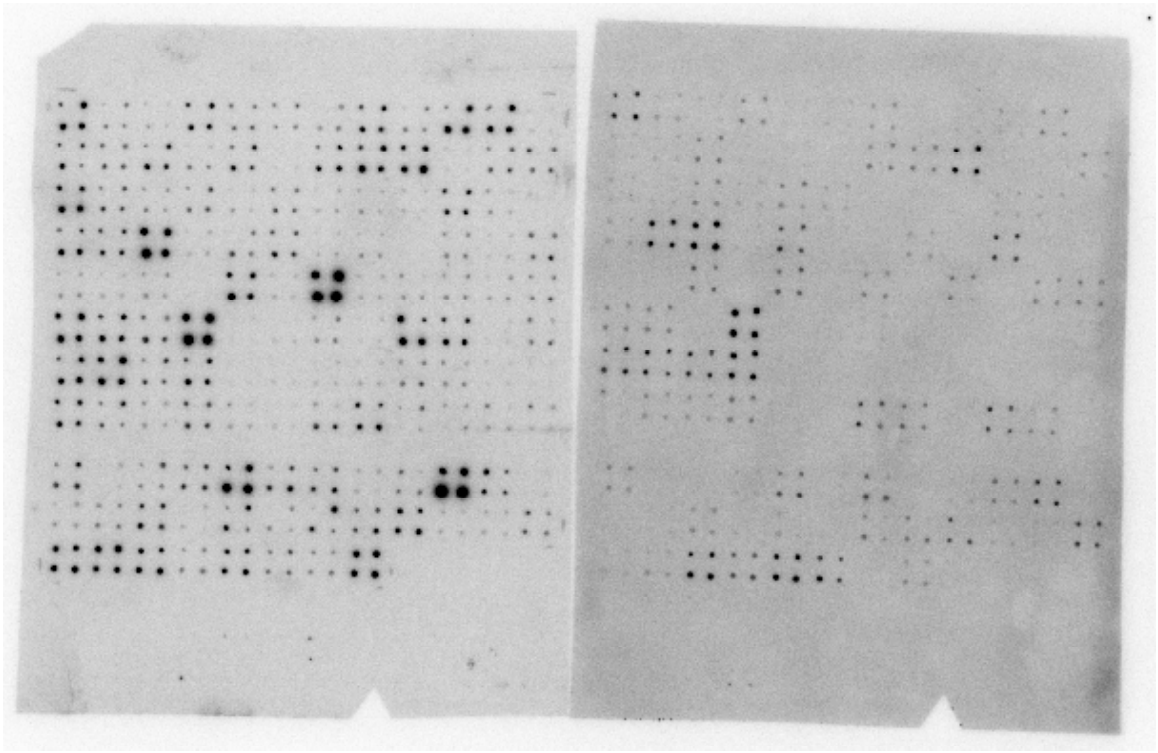


Fig. 27. Second round macroarrays from Palisades tissue taken on day two. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

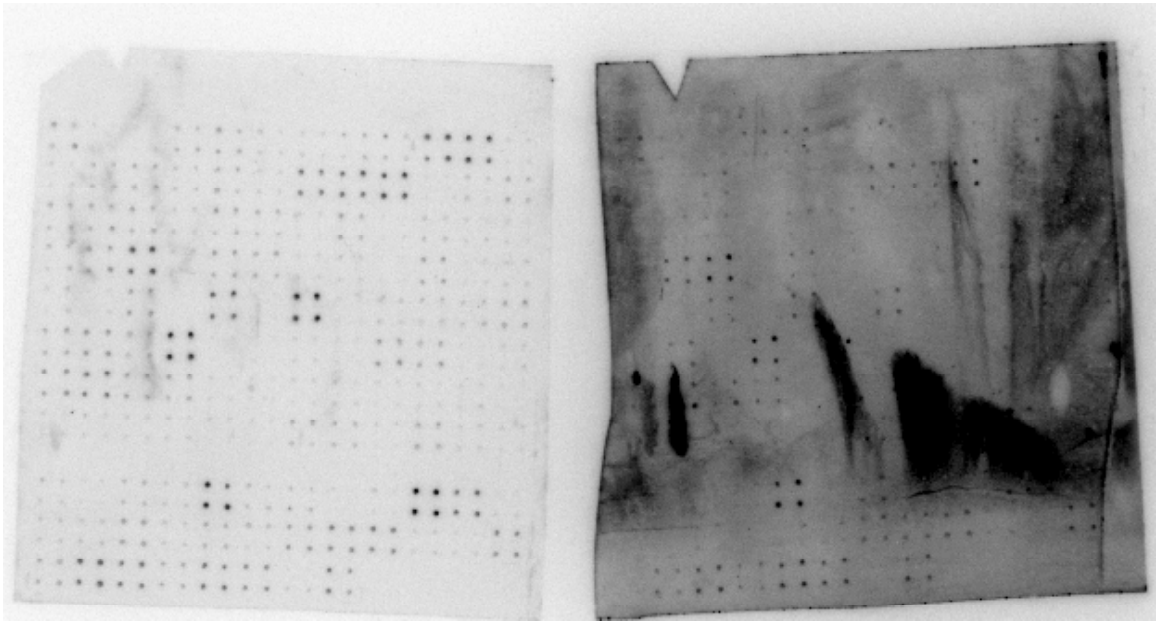


Fig. 28. Second round macroarrays from Palisades tissue taken on day eight. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

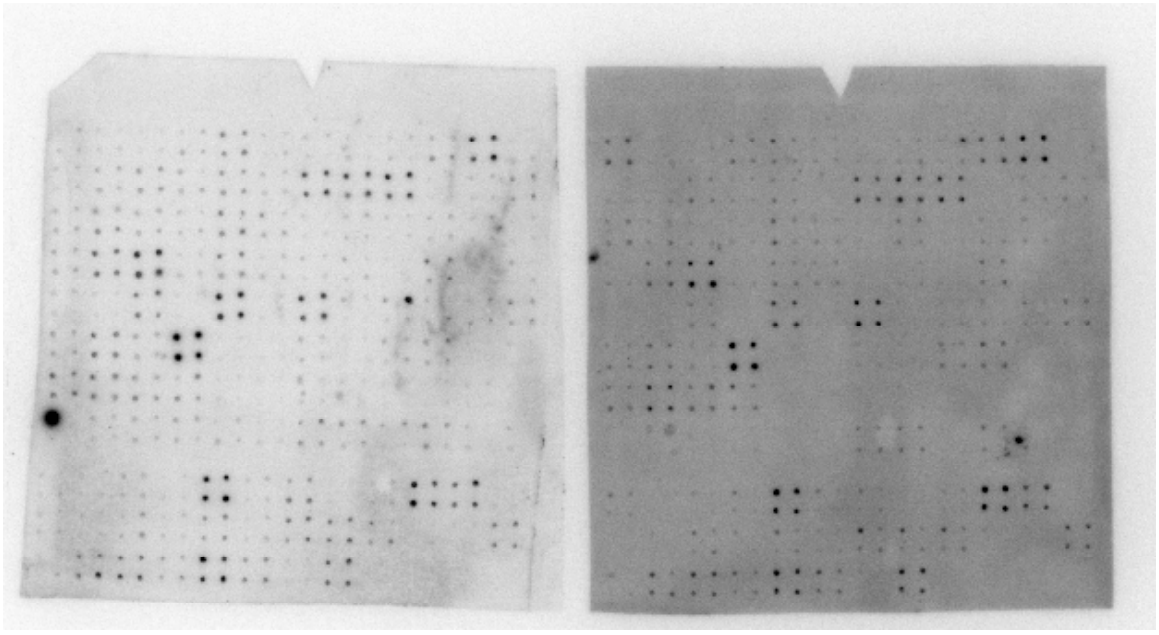


Fig. 29. Second round macroarrays from Palisades tissue taken on day fourteen. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

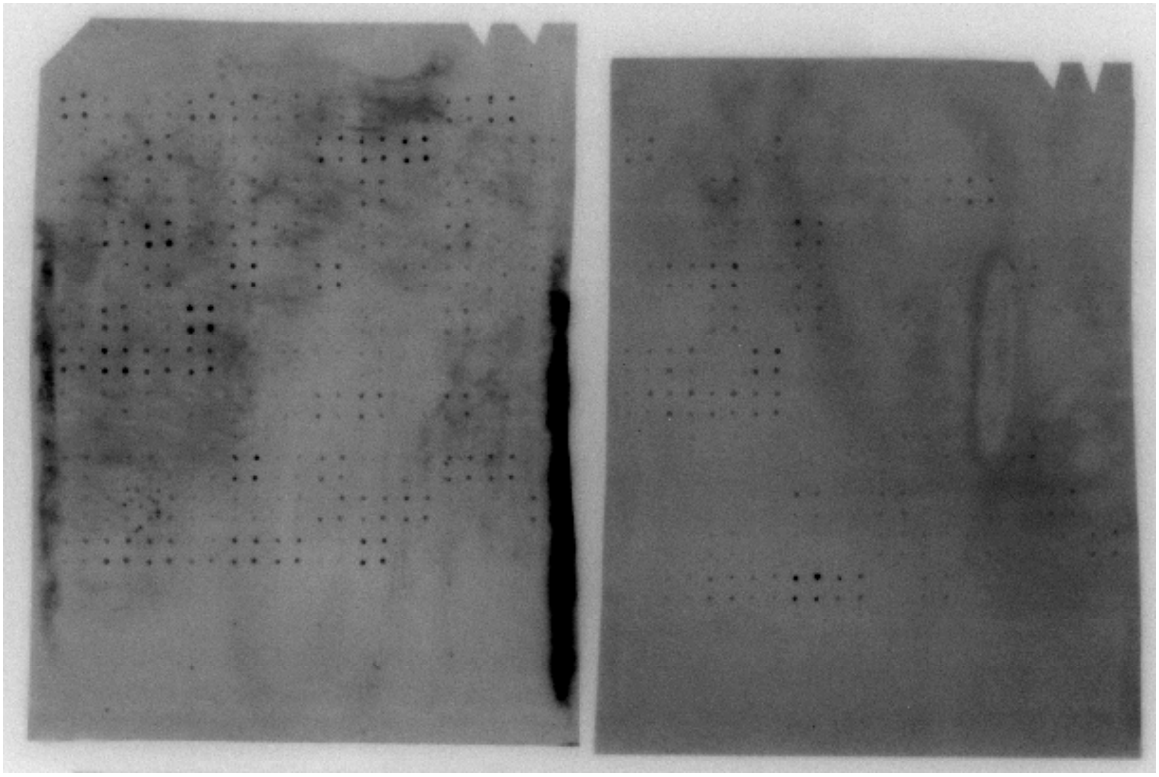


Fig. 30. Second round macroarrays from Palisades tissue taken on day twenty. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

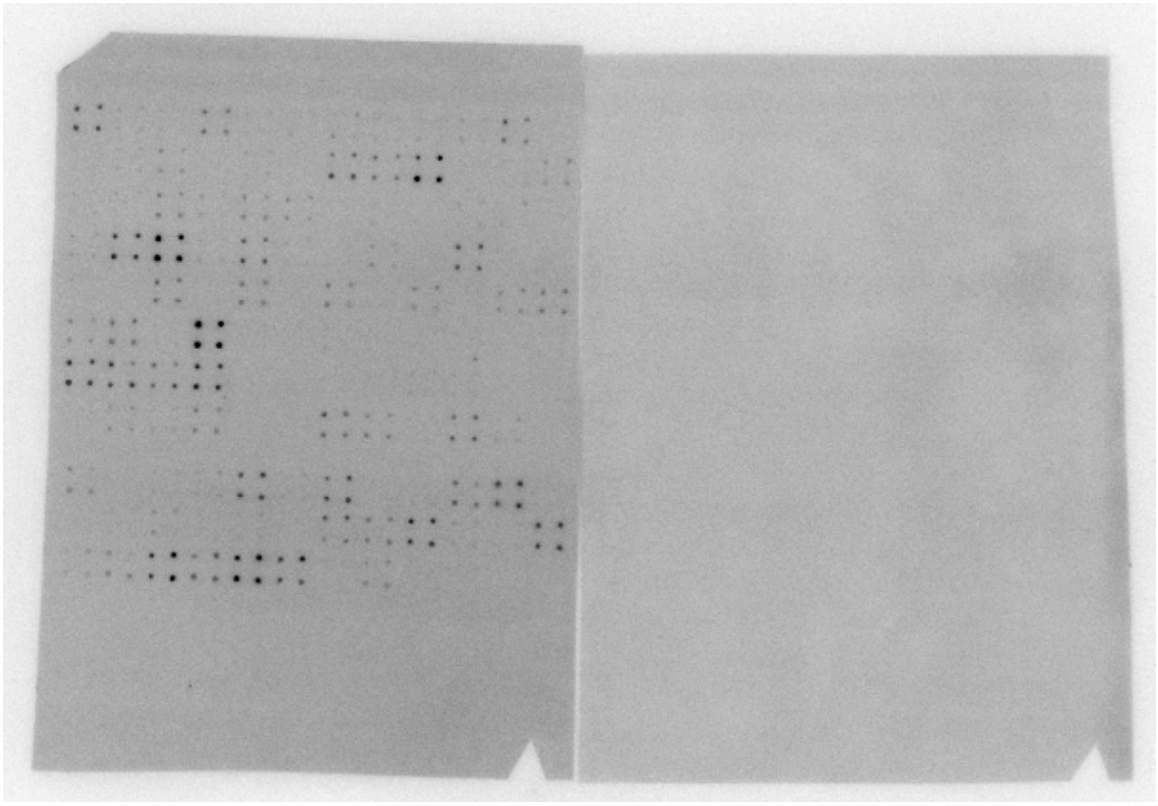


Fig. 31. Second round macroarrays from DALZ 8504 tissue taken on day two. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

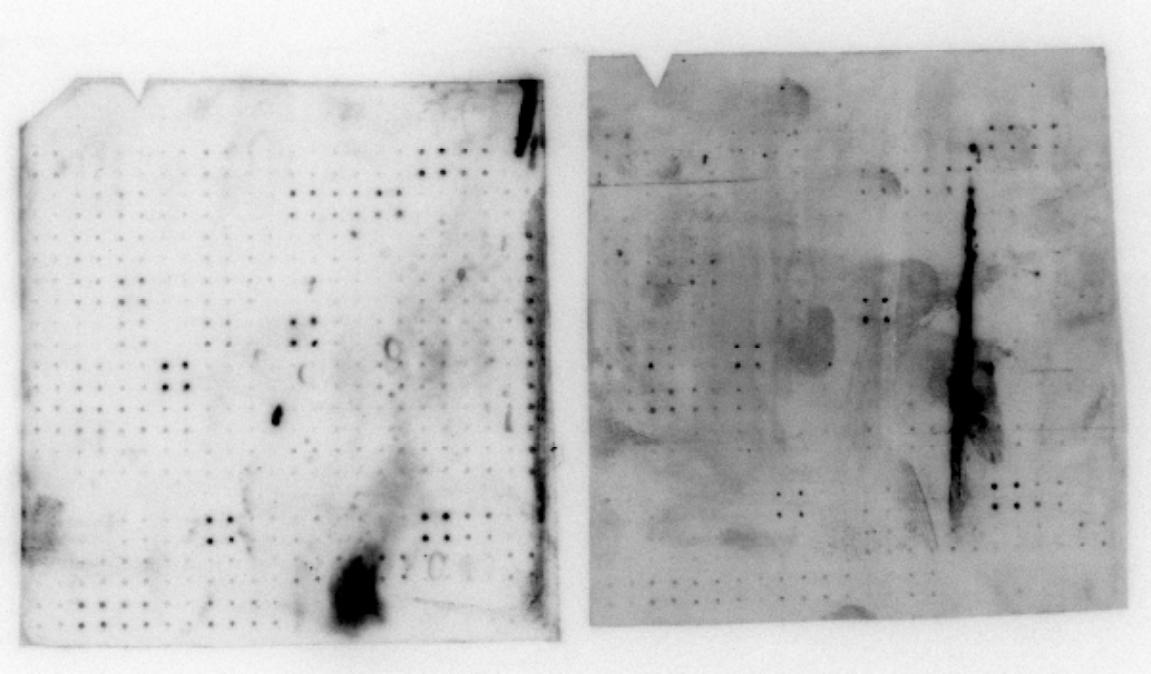


Fig. 32. Second round macroarrays from DALZ 8504 tissue taken on day eight. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

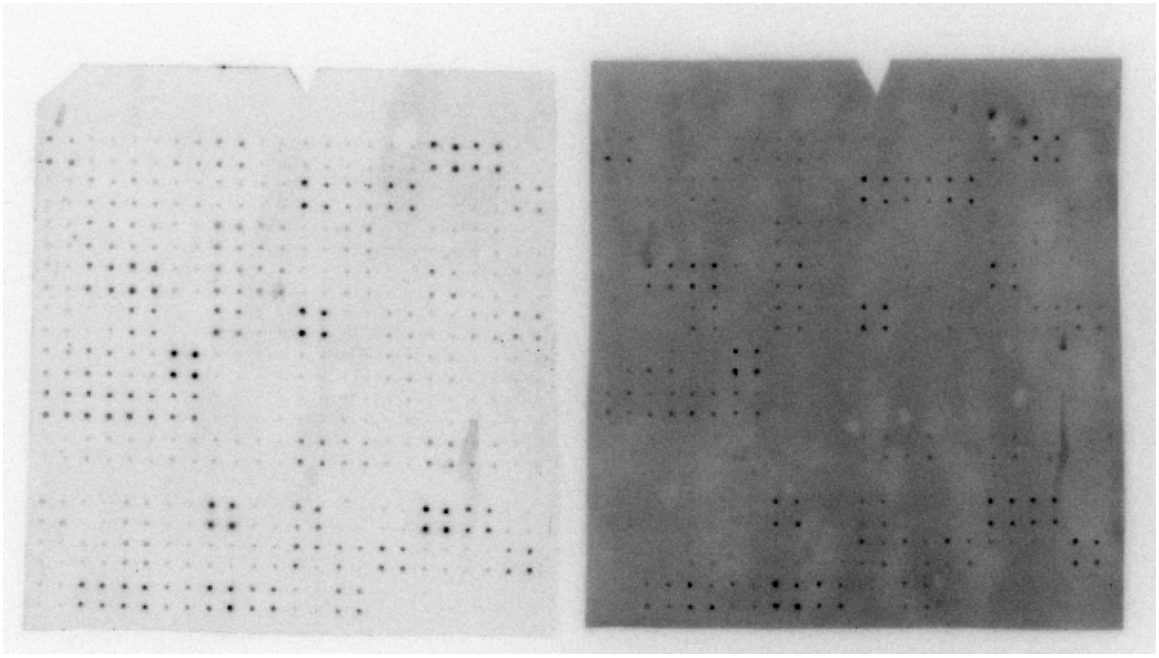


Fig. 33. Second round macroarrays from DALZ 8504 tissue taken on day fourteen. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

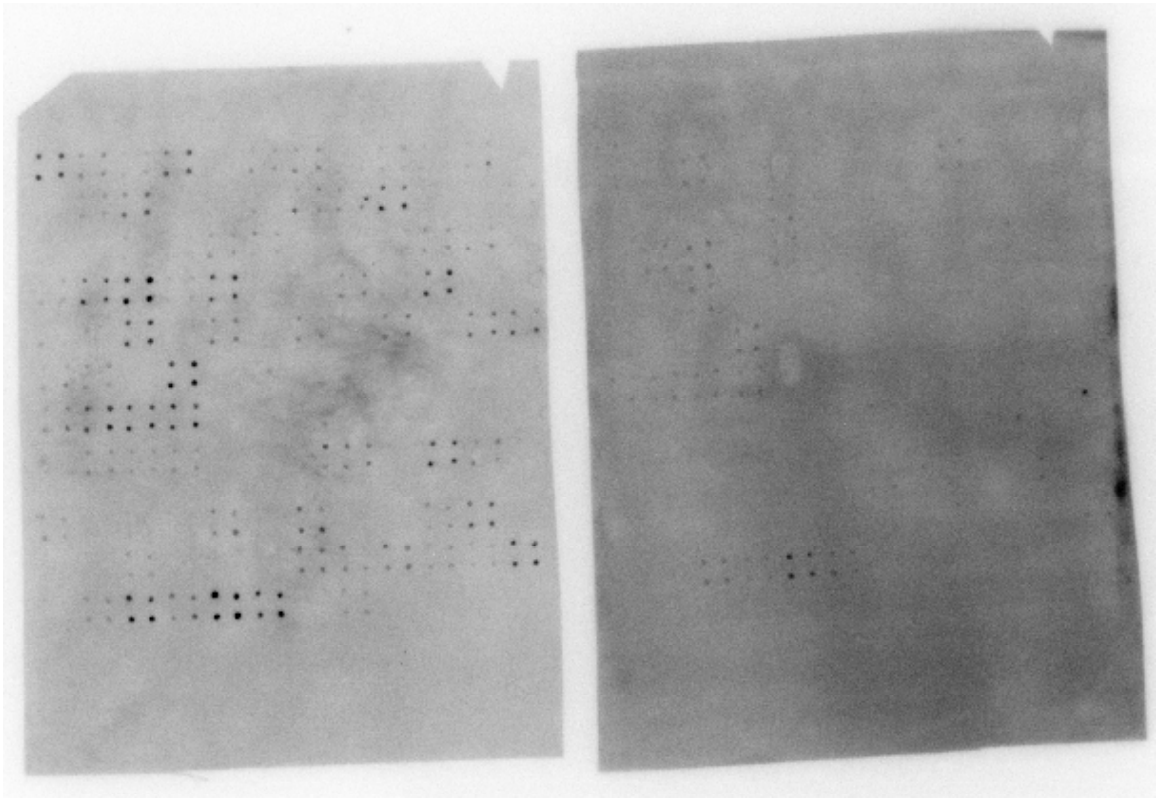


Fig. 34. Second round macroarrays from DALZ 8504 tissue taken on day twenty. Control RNA was hybridized to the left membrane and treatment RNA to the right membrane. Gene order flows from left to right and top to bottom, as a paper would be read, with gene 1 being the upper left four spots in both membranes.

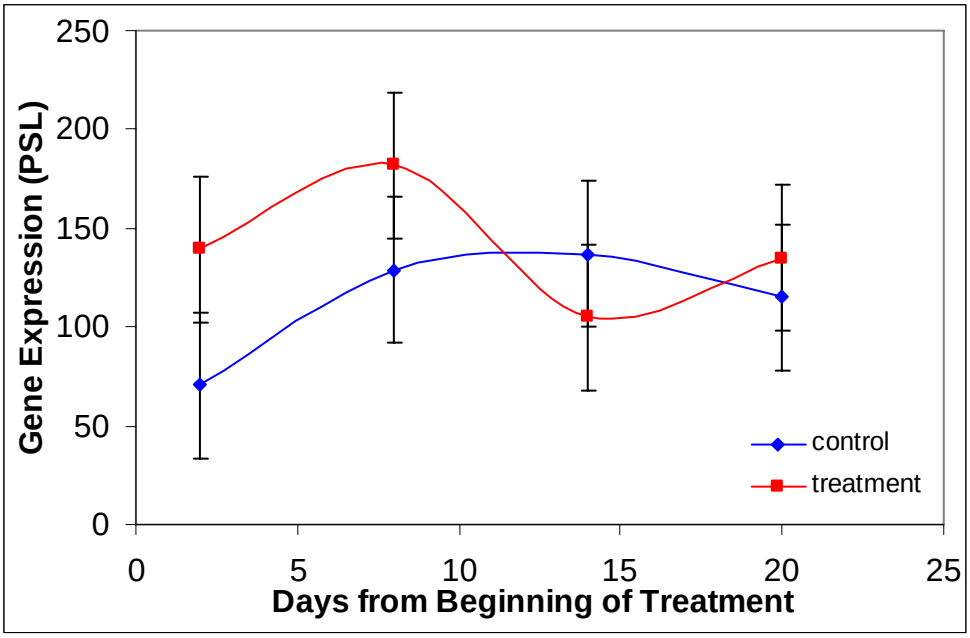


Fig. 35. Cavalier gene expression from the second round of macroarrays. Median gene expression of 75 Cavalier genes that showed significant differences between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

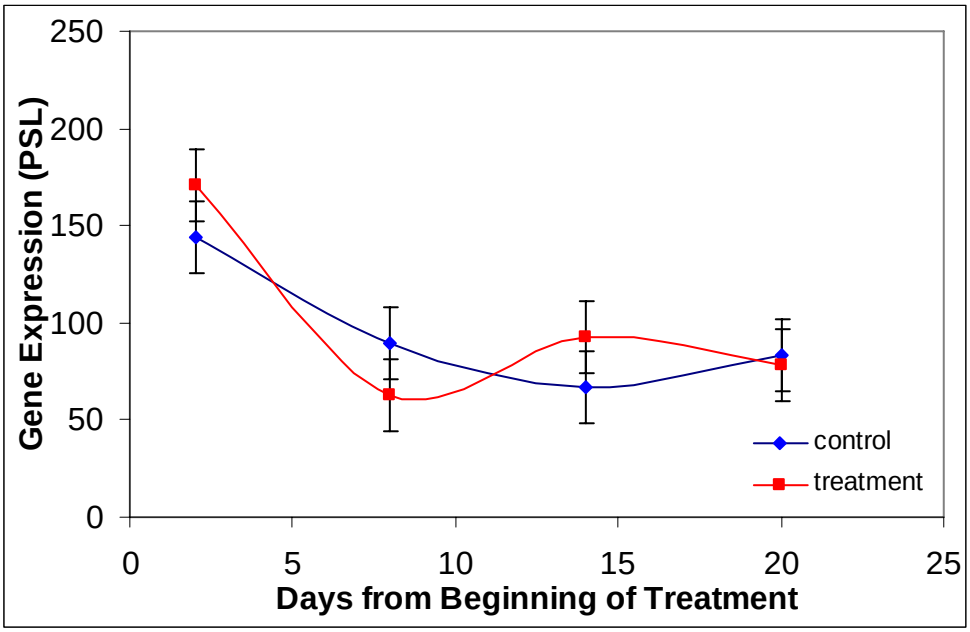


Fig. 36. Diamond gene expression from the second round of macroarrays. Median gene expression of 91 Diamond genes that showed significant differences between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

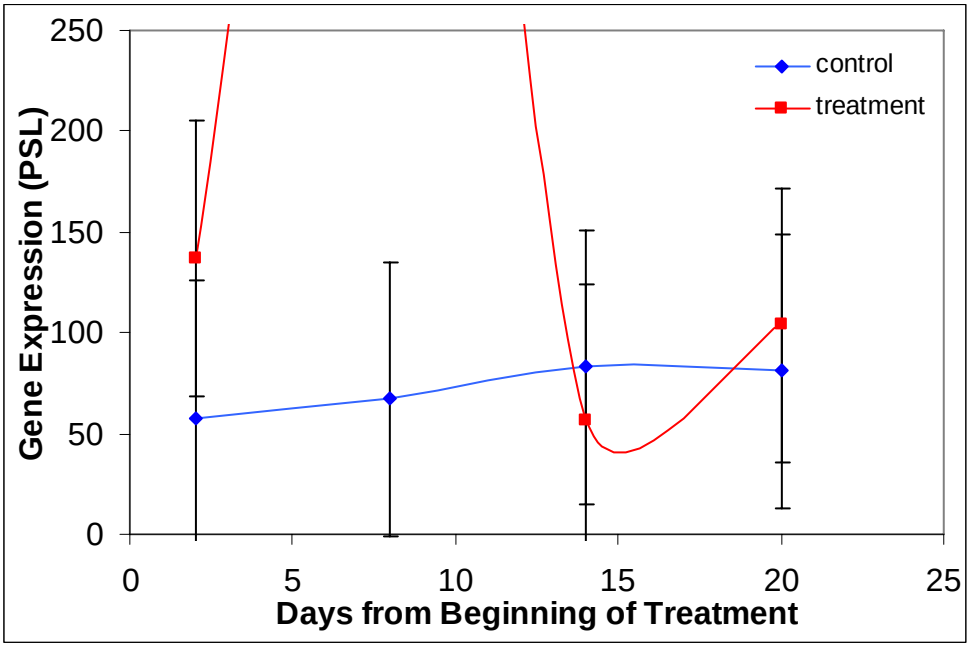


Fig. 37. Palisades gene expression from the second round of macroarrays. Median gene expression of 107 Palisades genes that showed significant differences between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

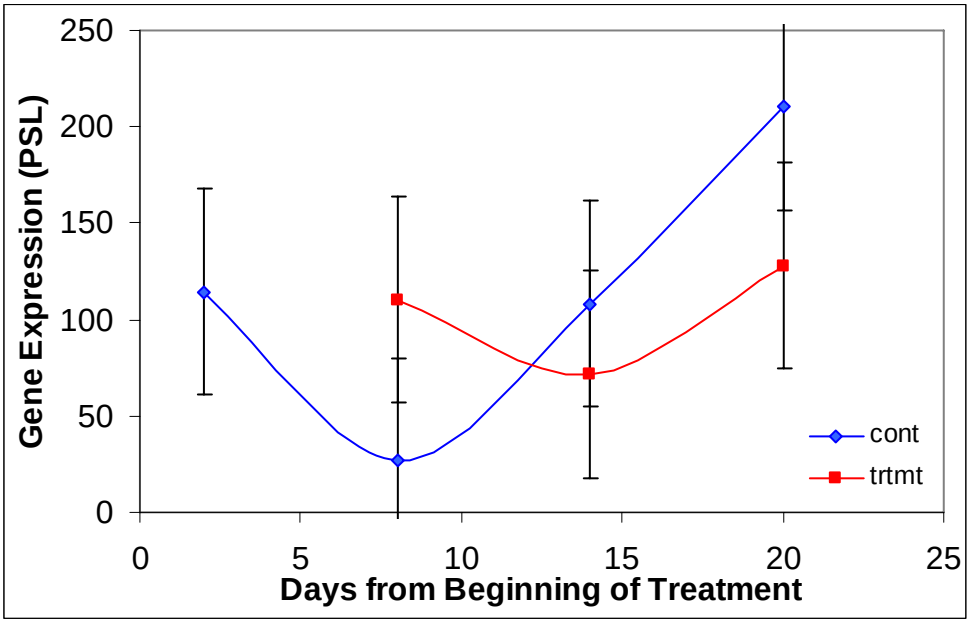


Fig. 38. DALZ 8504 gene expression from the second round of macroarrays. Median gene expression of 90 DALZ 8504 genes that showed significant differences between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

Of the genes that showed significant quantitative differences between days or treatments, 44, 7, 86, and 59 genes appeared to be up-regulated in response to the water stress treatment in Cavalier, Diamond, Palisades, and DALZ 8504 respectively. Mean (median for DALZ 8504 due to a few extremely high values) gene expression of the four cultivars for up-regulated genes are shown in Fig. 39-42. As only seven Diamond genes appeared to be up-regulated, the values in Fig. 40 should be viewed with caution.

Of the genes that showed significant quantitative differences between days or treatments ($P<0.05$), some did not show any significant visual response on any of the membranes (Fig. 19-34). Thirty-two, 29, 43, and 25 genes (129 total) showed both significant quantitative and visual differences between treatments or days for Cavalier, Diamond, Palisades, and DALZ 8504, respectively (Table 17). The 129 genes of interest from Table 17 consisted of 59 of the 115 genes on the membranes. Of the 59 genes of interest from Table 17, several groups can be made. Nine of the 59 genes were expressed in all cultivars (genes 10, 38, 39, 51, 53, 55, 64, 91, and 106). Three of the 59 were only expressed in the two *Z. matrella* cultivars (Cavalier and Diamond; genes 5, 101, and 113), while five genes were only expressed in the two *Z. japonica* cultivars (Palisades and DALZ 8504; genes 19, 20, 21, 41, and 75). Eleven of the 59 genes were only expressed in Cavalier (the water stress tolerant *Z. matrella*; genes 6, 9, 12, 14, 16, 26, 31, 60, 85, 93, and 107), while eight of the 59 genes were only

Table 17. Genes that showed both quantitative and visual differences.

Gene	Cavalier	Diamond	Palisades	8504
1		X	X	X
4		X	X	X
5	X	X		
6	X			
8	X	X	X	
9	X			
10	X	X	X	X
11	X		X	X
12	X			
13			X	
14	X			
15			X	
16	X			
17		X	X	
19			X	X
20			X	X
21			X	X
25		X	X	
26	X			
31	X			
34			X	
37			X	
38	X	X	X	X
39	X	X	X	X
41			X	X
42			X	
46	X		X	X
51	X	X	X	X
53	X	X	X	X
55	X	X	X	X
60	X			
61		X	X	
62		X	X	X
63	X		X	
64	X	X	X	X
69	X		X	
70	X	X	X	
73		X	X	X
74	X	X	X	
75			X	X
76		X	X	X
85	X			

Table 17 (cont.)

Gene	Cavalier	Diamond	Palisades	8504
88	X		X	
91	X	X	X	X
92		X	X	
93	X			
94	X		X	X
97			X	
100			X	
101	X	X		
102		X	X	
103		X	X	X
105		X		
106	X	X	X	X
107		X	X	X
107	X			
111			X	
113	X	X		
115		X		X

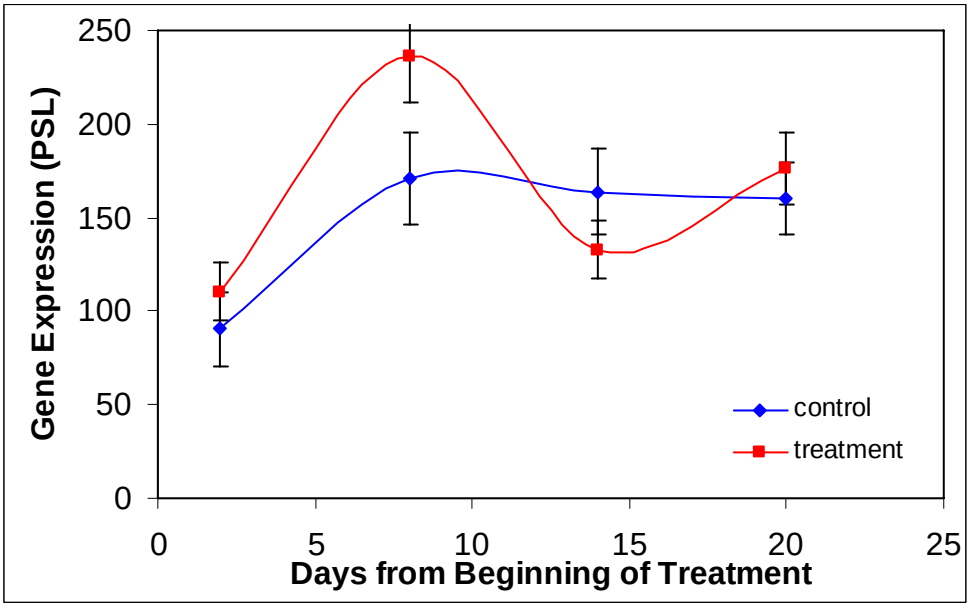


Fig. 39. Cavalier up regulated gene expression from the second round of macroarrays. Mean gene expression of 44 Cavalier genes that showed significant up-regulation between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

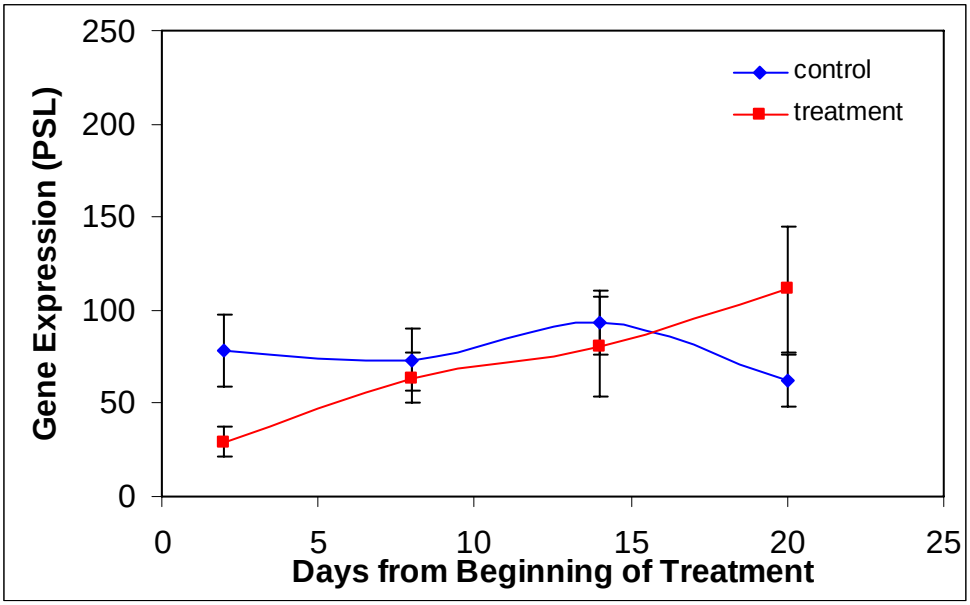


Fig. 40. Diamond up regulated gene expression from the second round of macroarrays. Mean gene expression of seven Diamond genes that showed significant up-regulation between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

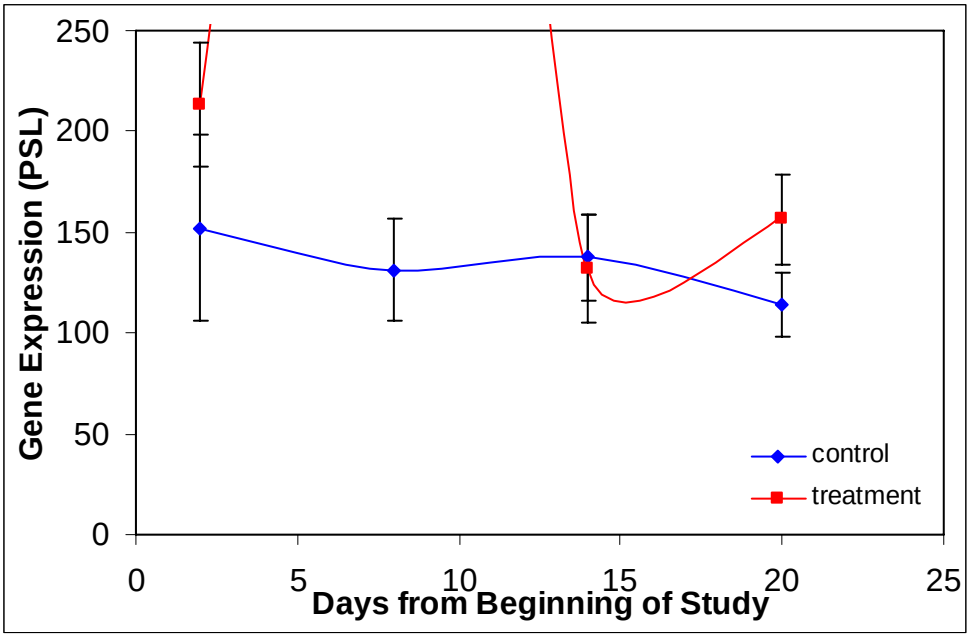


Fig. 41. Palisades up regulated gene expression from the second round of macroarrays. Mean gene expression of 86 Palisades genes that showed significant up-regulation between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

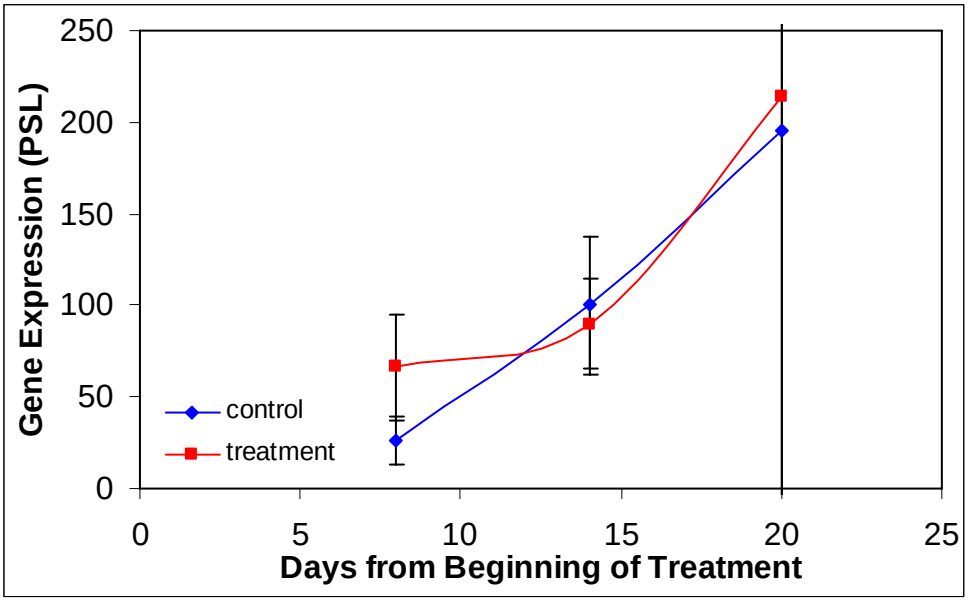


Fig. 42. DALZ 8504 up regulated gene expression from the second round of macroarrays. Median gene expression of 59 DALZ 8504 genes that showed significant up-regulation between days or controls (diamonds) and treatments (squares). Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.

expressed in Palisades (the water stress tolerant *Z. japonica*; genes 13, 15, 34, 37, 42, 97, 100, and 111). Three of the 59 genes were expressed only in the water stress tolerant cultivars (Cavalier and Palisades; genes 63, 69, and 88). Three of the 59 genes (genes 11, 46, and 94) were expressed in the three most water stress tolerant cultivars (Cavalier, Palisades, and DALZ 8504), and were absent in the most water stress susceptible cultivar (Diamond).

Of the 129 genes of interest, 102 showed an interaction between day and treatment (Tables 13-16). This is not surprising as 115 genes are going to respond to gradual soil drying at different times and to different degrees. The interaction is of interest if it causes the effect of treatment to be non-significant. Of the 129 genes of interest, only 31 showed a significant interaction with non-significant treatment effects (Tables 13-16). Of those 31 genes that showed a significant interaction with non-significant effects of treatment, only 15 were present in the water stress tolerant groupings from Table 17. Of those 15 interactions, five contained one value that was much different than the others (Cavalier gene 10, Palisades gene 42, and DALZ 8504 genes 1, 19, and 21), four consisted of a single significant cross-over event (Diamond genes 38, 39, and 51, and DALZ 8504 gene 64), and six consisted of multiple cross-over events (Cavalier gene 85, Diamond genes 55 and 106, Palisades genes 97 and 111, and DALZ 8504 gene 10; Fig. 43).

Northern analyses were conducted on six Cavalier and six Palisades genes of interest (genes 15, 30, 39, 64, 65, and 83). Data was normalized for RNA quantities on the membranes and mean northern and macroarray results from the six genes are shown

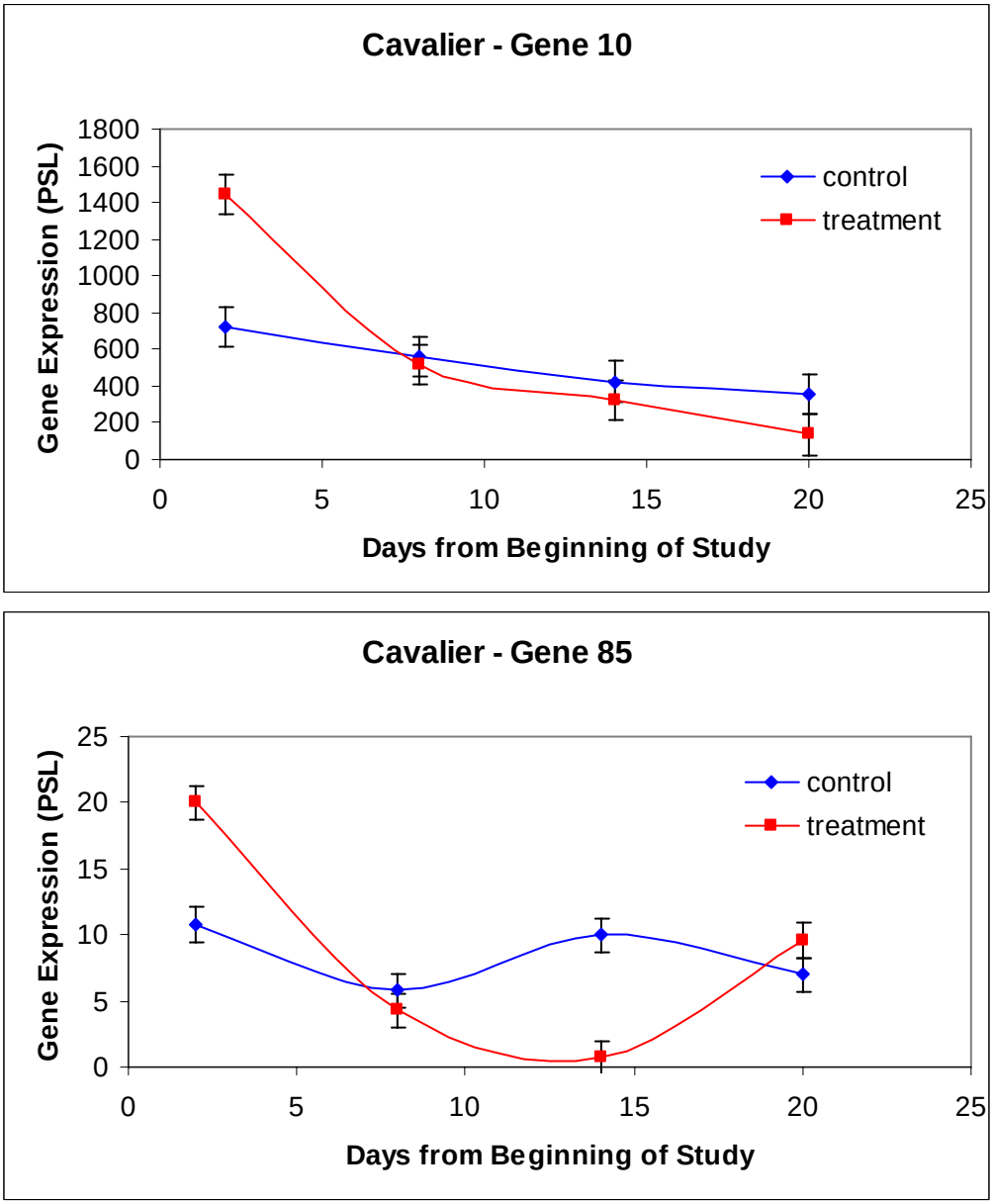
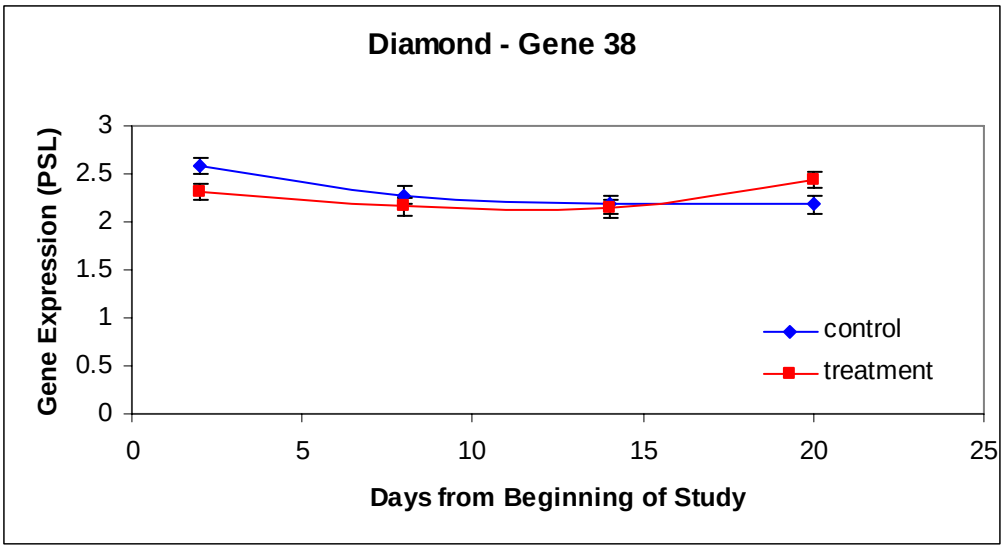
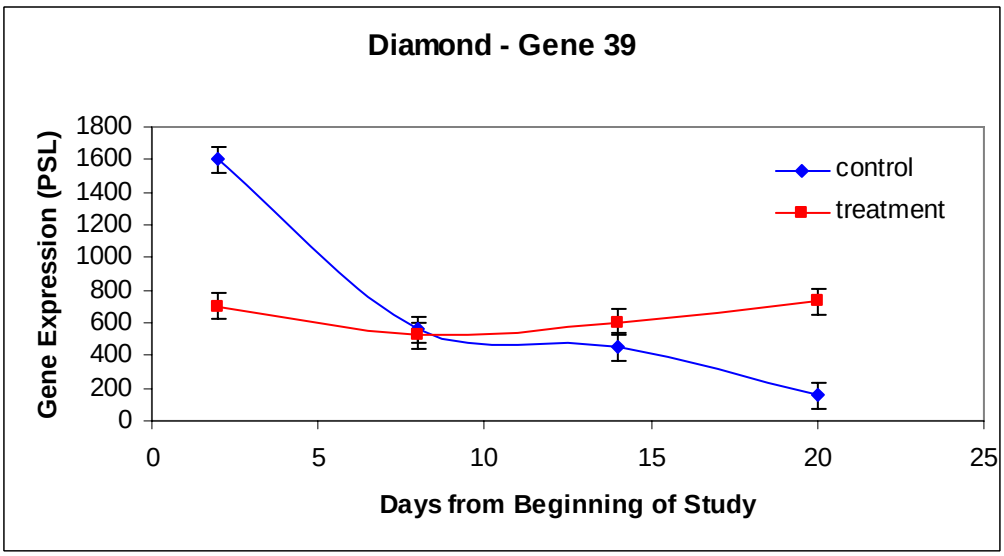


Fig. 43. Interaction plots of two-way interactions. Interaction plots between gene expression of control (diamonds) and treatment (squares) tissue for the 15 genes of interest that were found among the water stress tolerant groupings from Table 17. Data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12.


Diamond - Gene 38
Gene Expression (PSL)
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment


Diamond - Gene 39
Gene Expression (PSL)
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

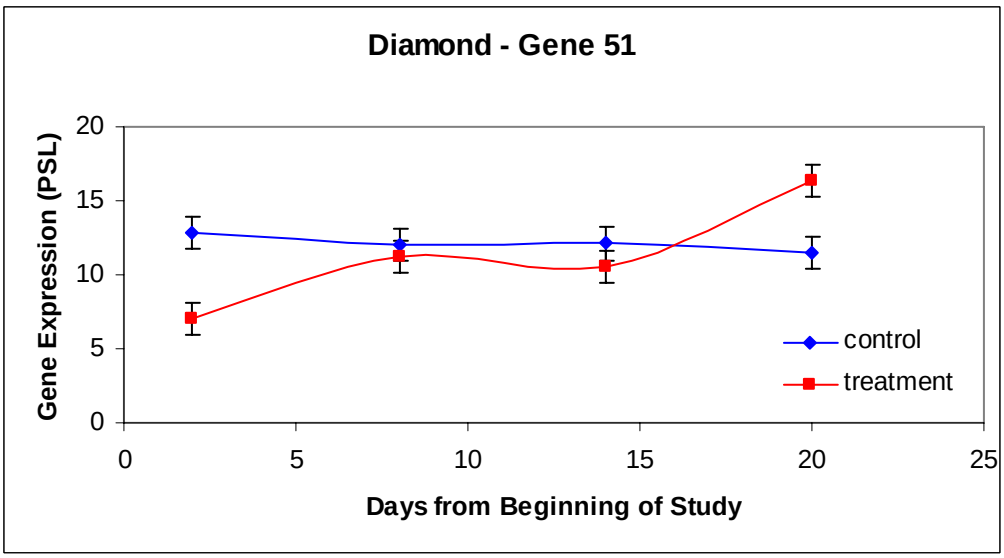
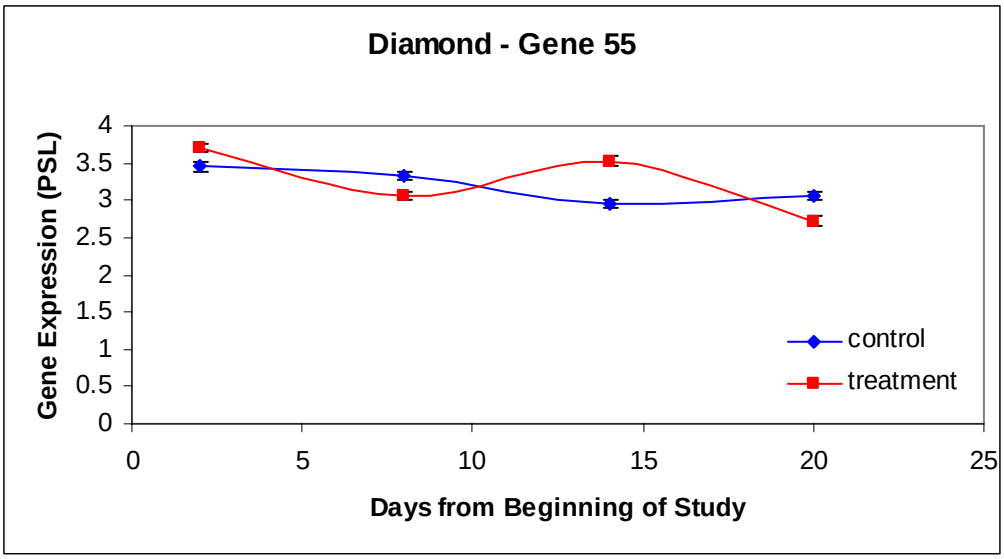
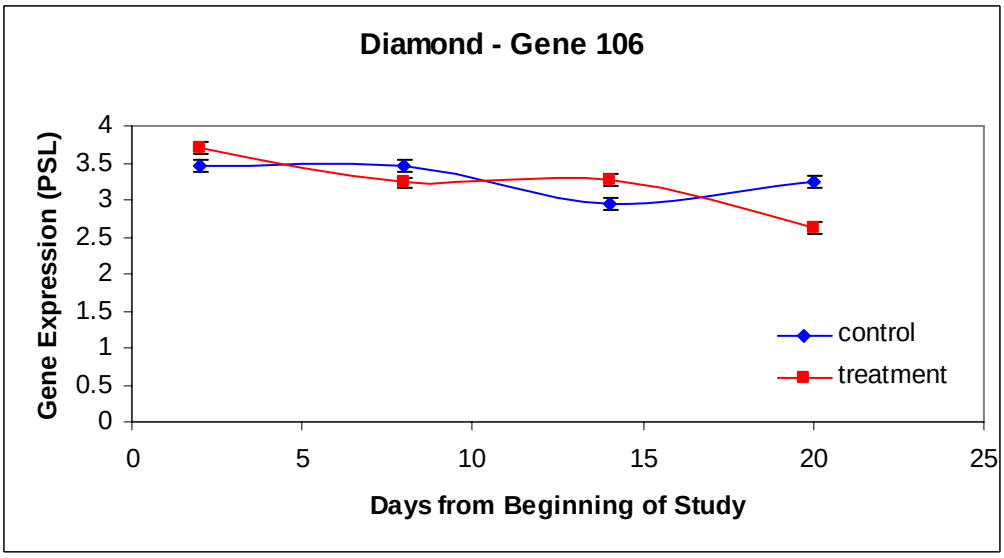

Diamond - Gene 51
Gene Expression (PSL)
20
15
10
5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

Fig. 43 (cont.)


Diamond - Gene 55
Gene Expression (PSL)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment


Diamond - Gene 106
Gene Expression (PSL)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

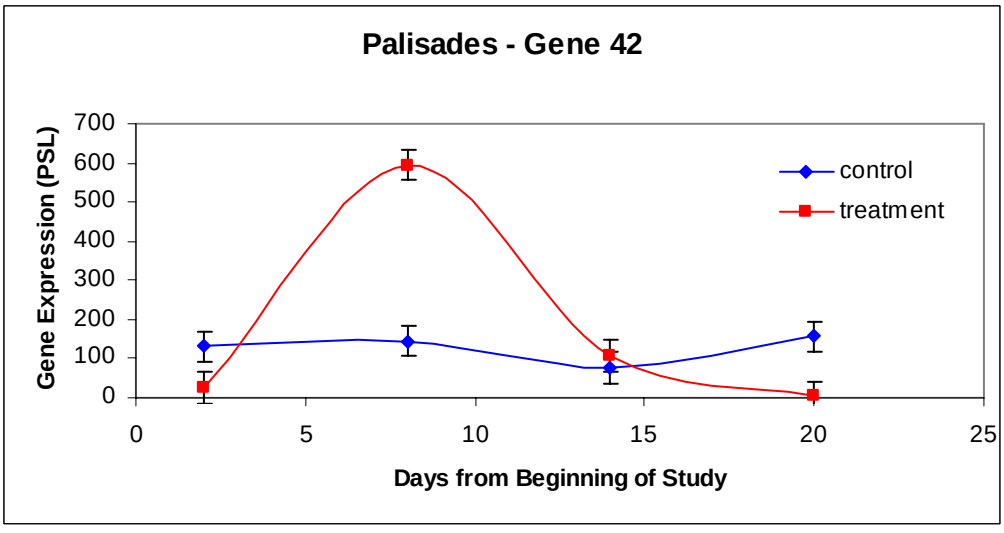
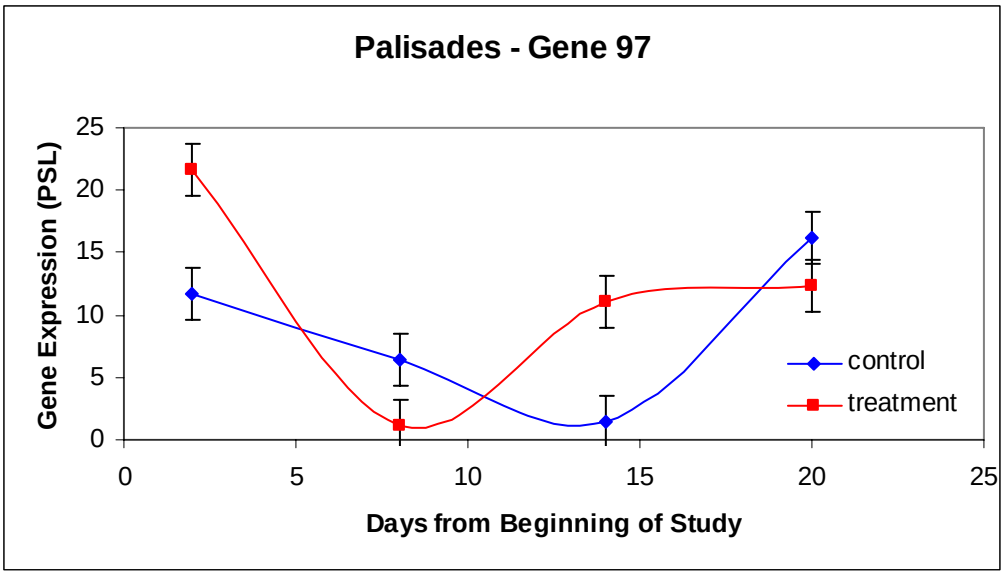
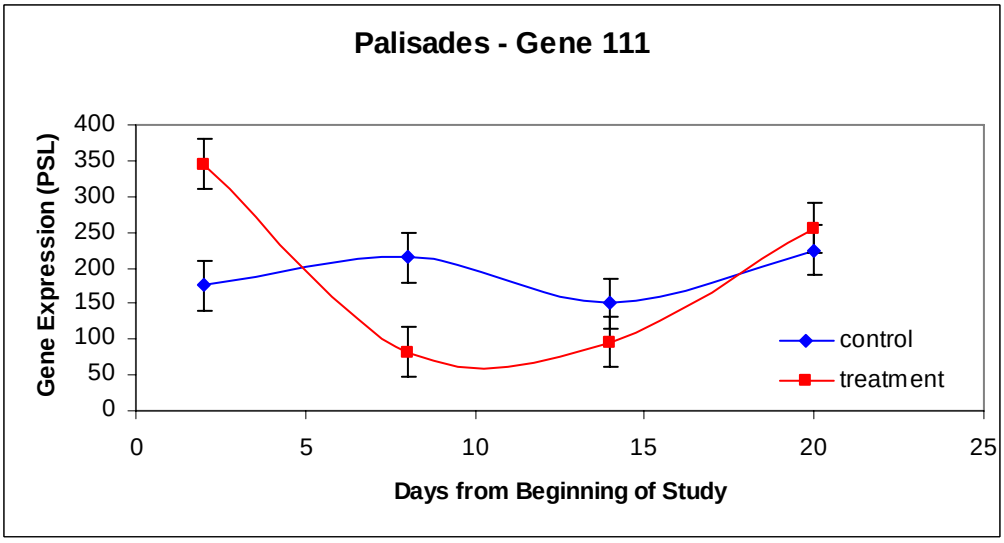

Palisades - Gene 42
Gene Expression (PSL)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

Fig. 43 (cont.)


Palisades - Gene 97
Gene Expression (PSL)
25
20
15
10
5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment


Palisades - Gene 111
Gene Expression (PSL)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

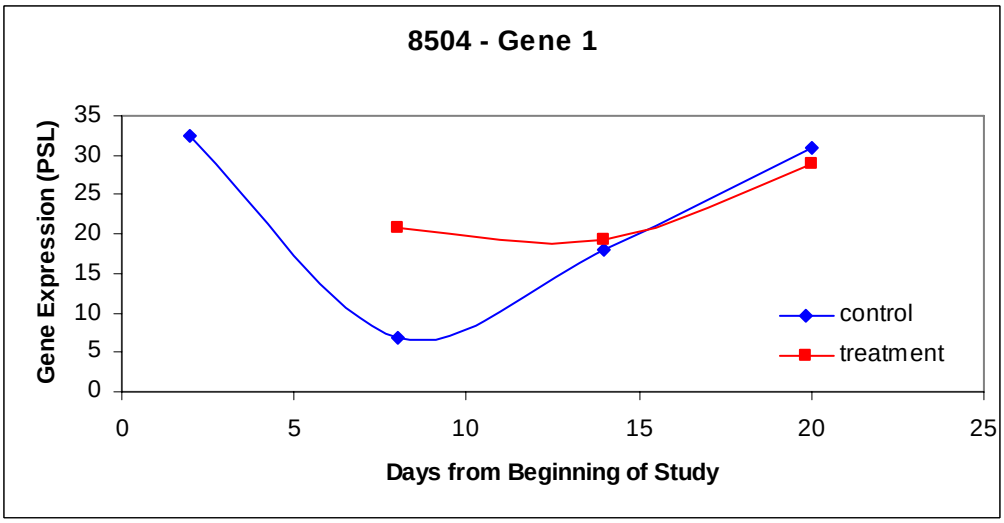
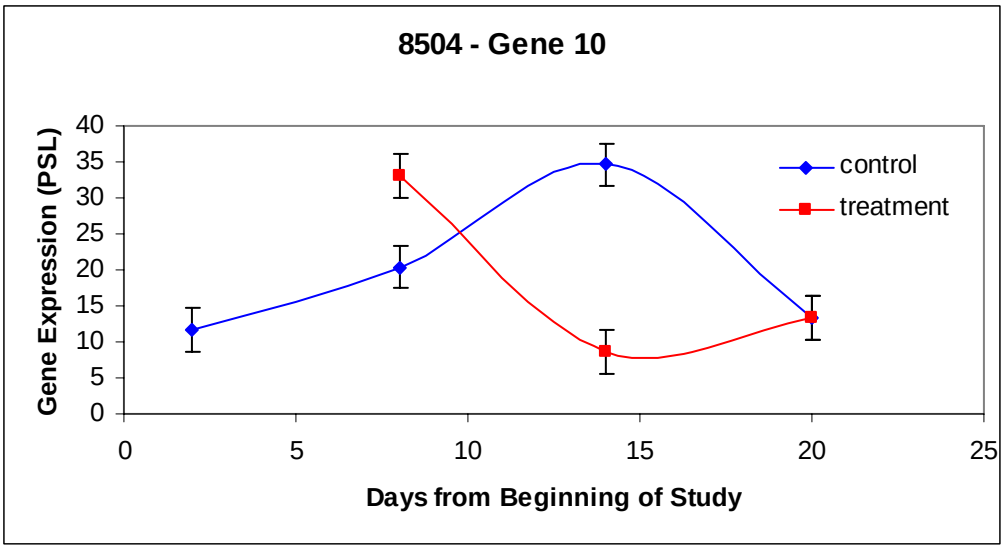
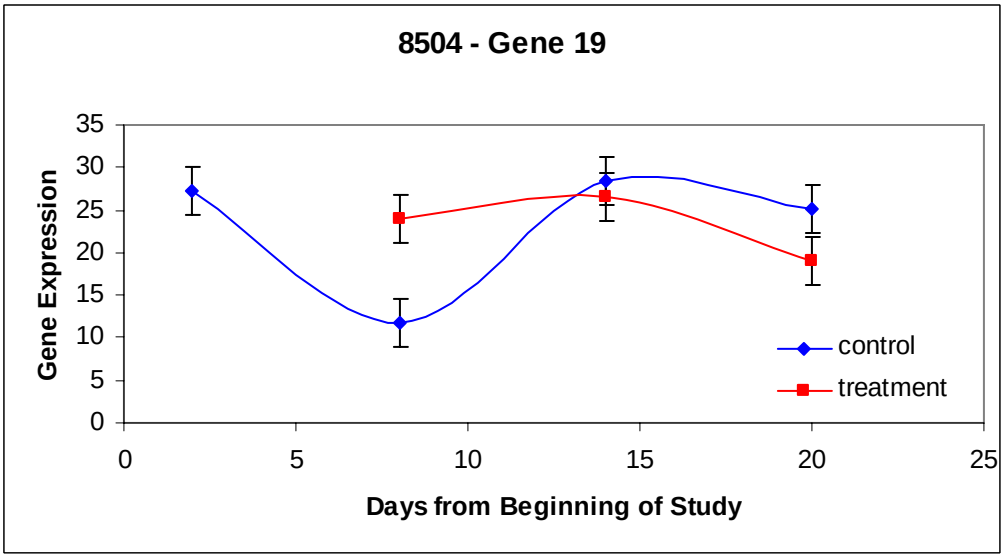

8504 - Gene 1
Gene Expression (PSL)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

Fig. 43 (cont.)


8504 - Gene 10
Gene Expression (PSL)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment


8504 - Gene 19
Gene Expression
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

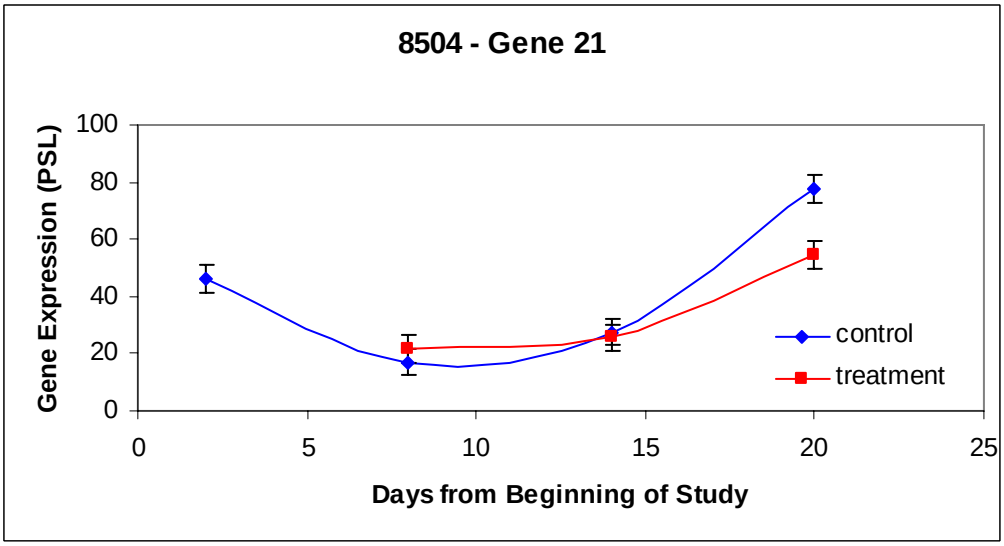

8504 - Gene 21
Gene Expression (PSL)
100
80
60
40
20
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

Fig. 43 (cont.)

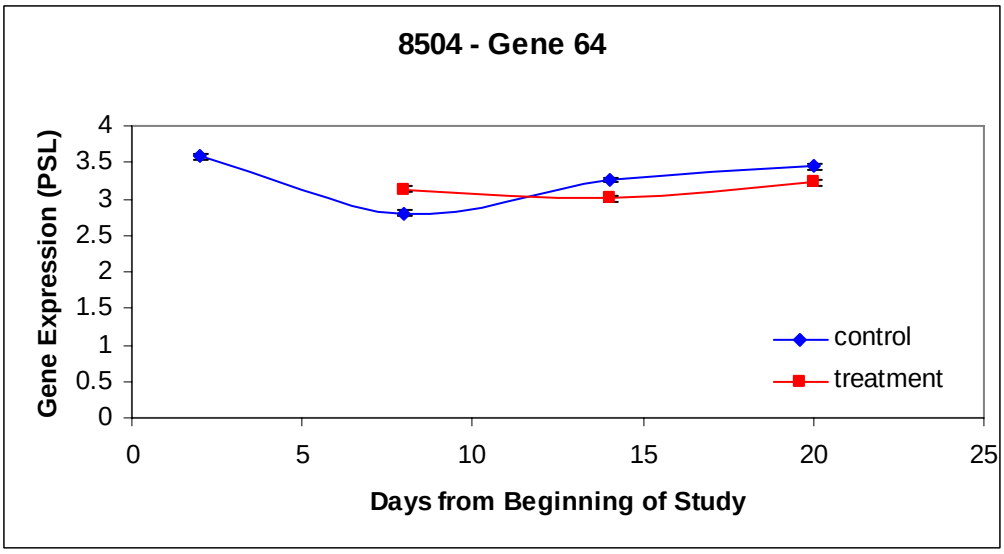

8504 - Gene 64
Gene Expression (PSL)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Days from Beginning of Study
control
treatment

Fig. 43 (cont.)

in Fig. 44 and 45. The general trends of the graphs in Fig. 44 and 45 are similar for both Cavalier and Palisades, indicating that the general responses of both methods are consistent. The apparent discrepancy between northern and macroarray analyses on day two may be a function of overestimation of treatment expression in the macroarrays. RNA quantities and individual northerns are shown in Fig. 46-52. It is interesting to note that both the macroarray and northern analyses in Fig. 44 showed an increase in gene expression of control plants from day 2 to day 8. This further supports the results of the water relations and water stress signaling experiments that suggested that non-stressed plants had lower TWDW in response to water stress signals.

T-DNA Insert Experiment

The *A. thaliana* knockout experiment was conducted using *A. thaliana* orthologous genes to genes 32 and 71 from the gene expression experiment. These genes were chosen because they had not been identified as water stress responsive genes and they appeared to not be part of a large gene family in the *A. thaliana* genome. Being unique reduced the likelihood of functional redundancy which could mask the effects of knocking out those genes. Gene 32 was identified as an expressed protein (E-value of $3x10^{-42}$) and gene 71 was identified as a bZIP protein (E-value of $2x10^{-22}$) (Table 8). The SALK lines with T-DNA insertions in *A. thaliana* orthologs of these two genes were SALK_034149 for gene 32 and SALK_096651 for gene 71. Neither SALK line, submitted to water stress, showed any reduction in water stress tolerance (measured as days to wilting) when compared to wild type *A. thaliana* which had fully functional orthologs of genes 32 and 71. The transition from visually healthy to wilted was very abrupt and easily measured.

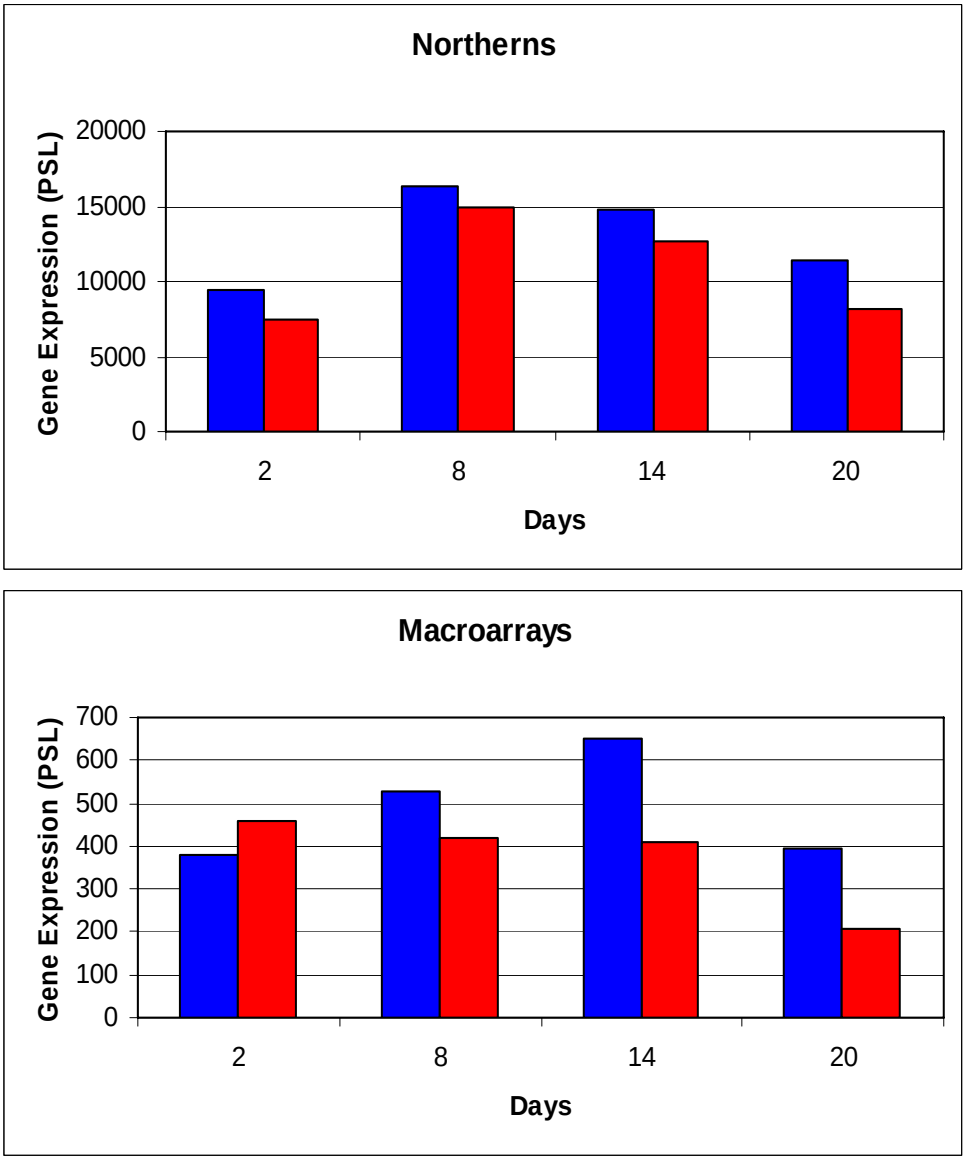


Fig. 44. Mean Cavalier gene expression of genes 15, 30, 39, 64, 65, and 83 for northerns and macroarrays. Controls are blue and treatments are red. Macroarray data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12. Northern data were normalized as described previously.

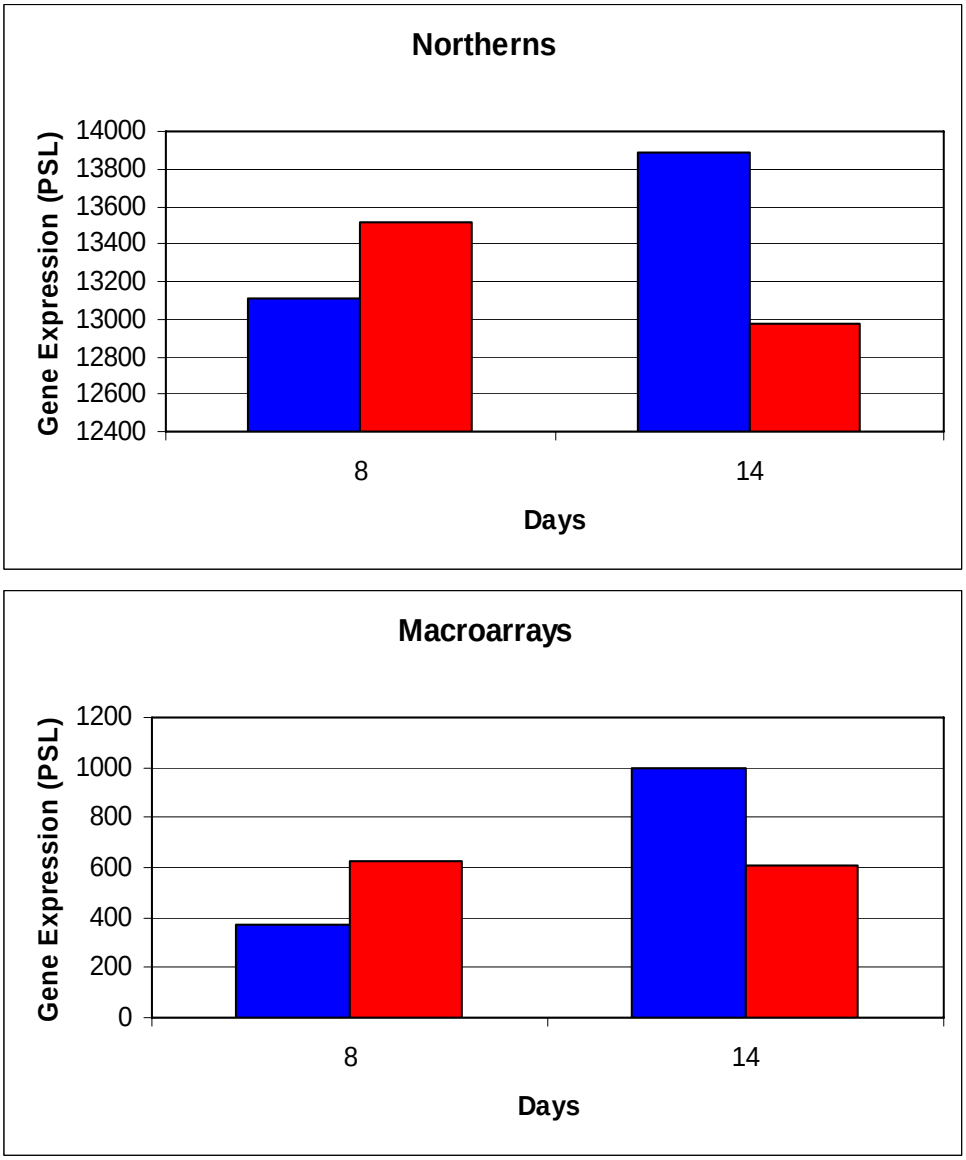


Fig. 45. Mean Palisades gene expression of genes 15, 30, 39, 64, 65, and 83 for northerns and macroarrays. Controls are blue and treatments are red. Macroarray data were normalized using constitutive genes from Table 11 and scintillation counts from Table 12. Northern data were normalized as described previously.

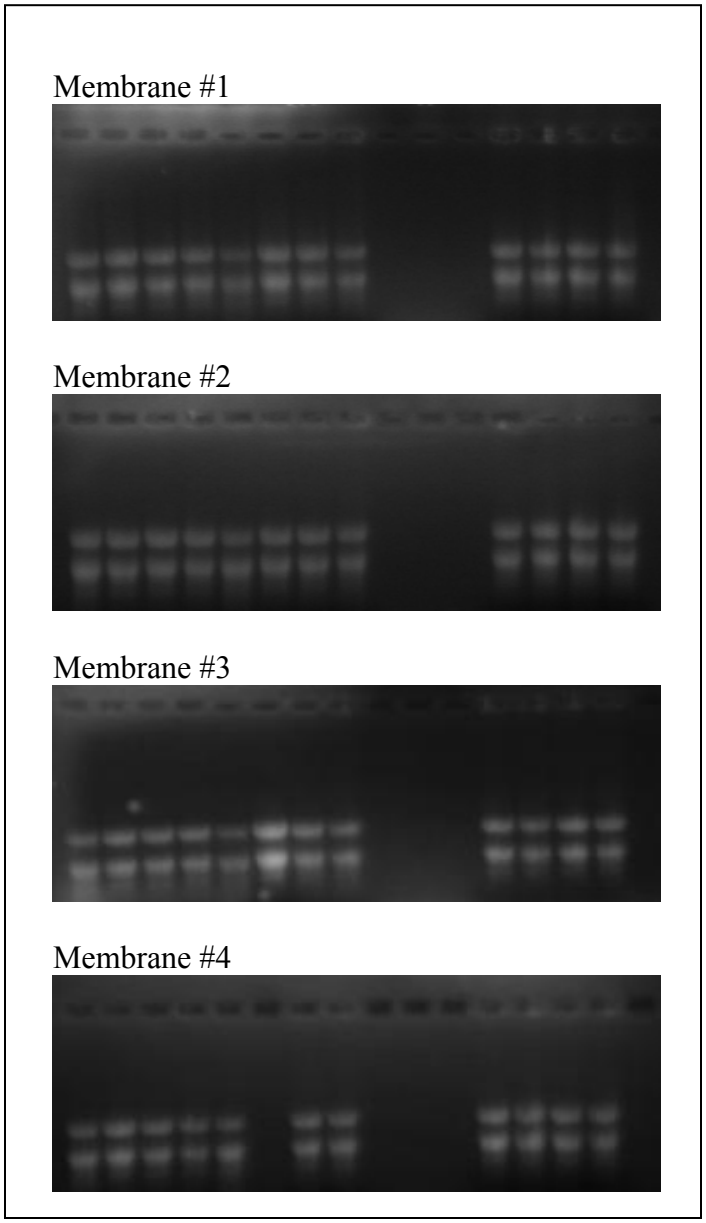


Fig. 46. RNA quantities of the four membranes used in northern analyses. From left to right the first eight wells are Cavalier RNA (control days 2, 8, 14, and 20 followed by treatment days 2, 8, 14, and 20). The last 4 wells are Palisades RNA (control days 8 and 14 followed by treatment days 8 and 14).

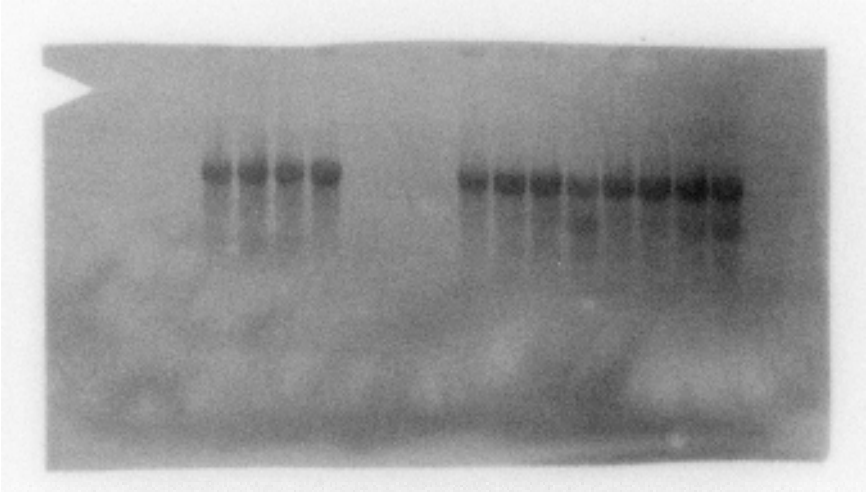


Fig. 47. Northern analysis of gene 15 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #2 was used in this northern.

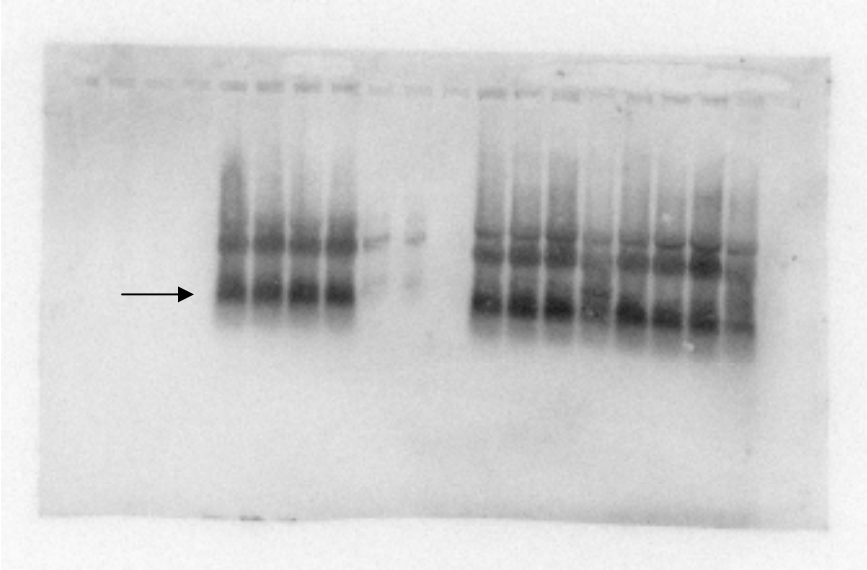


Fig. 48. Northern analysis of gene 30 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #1 was used in this northern and the bottom band was quantified (arrow).

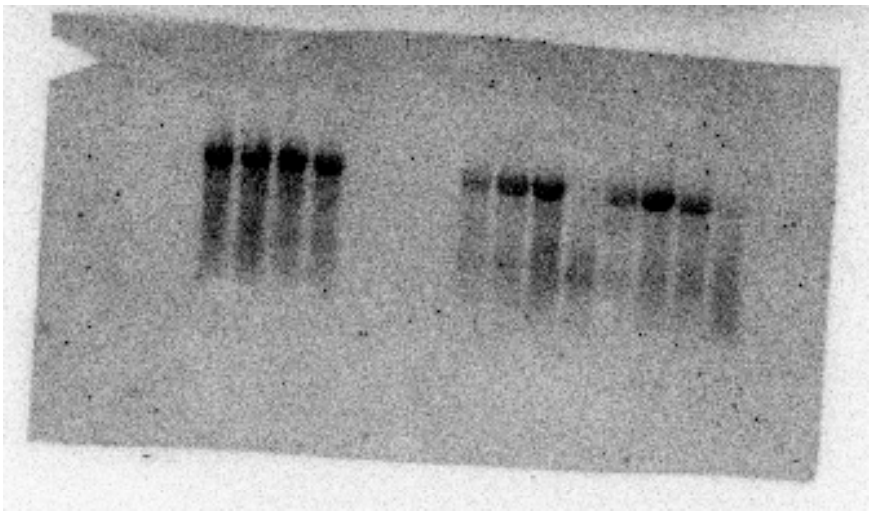


Fig. 49. Northern analysis of gene 39 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #2 was used in this northern after the radioactivity from gene 15 had deteriorated (about 6 weeks).

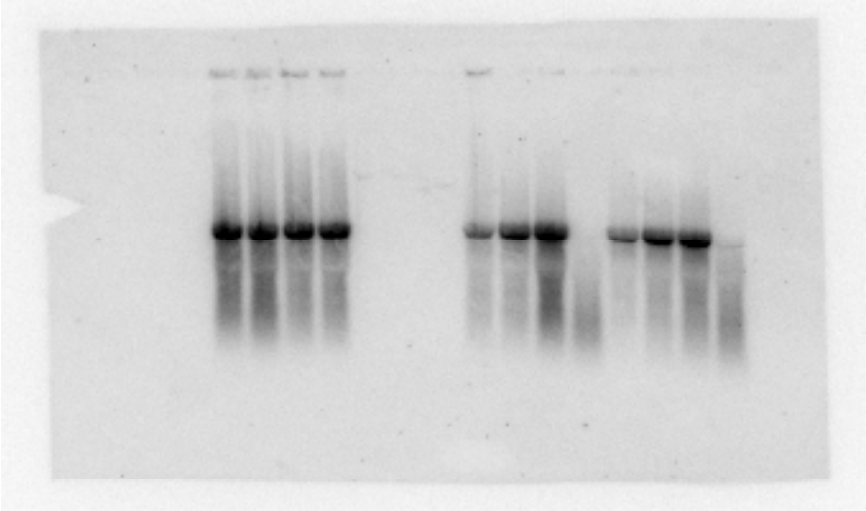


Fig. 50. Northern analysis of gene 64 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #3 was used in this northern after the radioactivity from gene 65 had deteriorated (about 6 weeks).

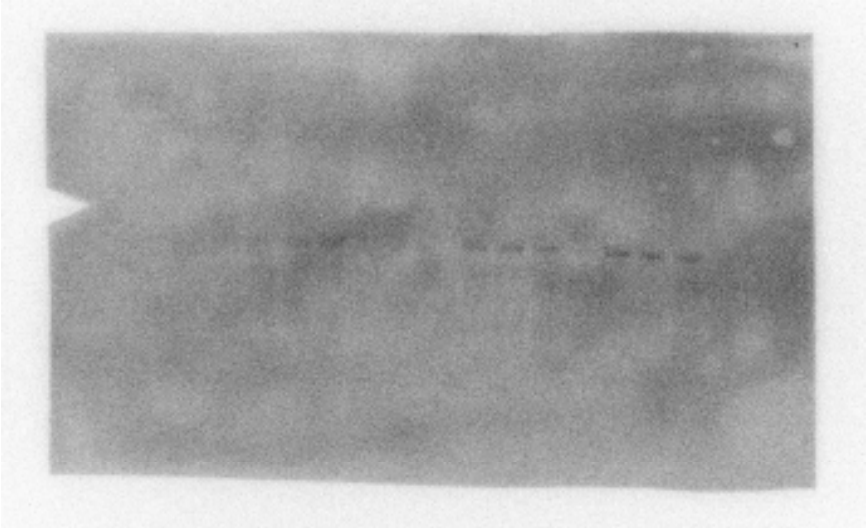


Fig. 51. Northern analysis of gene 65 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #3 was used in this northern.

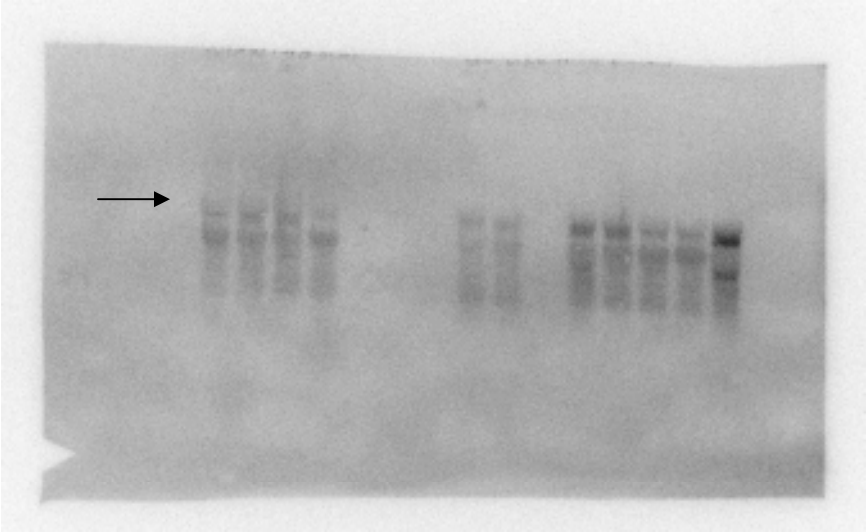


Fig. 52. Northern analysis of gene 83 for Cavalier and Palisades RNA. From left to right the first four wells are Palisades RNA (treatment days 14 and 8 followed by control days 14 and 8). The last eight wells are Cavalier RNA (treatment days 20, 14, 8, and 2 followed by control days 20, 14, 8, and 2). Membrane #4 was used in this northern and the top band was quantified (arrow).

For gene 32, all wild type and SALK_034149 plants were visually healthy after eight days of water stress, but all were wilted after nine days of water stress in repetition one. In repetition two, all wild type and SALK_034149 plants were visually healthy after eight days of water stress, three wild type and three SALK_034149 plants were wilted after nine days, and all plants were wilted after ten days of water stress.

For gene 71, all wild type and SALK_096651 plants were visually healthy after seven days of water stress, three wild type and six SALK_096651 plants were wilted after eight days, and all plants were wilted after nine days of water stress in repetition one. In repetition two, all wild type and SALK_096651 plants were visually healthy after six days of water stress, one wild type and one SALK_096651 plants were wilted after seven days, all but one wild type and all SALK_096651 plants were wilted after eight days, and all plants were wilted after nine days of water stress.

DISCUSSION

Water Relations

The water potential curves for all four cultivars under both non-stressed and water stressed treatments are consistent with other reports of a decrease in plant tissue water, osmotic and/or turgor potential in response to water stress (West et al., 1990; White et al., 1992a; Perdomo et al., 1996; Qian and Fry, 1997; Wang and Huang, 2003; Wang et al., 2003). As the major difference in the curves of repetitions one and two is a shift towards the left in repetition two (experienced water stress sooner), and that shift is consistent across species, the cultivar differences will be discussed with pooled data from both repetitions (Fig. 9 and 10). There were significant cultivar differences and interesting species trends in turgor potential with Diamond losing turgor first (day 14), followed by Cavalier (day 18), while Palisades and DALZ 8504 maintained turgor until day 22. However, once full turgor pressure was lost, the rate of decline in turgor pressure was similar for all cultivars (about 0.06 MPa d^{-1} for all cultivars; Fig. 9), indicating that the key to improving water stress tolerance may be delaying the onset of water stress responses like turgor loss, leaf rolling, or wilting.

Leaf rolling was another parameter used to indicate onset of water stress responses in this experiment with Diamond and Cavalier rolling by day 15, DALZ 8504 by day 19, and Palisades by day 24. Leaf rolling is a water stress tolerance mechanism to reduce transpiration by increasing the boundary layer surrounding leaves. Leaf rolling or wilting have been used to indicate water stress tolerance in many species (Price et al., 1997; White et al., 1992a; Sonesson and Eriksson, 2003; Chandra Babu et al., 2004;

Ebdon and Kopp, 2004, Guenni et al., 2004; Pellegrineschi et al., 2004; Ober et al., 2005). Leaf rolling has also been the focus of molecular research. Price et al. (1997 and 2002) identified quantitative trait loci for leaf rolling in rice, rolled leaf genes have been characterized (Singh and Mackill, 1991; Hu et al., 2002) and transgenic plants containing HVA1 and DREB1A genes have been shown to delay leaf rolling in rice and wheat (Chandra Babu et al., 2004; Pellegrineschi et al., 2004). There was a nine day difference between Palisades and both *Z. matrella* cultivars (Cavalier and Diamond), and a five day difference between Palisades and DALZ 8504 in leaf rolling. These differences in zoysiagrass leaf rolling should provide ample genetic diversity to delay leaf rolling in Cavalier, Diamond, and DALZ 8504 through conventional breeding or through molecular techniques now that *Agrobacterium* transformation of zoysiagrass is available (Toyama et al., 2003).

Surprisingly, loss of turgor did not seem to be very closely correlated with leaf rolling. The leaves of Diamond lost full turgor and rolled at about the same time, while Cavalier and DALZ 8504 leaves rolled 3 d before complete turgor loss. Palisades leaves rolled 2 d after it lost full turgor. Since turgor measurements were taken predawn and the leaf rolling was observed around midday, these results may indicate cultivar differences in rehydration ability. Diamond maintained the lowest full turgor potential of the cultivars under both stressed and non-stressed treatments (Fig. 3-6, 9). Mean turgor potential (days 14 through 32 of both repetitions) of well watered Diamond was 0.85 MPa, while Cavalier, Palisades, and DALZ 8504 averaged 0.92, 0.92, and 1.12, respectively (Fig. 3-6). Having the lowest full turgor may have prohibited Diamond from recovering from midday leaf rolling due to a smaller cushion existing between full and

zero turgor. This, combined with Diamond having the highest turgor loss point (Ψ_{LO}) of the four cultivars (Tables 1 and 2; White et al., 2001), may have caused Diamond cells to cavitate sooner than the other three cultivars. Sufficient cells may have cavitated to prevent recovery of positive turgor by Diamond by the following morning.

Leaves of Cavalier and DALZ 8504 started rolling 3 d prior to loss of full turgor, which may have contributed to turgor maintenance for those 3 d. Cavalier and DALZ 8504 had higher average turgor potential (0.92 and 1.12 MPa, respectively) and more negative turgor loss points (Tables 1 and 2; White et al., 2001) than Diamond. This may have permitted Cavalier and DALZ 8504 to utilize leaf rolling more extensively in maintaining turgor. It is interesting that DALZ 8504 had much higher average turgor potential and a more negative turgor loss point than Cavalier (Tables 1 and 2; White et al., 2001), which may explain why the leaves of DALZ 8504 lost turgor and rolled 4 d after Cavalier.

Some of the most interesting water relations differences occurred among Palisades and DALZ 8504. The leaves of Palisades did not roll in response to water stress for 24 days (5 more days than DALZ 8504), even though both cultivars reached zero turgor on the same day (day 22). Leaf rolling in Palisades may not be turned on until full turgor is lost which would not be the case with DALZ 8504 or the *Z. matrella* cultivars (Cavalier and Diamond). The leaves of DALZ 8504 rolled on day 19 at a turgor potential of 0.93 MPa, while the leaves of Palisades did not roll until day 24 when the turgor potential was 0.75 MPa (Fig. 9). Genetic alterations that delay visual symptoms of water stress by decreasing the turgor potential at which leaf rolling is turned on might

contribute to longer intervals between irrigation events while maintaining turf appearance.

There were differences in osmotic potential between the four cultivars, although none of those differences occurred at positive turgor potentials (Fig. 10). Although not significant, prior to reaching zero turgor, it appears as though the osmotic potentials of *Z. matrella* cultivars (Cavalier and Diamond) decreased more rapidly than the *Z. japonica* cultivars (Palisades and DALZ 8504; Fig. 10), which contributed to the significant differences in turgor potential among the four cultivars (Fig. 9).

These results generally support previous studies on relative water stress tolerance of zoysiagrass cultivars (White et al., 1993a; Marcum et al., 1995; National Turfgrass Evaluation Program, 1995; White et al., 2001). One of the most obvious explanations for the cultivar differences seen in this experiment is root characteristics. Marcum et al. (1995) studied rooting characteristics of 25 zoysiagrass cultivars (three of the four cultivars in this experiment were present in their study) and found significant correlation between percent green cover and maximum root depth, total root mass, and number of roots in deep soil. Of the three cultivars from this experiment, Marcum et al. (1995) found that Palisades had a deeper average maximum rooting depth (318 mm versus 255 and 246 mm for Cavalier and Diamond, respectively), more total root mass (457 mg versus 278 and 270 mg for Cavalier and Diamond, respectively), and more roots in deep soil (8 roots in the 300-500 mm soil depths versus 2.5 and 1.3 for Cavalier and Diamond, respectively) than Cavalier and Diamond. Observations from this experiment concur with the study by Marcum et al. (1995) in that Palisades had the greatest root mass, followed closely by DALZ 8504 and then Cavalier, with Diamond having the least root

mass (unpublished data). Marcum et al. (1995) reported that total root mass and average maximum rooting depth were equally indicative of water stress resistance in zoysiagrass. High total root mass would allow a plant to extract more water from a given soil volume while maximizing rooting depth would allow a plant to extract water from a larger soil volume. In this experiment, maximum rooting depth was 28 cm so differences in total root mass would be the only possible root system morphological contribution to cultivar differences in water stress tolerance. Palisades and DALZ 8504 may have been able to extract more water from the soil volume than Cavalier and Diamond, which would explain the differences in loss of turgor. However, that does not explain the five day difference in leaf rolling between Palisades and DALZ 8504. The cultivars were grown in the same container to theoretically minimize or prevent root system characteristics from influencing plant water status because root systems of all cultivars would be exposed to the same soil water potential (Thomas, 1987). Total root mass and deep rooting are very important, but are not the only water stress avoidance mechanism used by zoysiagrass.

Deep rooting is a common water stress avoidance mechanism for many species, including turfgrass (Sifers and Beard, 1992; White et al., 1993b; Marcum et al., 1995; Huang et al., 1997; Qian et al., 1997; Ervin and Koski, 1998; Bastiah, 1999; Stone et al., 2002; Bonos et al., 2004; Ebdon and Kopp, 2004; Stewart et al., 2004, Johnson, 2005). Having deep roots allows plants to use water from a larger soil volume and can thereby maintain plant function. Shallow rooted species like Kentucky bluegrass and Diamond zoysiagrass can be efficient at extracting soil water, but if roots are only within the upper 30 cm of soil, normal plant function, growth, and survival may be jeopardized once water

is depleted in that soil volume. Johnson (2005) conducted soil moisture studies on buffalograss, Kentucky bluegrass, and tall fescue. He reported that Kentucky bluegrass did not extract water from deeper than 50 cm, whereas buffalograss and tall fescue extracted water from as deep as soil measurements were taken (100 cm). Buffalograss and tall fescue are more water stress tolerant than Kentucky bluegrass (Turgeon, 2002), and may accomplish this through deep root systems that allow buffalograss and tall fescue to avoid water stress (Carrow, 1996b; Qian et al., 1997; Ervin and Koski, 1998; Huang, 1999; Voliare and Lelievre, 2001).

In addition to extracting water from a larger soil volume, deep roots may allow some turf species to redistribute soil water through the phenomenon of hydraulic lift. Hydraulic lift was first observed by Richards and Caldwell (1987) and is a survival mechanism where water is transported from deep roots through shallow roots and into shallow soil at night and then reabsorbed by the plant the following day. Huang (1999) observed diurnal fluctuations in soil water content of shallow soils (0 to 20 cm) when buffalograss and zoysiagrass were supplied with water at deeper soil depths (40 to 80 cm), with the deep-rooted buffalograss exhibiting more pronounced fluctuations than the shallow-rooted zoysiagrass. Hydraulic lift has also been observed in bermudagrass (Baker and Van Bavel, 1986) and other grasses (Yoder and Nowak, 1999; Espelata et al., 2004). As Huang (1999) observed differences between buffalograss and zoysiagrass hydraulic lift, it should be possible to increase zoysiagrass hydraulic lift by selecting for deep rooting. Hydraulic lift was not measured, but may also have contributed to the results of Marcum et al. (1995) as some cultivars had 20 percent of their roots at or below 30 cm.

Another interesting correlation that this experiment confirms is that of zoysiagrass water stress tolerance and leaf characteristics. In this experiment, Palisades was the most water stress tolerant followed by DALZ 8504, then Cavalier, with Diamond being the least water stress tolerant. Both Palisades and DALZ 8504 (*Z. japonica* cultivars) have wide leaves while Cavalier and Diamond (*Z. matrella* cultivars) have narrow leaves (White et al., 1993a). Within each species, Cavalier has longer leaves than Diamond and Palisades has longer leaves than DALZ 8504 (White et al., 1993a). These leaf characteristics seem to be fairly closely correlated to supplemental irrigation requirement (water stress tolerance) with wide leaved cultivars requiring less irrigation than narrow leaved cultivars (White et al., 1993a). Also, within leaf width categories, long leaved cultivars required less irrigation than short leaved cultivars (White et al., 1993a). When the data from White et al. (1993a) are graphed (leaf characteristics against irrigation requirement), the correlation is very good (r^2=0.78), indicating that selection for wider, longer leaves in zoysiagrass may improve water stress tolerance.

The water potential curves and leaf rolling scores from this experiment have illuminated cultivar differences in water stress response. Many possible explanations exist for these differences, some of which, have been addressed. Differences in the full turgor pressure, turgor loss point, turgor pressure at which leaf rolling occurred, root characteristics, and leaf characteristics likely explain these cultivar differences. Of the four cultivars, Palisades was the most water stress tolerant and had the most negative turgor loss point, leaves rolled after turgor loss, had more and deeper roots, and wider and longer leaves (White et al., 1993a; Marcum et al., 1995; White et al., 2001). On the other end of the spectrum, Diamond was the least water stress tolerant and had the lowest full

turgor pressure, the least negative turgor loss point, leaves rolled at positive turgor, had fewer and shallower roots, and narrower and shorter leaves (White et al., 1993a; Marcum et al., 1995; White et al., 2001). The delay of turgor loss and improved water stress tolerance of zoysiagrass should be improved through selection for delayed leaf rolling, wide leaves, long leaves, deep rooting, or high root mass.

Water Relations Characteristics and Water Stress Preconditioning

The main effects of cultivar, days, and treatment were fairly consistent between both the initial water relations characteristics experiment and the preconditioning experiments and as preconditioning had no effect on water relations characteristics, the two experiments will be discussed together. Preconditioning has been shown to improve subsequent water stress tolerance (Oosterhuis and Wullschleger, 1987; Zwiazek and Blake, 1989; Zine El-Abidine et al., 1994; Ruiz-Sanchez et al., 2000; Guarnaschelli et al., 2003). In this experiment, the preconditioned plants were exposed to one cycle of water stress for 21 days, rewatered once, and then immediately exposed to soil drying along with the non-preconditioned plants. The lack of preconditioning effect may have been due to the fact that plants were exposed to only one preconditioning cycle and/or that no substantial recovery time was present before the next soil drying cycle. Most of the reports of preconditioning enhanced performance used several water stress cycles, or an extended period of reduced irrigation to precondition plants (Agnew and Carrow, 1985; Oosterhuis and Wullschleger, 1987; Zine El-Abidine et al., 1994; Jiang and Huang, 2000; Ruiz-Sanchez et al., 2000; Jiang and Huang, 2001a; Guarnaschelli et al., 2003). This may be significant as enhanced rooting has been an explanation for the positive effects of preconditioning in turgrass (Jiang and Huang, 2000; Jiang and Huang, 2001a). In the

present experiment, there may have been an insufficient number of cycles to positively affect the water relations characteristics or insufficient recovery time for enhanced rooting to occur in these cultivars. Another common explanation for the positive effects of preconditioning is solute accumulation (Oosterhuis and Wullschleger, 1987; Zwiazek and Blake, 1989; Jiang and Huang, 2000; Ruiz-Sanchez et al., 2000; Jiang and Huang, 2001a; Guarnaschelli et al., 2003). In the present experiment, the length of the preconditioning treatment (21 days may have caused too much stress) combined with insufficient recovery time may have hindered significantly more solute accumulation in the preconditioned plants than the non-preconditioned plants. Preconditioning would most likely enhance zoysiagrass water stress tolerance if plants were preconditioned with more intervals of a shorter duration.

In general, cultivar differences existed for all the water relations characteristics except TWDW. *Z. matrella* cultivars (Cavalier and/or Diamond) showed less water stress tolerance (lower β, RWC_0, and ε, with less negative ψ_{L0} and $\psi_{\pi 100}$) than the *Z. japonica* cultivars (Palisades and DALZ 8504). These findings generally support those of White et al. (2001), who reported significant cultivar differences in β, RWC_0, ψ_{L0}, $\psi_{\pi 100}$, and ε, with no cultivar differences in TWDW. Results from White et al. (2001) also indicated that the two *Z. matrella* cultivars (Diamond and Cavalier) had lower β, RWC_0, and ε, and less negative ψ_{L0} and $\psi_{\pi 100}$ than the two *Z. japonica* cultivars (Palisades and DALZ 8504). Non-stressed and all water stressed treatments responded similarly to water stress. Since White et al. (2001) did not report water relations characteristics of non-stressed control plants, the response of non-stressed plants observed in this experiment cannot be corroborated.

As β is the proportion of water present in cell walls, it increases in response to stress (Wilson et al., 1980; White et al., 1992a; White et al., 1992b; Martin et al., 1997; White et al., 2001) as a result of cell wall thickening. Cell wall thickening is a water stress response of some species (Cutler et al., 1977; Wilson et al., 1980; Utrillas and Alegre, 1997). As cell walls thicken the volume inside the cells decreases, which decreases the osmotic potential and contributes to osmotic adjustment (Cutler et al., 1977; Wilson et al., 1980). In this experiment, β (combined means for both the water relations experiment and preconditioning experiments) was significantly lower before stress (0.24 g g^{-1}) than after stress (0.29 g g^{-1}), indicating that cell wall thickening occurred. However, it is questionable if this small of an increase had much of an effect on $\psi_{\pi 100}$ (Wilson et al., 1980; White et al., 2001).

Another effect of cell wall thickening in response to water stress is increased ε (Wilson et al., 1980; Auge et al., 1990; Khalil and Grace, 1992; White et al., 1992a; White et al., 1992b; Schultz and Matthews, 1993; Martin et al., 1997; Marur, 1999; Chartzoulakis et al., 2000; White et al,. 2001). As cells thicken they become more rigid (higher ε values), which accounts for small decreases in cell water content even though plant water potential may be dramatically lower. This allows plants to extract more soil water due to an increase in the water potential gradient between soil, roots, and leaves without losing much cell water (Kramer and Boyer, 1995; Dichio et al., 1997). In this experiment, ε (combined means for both the initial water relations experiment and preconditioning experiment) was lower before stress (5.24 MPa) than after stress (5.7 MPa). This increase in cell rigidity is an interesting response as reductions in cell rigidity (more elastic) have also been observed in response to water stress (Meinzer et al., 1988;

Conover and Sovonick-Dunford, 1989; Blake and Bevilacqua, 1991; Jensen et al., 1992; White et al., 2000). Reduced ε (more elastic cells) allows cell wall flexing to maintain turgor potential (Kozlowski et al., 1990). Whether cells become more elastic or rigid appears to be species specific and this experiment concurs with White et al. (2001) indicating that zoysiagrass cells become more rigid (cell wall thickening) in response to water stress.

Increases in RWC_0 are also associated with cell wall thickening in response to water stress (White et al., 1992a; White et al., 1992b; Kloeppel et al., 1994). Although not significant, White et al. (2001) reported increases in RWC_0 in response to water stress in zoysiagrass. High values of RWC_0, as well as β and ε, are indicative of water stress tolerance in zoysiagrass (White et al., 2001). In this experiment, RWC_0 (combined means for both the water relations experiment and preconditioning experiments) was significantly lower before stress (0.66 g g^{-1}) than after stress (0.70 g g^{-1}). As was mentioned previously, rigid cells can reduce their water potential drastically with small reductions in water content. This means that rigid cells will approach zero turgor with smaller reductions in water content than elastic cells and therefore have a higher RWC_0.

Leaf water potential at zero turgor reflects how well a plant manages water stress since more negative ψ_{L0} allows a plant to maintain turgor longer during water stress (White et al., 2001). In this experiment, ψ_{L0} was significantly more negative before stress (-2.24 MPa) than after stress (-2.17 MPa) in the water relations experiment and not significantly different before (-2.01 MPa) and after stress (-2.05 MPa) in the preconditioning experiment. As these data are not consistent, the water relations experiment numbers are barely significant (P=0.04), and the water release experiment

numbers are contrary to what should happen (ψ_{L0} should become more negative in response to water stress; Wilson et al., 1980; Jensen and Henson, 1990; White et al., 1992a; White et al., 2001), the before stress value for ψ_{L0} is suspect and the results from the preconditioning experiment will be the focus of further discussion. The difference between before and after stress is 0.04 MPa, which is much smaller than the difference of 0.27 MPa observed by White et al. (2001). However, this difference in zoysiagrass is similar to the differences in ψ_{L0} of tall fescue observed by White et al. (1992a and 1992b). White et al. (1992a) reported a significant reduction (0.04 MPa) of ψ_{L0} in response to water stress, while White et al. (1992b) reported a non-significant reduction (0.05 MPa) of ψ_{L0} in response to water stress. Although not significant, the 0.05 MPa reduction of ψ_{L0} in this experiment, combined with the results of White et al. (2001), show that zoysiagrass does respond to water stress by reducing ψ_{L0}.

Negative shifts of $\psi_{\pi 100}$ in response to water stress indicate active osmotic adjustment (Wilson et al., 1980, Jensen and Henson, 1990; Jensen et al., 1992; White et al., 1992b; Marur, 1999; Guarnaschelli et al., 2003). Osmotic adjustment has been shown in turfgrass species (Qian and Fry, 1997; Jiang and Huang, 2001a; Wang et al., 2003). Active osmotic adjustment occurs through increases in β, decreases in TWDW, and/or solute accumulation (Wilson et al., 1980). Osmotic adjustment during water stress maintains positive turgor as leaf water potential decreases (Nilsen and Orcutt, 1996). In this experiment, $\psi_{\pi 100}$ was slightly less negative before stress (-1.24 MPa) than after stress (-1.26 MPa) in the water relations experiment and significantly less negative before stress (-1.12 MPa) than after stress (-1.19 MPa) in the preconditioning experiment. This is not conclusive evidence for osmotic adjustment in zoysiagrass. Qian and Fry (1997)

reported osmotic adjustment in zoysiagrass, but White et al. (2001) found no significant osmotic adjustment of zoysiagrass in response to water stress. It is also interesting to note that significant osmotic adjustment has been present ($P<0.001$) and absent (non-significant) in the same three cultivars of tall fescue, submitted to the same water stress treatment, studied at the same time, and reported by the same author (White et al,. 1992a; White et al., 1992b). The apparent disparities in zoysiagrass and tall fescue are most likely due to differences in variance as the means for osmotic adjustment were identical in both tall fescue studies (White et al., 1992a; White et al., 1992b). In this experiment, mean osmotic adjustment for the two experiments was 0.05 MPa. White et al. (2001) reported a non-significant mean osmotic adjustment of 0.10 MPa for the same four cultivars. Osmotic adjustment appears to be functioning in zoysiagrass, but may not be as strong of a water stress response as in other species.

Turgid weight/dry weight ratios measure the change in cell wall thickness or constituents (Cutler et al., 1977; Wilson et al., 1980; Liu and Stutzel, 2002). In this experiment, TWDW showed the most consistent and significant response in both the water release and preconditioning experiments (both P values were less than 0.001) between before and after water stress. As there were no significant cultivar differences in either experiment, it appears that reducing TWDW is a fairly consistent water stress response in zoysiagrass. In this experiment, TWDW (combined means for both water release and preconditioning experiments) was significantly higher before stress (3.21) than after stress (2.91). Reductions in TWDW are a common water stress response (Nus and Hodges, 1985; Myers and Neales, 1986; Rascio et al., 1988; Khalil and Grace, 1992; White et al., 1992a; White et al., 1992b; Liu and Stutzel, 2002; Guarnaschelli et al., 2003;

Martinez et al., 2004). It is interesting that White et al. (2001) found no significant difference in TWDW between before and after water stress, and yet found significant differences in β and ε, as all three are associated with cell wall thickening. Although not significant, TWDW of the four cultivars from this experiment did decrease in the study by White et al. (2001) from 2.59 before to 2.33 after water stress. The change in TWDW in response to stress is similar for both studies (0.3 reported in this experiment and 0.26 reported by White et al. (2001)), indicating that the apparent disparity between the two studies is, most likely, an artifact of variance of the data and not contradictory.

Reductions in TWDW and increases in β, ε, and RWC_0 are all related to cell wall thickening. Thicker cell walls have higher dry weights which reduces their TWDW. Thicker cell walls mean more water bound in those walls (higher β). Thicker cell walls are also more rigid than thin cell walls (higher ε) and high rigidity allows for dramatic reductions in turgor potential with small decreases in cell water content. This means that when rigid cells reach zero turgor, they will have higher water content than more elastic cells. As was mentioned previously, species, like zoysiagrass, can respond to water stress by increasing cell wall thickness (lower TWDW, higher β, ε, and RWC_0). Another major mechanism for avoiding water stress seems to be cell wall relaxation (increase TWDW, decrease ε and RWC_0). This permits turgor maintenance and may be associated with shorter and/or faster water stress. da Silva and Arrabaca (1999) reported that slowly dehydrated bristlegrass responded as zoysiagrass by increasing ε and decreasing TWDW (cell wall thickening). However, when bristlegrass was dehydrated rapidly, it responded conversely by decreasing ε and increasing TWDW. Martin et al. (1997) also found that increases in β and ε were more pronounced in slowly stressed than in rapidly stressed

wheat. It may be that cell wall relaxation is a short term water stress response to maintain turgor, cell wall thickening is a longer term water stress response but a species can use both (da Silva and Arrabaca, 1999), or one can be used to varying degrees (Martin et al., 1997) depending on the severity and/or length of water stress. Apparent disparities within a species could very well be explained by differential rates of soil drying (da Silva and Arrabaca, 1999) or be attributed to differential variance between studies (White et al., 1992a; White et al., 1992b).

As all four cultivars responded to water stress (measured by significant differences in β, RWC_0, ψ_{L0}, $\psi_{\pi 100}$, ε, and TWDW), it is astonishing that there were no differences among non-stressed and water stressed plants for any of the six parameters of either the water relations or preconditioning experiments. This means that the control plants were responding in the same manner to the water stress as the water stressed plants. This phenomenon is most easily seen in the TWDW values from Tables 1 and 2. The control plants of every single cultivar from both experiments had significantly higher TWDW before water stress was imposed on plants in adjacent tubs than after water stress had been imposed on plants in adjacent tubs. The Diamond values from Table 1 were not significant, but the difference in TWDW between before and after was similar to the significant differences of Cavalier and DALZ 8504. Control tubs were not water stressed (Fig. 1 and 11) and had no physical contact with water stressed plants in immediately adjacent tubs. Significant decreases in TWDW of control plants when adjacent plants are water stressed have been observed in other studies (Jensen et al,. 2000; Liu and Stutzel, 2002; Martinez et al., 2004). Liu and Stutzel (2002) measured TWDW of well watered and water stressed amaranth during water stress and after re-irrigating water stressed

plants. Well watered amaranth plants showed significant decreases in TWDW which increased once water stressed neighbors were re-watered (Liu and Stutzel, 2002). The study by Liu and Stutzel (2002) concurs with the results from the present water stress signaling experiment that demonstrated increases in TWDW of non-stressed Kentucky bluegrass after adjacent, water stressed plants were removed. The TWDW provide the most convincing evidence of non-stressed plants responding to neighboring water stressed plants, but the similarity among non-stressed and water stressed plants for β, RWC_0, ψ_{L0}, $\psi_{\pi 100}$, and ε is also strong evidence that the water stressed plants were communicating with the control plants. Since the non-stressed and water stressed plants had no physical contact the most likely explanation for the water stress response of well watered plants is through airborne signaling, which will be discussed in more detail subsequently.

The water relations and preconditioning experiments yielded insights into how zoysiagrass responds to stress. As there were significant cultivar differences for five of the six water relations characteristics measured, improvements in water stress tolerance should be attainable by increasing, or selecting for, high β, highRWC_0, high ε, low ψ_{L0}, low $\psi_{\pi 100}$, and/or low TWDW (White et al., 2001). Zoysiagrass appears to respond to slow soil drying by increasing cell wall thickness and osmotically adjusting, which allows zoysiagrass to maintain turgor and relative water content while maximizing soil water extraction. Perhaps the most interesting finding from these studies was the apparent response of non-stressed plants to water stress imposed on adjacent plants. There was no difference between control and treatment plants in any of the six water relations characteristics measured in either the water relations or preconditioning

experiments. These data, along with the significant decrease in TWDW of well watered Kentucky bluegrass, adjacent to stressed Kentucky bluegrass, and subsequent increase in TWDW once the water stressed plants were removed, are strong evidence that airborne water stress signaling is occurring in both cool- and warm-season turfgrasses.

Water Stress Signaling

As very little, if any, literature exists on plant water stress signaling, the results from the water stress signaling experiment, combined with the results from the water relations and preconditioning experiments warrant further exploration on this subject. The only parameter that changed throughout the entire experiment was the presence or absence of adjacent water stressed plants. The well watered plants were not clipped so leaf area also increased. If increased leaf area were the cause of the difference in TWDW between the before and after stress measurements, then the recovery measurements should not have increased because there was even more leaf area when those measurements were taken than when the after stress measurements were taken. Significant decreases in TWDW of control plants in proximity to water stressed plants have been reported for field quinoa (Jensen et al., 2000), saltbush (Martinez et al., 2004), and vegetable amaranth (Liu and Stutzel, 2002), with increases in TWDW occurring once water stressed plants were re-watered (Liu and Stutzel, 2002). Hence, the results from zoysiagrass and Kentucky bluegrass are not unique and beg the question, "Why?"

It was surprising that no differences in TWDW were observed between any of the three well watered groups, indicating that plants at a distance of one meter were receiving sufficient signal to cause a response equal to that of the well watered plants immediately adjacent to the water stressed plants. This may indicate that well watered plants are fairly

sensitive to the signal and/or that the water stressed plants are sending out a fairly strong signal. Farmer (2001) reported no detectable levels of methyl jasmonate at a distance of 3 m from sagebrush, indicating that the well watered plants from the present study were likely within an appropriate distance from the water stressed plants to receive a signal.

Plants communicate through airborne signals in response to their surroundings. In recent years, a widely published phenomenon is that of airborne signals inducing pest defense responses in some plants (Farmer and Ryan, 1990; Miksch and Boland, 1996; Shulaev et al., 1997; Arimura et al., 2000; Tscharntke et al., 2001; Hudgins and Franseschi, 2004). Farmer and Ryan (1990) observe that unsprayed tomato plants increased proteinase inhibitors, which are herbivore defense proteins that interfere with herbivore digestion, when neighboring plants were sprayed with methyl jasmonate. Three of the most commonly studied volatile compounds that have been shown to induce plant stress/defense responses are ethylene (review by Morgan and Drew, 1997), methyl jasmonate (Farmer and Ryan, 1990; Pan and Gu, 1995; Miksch and Boland, 1996; Wang, 2000; Tscharntke et al., 2001; Hudgins and Franseschi, 2004), and methyl salicylate (Shulaev et al., 1997). Methyl jasmonate and methyl salicylate are the methylated (volatile) forms of jasmonic and salicylic acids. Application of methyl jasmonate, jasmonic and salicylic acids, have been shown to improve water stress tolerance (Yao et al., 1999; Senaratna et al., 2000; Wang, 2000; Hamada and Al-Hakimi, 2001; Munn-Bosch and Penuelas, 2003; Singh and Usha, 2003; Gao et al., 2004; Huang et al., 2004). A common salicylic/jasmonic acid induced mechanism for improved abiotic stress tolerance is enhanced antioxidant activity, measured as increases in photochemical efficiency and/or antioxidant levels (Yao et al,. 1999; Senaratna et al., 2000; Wang, 2000;

Munne-Bosch and Penuelas, 2003; Singh and Usha, 2003; Ervin et al., 2005). Salicylic acid has been associated with improved tolerance and antioxidant activity for many abiotic stresses including water stress (Senaratna et al,. 2000; Hamada and Al-Hakimi, 2001; Singh and Usha, 2003; Munne-Bosch and Penuelas, 2003), heat stress (Senaratna et al., 2000; Ervin et al., 2005), salt stress (Hamada and Al-Hakimi, 2001), and high UV-B (Schmidt and Zhang, 2001; Ervin et al., 2004). In turfgrass, Jiang and Huang (2001b) reported that both heat and water stress injuries were associated with decreases in antioxidant activities of tall fescue and Kentucky bluegrass. Several other studies have reported improved tolerance and/or antioxidant activity of Kentucky bluegrass, tall fescue, and creeping bentgrass to heat or UV-B stress through applications of salicylic acid (Schmidt and Zhang, 2001; Ervin et al., 2004; Ervin et al,. 2005). As water stress decreases antioxidant activities in tall fescue and Kentucky bluegrass, it is likely that salicylic acid applications would have a positive effect on water stress tolerance of turfgrasses. Jasmonic acid, or methyl jasmonate, have also been shown to improve water stress tolerance (Pan and Gu, 1995; Li et al., 1998; Yao et al., 1999; Wang, 2000; Bandurska et al., 2003; Gao et al., 2004; Lan et al., 2004). Jasmonic acid has been shown to increase abscisic acid (Pan and Gu, 1995; Bandurska et al., 2003; Rakwal and Komatsu, 2004) antioxidant (Yao et al., 1999; Wang, 2000), and solute (Pan and Gu, 1995; Gao et al., 2004) levels in response to water stress.

Jasmonic and salicylic acids have been discussed previously with respect to water stress, and one, or both, likely plays an important role as a signaling molecule in water stress tolerance of some species through increasing antioxidant activities and/or stomatal control. If jasmonic or salicylic acid is a major signaling molecule in water stress

responses, it is plausible that methyl jasmonate or salicylate is an airborne signaling molecule for water stress. This would explain the consistent response of well watered zoysiagrass and Kentucky bluegrass plants in three experiments conducted at three different times of year and in two disparate locations (Texas and Idaho) to water stress imposed on neighboring plants. As jasmonic and salicylic acids seem to be antagonistic (Doares et al., 1995; Petersen et al., 2000; Kloek et al., 2001; Gong et al., 2003), it would be surprising if methyl jasmonate and methyl salicylate were simultaneous water stress signaling molecules. As both salicylic and jasmonic acid enhance antioxidant activities, their use as signaling molecules is most likely species or stress specific.

The water stress signaling experiment, combined with the results from the water relations and preconditioning experiments provide evidence that well watered turfgrass plants respond to airborne signals from adjacent water stressed plants. The fact that each well watered plant had no physical contact with water stressed plants precludes everything but an airborne signal. Airborne water stress signaling would be of great advantage to plants in arid and semi-arid environments as they could prepare for impending water stress. Gas chromatograph experiments will have to be conducted to confirm the presence and identification of any volatiles given off by water stressed plants, but an airborne water stress signal is the most reasonable explanation for the water stress responses of well watered plants observed in this experiment.

Gene Expression

There were no differences in soil moisture between the two tubs from which plant material was taken for the first and second rounds of macroarrays (Fig. 18), making it unlikely that the lack of difference between non-stressed and stressed plant tissue arrays

seen in the second round of macroarrays was due to differences in soil moisture. The only other difference between the two rounds was that RNA for the first round was pooled (days 8, 14, and 20) whereas RNA for the second round was not. Unless more treatment RNA was inadvertently added to the pooled RNA, this is an unlikely explanation. The only other difference between the two experiments was the omission of hundreds of cDNAs in the second round. In Table 10, the first round was the mean of 768 cDNAs while the second round was the mean of 115 genes. If collectively, the cDNAs that were omitted from the second round showed more expression in water stressed than non-stressed plants, that may explain some of the difference seen in the first and not the second round.

As there is very little, if any, literature on large scale gene expression in turfgrass, discussion will focus on literature in other monocots. A lot of the literature focuses on one species over a fairly short period of time (Ozturk et al., 2002; Rabbani et al., 2003; Zheng et al., 2004). Way et al. (2004) did study differential gene expression with a gradual water stress treatment (12 days), but only for one variety of wheat. Of the above-mentioned articles, only the article by Zheng et al. (2004) provided array data from more than two time points. Zheng et al. (2004) reported array data from maize leaves after 24, 48, 54, and 72 hours of water stress, with all plants being "deeply" wilted by 54 hours. Zheng et al. (2004) also reported that the magnitude of gene expression did not change after 54 hours. In this experiment, it is unlikely that changes in gene expression on day 20 were significant water stress responses for Cavalier and Diamond as they were severely wilted by that point. Zheng et al. (2004) also found that genes were turned on at specific times and may have been turned on at only one point and were turned off at

subsequent time points. This was also observed in the current experiment with some genes being turned on at only one time point (Tables 13-16). The results from Fig. 40-42 should therefore be considered as broad generalizations and individual genes should be the focus of more in depth discussion. However, Fig. 40-42 do show some interesting trends. The non-stressed and water stressed plant of the least water stress tolerant cultivars within each species (Diamond and DALZ 8504) responded similarly on the days where water stress was most likely affecting gene expression (days 8 and 14). The lack of generalized response of the two susceptible cultivars may contribute to their susceptibleness. As DALZ 8504 was more tolerant than Diamond, the barely non-significant difference seen on day 8 (Fig. 42) may actually contribute to the difference in water stress tolerance between the two cultivars. The lack of difference between non-stressed and water stressed Diamond and DALZ 8504 may also have been affected by the fact that they were not hybridized to their own cDNAs, but were hybridized to Cavalier and Palisades cDNAs. This is unlikely as there were five pairs of genes on the membranes where one gene was from the Cavalier cDNA library and the other was from the Palisades cDNA library. Mean gene expression for the five pairs of genes showed no significant difference between the cDNAs from the two libraries for any of the four cultivars (unpublished data).

It is interesting that such a ubiquitous physiological water stress response was observed in non-stressed plants yet only Cavalier demonstrated any generalized gene expression difference among days for non-stressed plants. This is especially interesting as the results from Fig. 44 and 45 show a steady increase in gene expression of non-stressed plants from day 2 to day 14. This illustrates the need to analyze individual genes

when exploring water stress response and using arrays to identify genes of interest. Generalized gene expression can be useful but care should be taken to assure that individual genes are not masked by the mean gene expression.

Of the 59 genes of interest that showed differential expression among the four cultivars (Table 17), there are several groups of genes that are of particular interest. Genes that are expressed in every cultivar except Diamond may help explain why Cavalier, Palisades, and DALZ 8504 had higher turgor potential and/or utilized leaf rolling more effectively. Genes that are expressed only in Cavalier and/or Palisades may help explain the differences between water stress tolerant and susceptible cultivars. Genes that are expressed only in Palisades and DALZ 8504 (*Z. japonica* cultivars) may help explain why those cultivars did not lose full turgor until 4 d after Cavalier and 8 d after Diamond. Genes that are only expressed in Palisades may help explain why that cultivar did not show any visual symptoms of water stress until 24 d after water stress was induced. Genes that were expressed in all four cultivars may be useful by comparing differences in induction levels between the four cultivars. As there is significant overlap in salinity and water stress gene expression (Seki et al,. 2002), many genes associated with salt stress found in this experiment may also play a role in the water stress response.

There were only three genes that were turned on in every cultivar except Diamond (Table 17). Genes 11, 46, and 94 (photosystem 1 assembly, tetracycline transporter, and novel proteins, respectively) were not turned on in response to water stress in Diamond. These may be of interest but a connection to decreased water stress tolerance is not immediately obvious.

Twenty-two of the 59 genes were only turned on in one or both of the most water stress tolerant cultivars, Cavalier and Palisades (Table 17). Of those 22 genes, ten have previously been associated with stress responses (genes 12 (hypothetical protein that may be related to a water stress inducible gene; Dubos et al., 2001), 13 (extensin that responds to osmotic stress; Dopico et al., 1998), 14 (manganese superoxide dismutase (ROS scavenger)), 15 (senescence associated protein), 31 (hydroxypyruvate reductase that responds to salt; Banzai et al., 2001), 42 (S-adenosylmethionine synthetase 2 that responds to salt stress; Espartero et al., 1994), 88 (oxidase that responds to salt stress; Kong et al., 2003), 93 (same senescence associated protein as gene 15), 100 (senescence/dehydration-associated protein), and 111 (elongation factor 1 that is cold regulated; Baldi et al., 2001)). The expression of antioxidant genes is of particular interest because this may indicate differences in the ability of cultivars to deal with oxidative stress. Many of the senescence associated proteins identified in this experiment were most likely superoxide dismutases based on BLASTx results. Higher levels of antioxidants or extended antioxidant presence have been associated with improved water stress tolerance in turfgrasses (Zhang and Schmidt, 2000; Ge et al., 2004). Li et al. (1998) also reported that water stress tolerant cultivars of maize seedlings had higher antioxidant activity than susceptible cultivars when treated with methyl jasmonate. As methyl jasmonate is an airborne signaling molecule, it is also possible that antioxidant activity in control plants increased along with the physiological parameters measured in this experiment.

Five of the 59 genes were only turned on in the two *Z. japonica* cultivars (Palisades and DALZ 8504) (Table 17). Of those five genes (19, 20, 21, 41, and 75),

genes 41 and 75 are both phospholipases that have been associated with systemic acquired resistance (Song and Goodman, 2002) and water stress (Frank et al., 2000), respectively. Gene 41 is a phospholipase C while gene 75 is a phospholipase D. Both phospholipases have been associated with abscisic acid mediation of guard cell activity (Jacob et al., 1999; Staxen et al., 1999; Wang 2001). Besides abscisic acid, phospholipase D has also been shown to mediate the action and production of jasmonic acid and ethylene involved in stress response (Wang, 2001). Abscisic acid activates phospholipase D, which in turn stimulates phosphatidic acid, which in turn induces stomatal closure (Jacob et al., 1999). Phospholipase D accumulation in response to water stress has been well documented (Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001). Enhanced phospholipase D activity has been shown to increase abscisic acid sensitivity (Sang et al., 2001) indicating that plants with higher phospholipase D activity can respond to a much smaller abscisic acid signal. Water stress tolerance has been associated with depressed abscisic acid accumulation in Kentucky bluegrass (Wang et al., 2004), maize (Landi et al., 2001), maple (Bauerle et al., 2004), and wheat (Innes et al., 1984). In turfgrass, Wang et al. (2004) reported that water stress tolerant cultivars of Kentucky bluegrass accumulated less abscisic acid than water stress sensitive cultivars. This is most likely because water stress tolerant cultivars can increase their phospholipase D and phosphatidic acid levels and therefore close their stomata with much less of an abscisic acid signal. Better stomatal control, through increased phospholipase activity and therefore increased sensitivity to abscisic acid, of Palisades and DALZ 8504 than Cavalier and Diamond during water stress may very easily explain the differences in the number of days before the two species lost full turgor. Diamond and Cavalier lost full

turgor on days 14 and 18 respectively while Palisades and DALZ 8504 did not lose full turgor until day 22. Enhanced water stress tolerance of Diamond and Cavalier may be attained through improving stomatal sensitivity to water stress and abscisic acid accumulation is very possibly a valid selection criteria for water stress tolerance in turfgrass.

Eight of the 59 genes were only turned on in Palisades, the most water stress tolerant cultivar (Table 17). Of those eight (genes 13, 15, 34, 37, 42, 97, 100, and 111), five have been associated with stress tolerance in other studies (genes 13 (extensin that responds to osmotic stress; Dopico et al., 1998), 15 (senescence associated protein), 42 (S-adenosylmethionine synthetase 2 that responds to salt stress; Espartero et al., 1994), 100 (senescence/dehydration-associated protein), and 111 (elongation factor 1 that is cold regulated; Baldi et al., 2001)). The most unique physiological aspect of Palisades water stress tolerance in this experiment was the delayed visual stress symptoms. One, or several, of these genes may be associated with leaf rolling, although that is not immediately apparent. A common adaptation to surviving under reduced water conditions is senescence (Taiz and Zeiger, 2002). As Palisades recovers better than the other three cultivars (White et al., 2001), the enhanced expression of senescence associated proteins may be a Palisades adaptation to minimize leaf area and help the plant survive until the water stress is alleviated and plant growth can resume.

Nine of the 59 genes were expressed in all four cultivars (Table 17). Of those nine (gene 10, 38, 39, 51, 53, 55, 64, 91, and 106), only gene 64 (phosphoenolpyruvate carboxykinase) has been associated with stress tolerance (cold inducible; Saez-Vasquez, 1995). As six of the nine showed no or very minimal similarity with any known

sequence in the BLASTx searches, these may indicate novel water stress inducible genes in zoysiagrass. When the mean gene expression for the nine genes is graphed for all four cultivars, there are no obvious differences as may have been expected (for example, Diamond having lower expression than Palisades) (unpublished graph compiled from information in Tables 13-16).

There were a large number of genes that were up regulated in well watered control plants between days two and eight. Cavalier had 41 genes that were significantly up regulated between day two and eight while Diamond, Palisades, and DALZ 8504 had 15, 22, and 9, respectively. There were only two genes that showed significant up regulation in control plants for three or all four cultivars. Gene 18, a systemin degrading enzyme, (Strassner et al., 2001) was significantly up regulated by day eight in every cultivar except DALZ 8504 and gene 36, an integral membrane protein, (Sasaki et al., 2002) was significantly up regulated by day eight in all four cultivars. Since integral membrane proteins are signal receptors, it is fascinating that a systemin degrading enzyme and a signal receptor protein are the only two genes turned on in the well watered control plants of almost all of the cultivars. As neither gene 36, nor the protein identified by Sasaki et al. (2002) have been characterized, only speculations as to its function can be made. It is possible that gene 36 is another systemin receptor (although one receptor has already been identified; Yin et al., 2002) and that systemin is signaling jasmonic acid induced water stress responses in well watered zoysiagrass plants. Gene 36 may also be a receptor involved in the antioxidant response involving salicylic acid and methyl salicylate (Taiz and Zeiger, 2002). The up regulation of a systemin degrading enzyme and a membrane receptor protein, along with the physiological responses discussed

previously by well watered zoysiagrass and Kentucky bluegrass, are compelling evidence of an airborne signal (most likely methyl jasmonate or methyl salicylate) given off by water stressed turfgrasses that elicits water stress responses in neighboring well watered turfgrass plants.

The *A. thaliana* knockout experiment was designed to target genes that have not previously been associated with water stress responses. Since there were no differences between the transgenic and wild-type seedlings in their response to water stress, a number of possible conclusions may be drawn. Either the genes do not drastically affect water stress tolerance, or the effect may be specific to monocots. Also, there may be some functional redundancy that is masking the knockout effect of the genes. As these genes were chosen because their sequence was unique in the *A. thaliana* genome, the most likely answer is that neither of the genes plays a major role in water stress tolerance of A. *thaliana*. Likewise, neither of the genes was present in Table 17 indicating that they were likely not as important as some of the genes present in Table 17 in water stress tolerance of zoysiagrass.

The gene expression experiments show some interesting results that confirm, and may explain, some of the differences observed in the water relations and preconditioning experiments. It appears that antioxidant activity is a zoysiagrass response to water stress. As antioxidant levels or presence have been associated with water stress tolerance, antioxidants may be used to identify, or select for, water stress tolerant zoysiagrass cultivars since there was significant variation in expression of genes coding for antioxidant activity among the four cultivars (Table 17). Another significant difference elucidated by the gene expression experiment was the species difference in phospholipase

gene expression. This difference may very well explain the marked difference in the number of days until full turgor was lost since phospholipase D is directly involved in stomatal control with higher levels of phospholipase D activity indicating enhanced abscisic acid sensitivity, stomatal control, and water stress tolerance. The most intriguing outcome of the gene expression experiment was the almost ubiquitous increase in the expression of genes that code for signaling related proteins including a systemin degrading enzyme and an integral membrane protein, or receptor, in non-stressed plants. The data from the gene expression and the water relations and preconditioning experiments are strong evidence of plant-to-plant communication between water stressed and non-stressed zoysiagrasses.

CONCLUSIONS

The water relations characteristics and leaf rolling scores illuminated cultivar differences in water stress response. Palisades and DALZ 8504 did not approach zero turgor until much later than Cavalier and Diamond. Diamond did not appear to utilize leaf rolling as a mechanism of water stress tolerance. Leaves of Cavalier and DALZ 8504 rolled prior to full turgor loss while the leaves of Palisades did not roll until after full turgor loss. Of the four cultivars, Palisades was the most water stress tolerant and had the most negative turgor loss point, and leaf rolled after loss of full turgor pressure. On the other end of the spectrum, Diamond was the least water stress tolerant and had the lowest full turgor pressure, the least negative turgor loss point, and leaf rolled at full turgor. Through selection for delayed leaf rolling, wide leaves, long leaves, deep rooting, or high root mass, researchers should be able to delay loss of full turgor and/or improve water stress tolerance of zoysiagrass.

The water relations characteristics and preconditioning experiments yielded insights into how zoysiagrass responds to stress. As there were significant cultivar differences between five of the six parameters measured, improvements in water stress tolerance should be attainable by increasing, or selecting for, high β, high RWC_0, high ε, low ψ_{L0}, low $\psi_{\pi 100}$, and/or low TWDW. Zoysiagrass responds to slow soil drying by increasing cell wall thickness and osmotically adjusting, which allows zoysiagrass to maintain turgor pressure and water content while maximizing soil water extraction.

Perhaps the most interesting finding from these studies was the apparent response of control plants to water stress imposed on adjacent plants. There was no difference

between control and treatment plants in any of the six parameters measured in either the water relations characteristics or preconditioning experiments. These findings, along with the decrease in TWDW of well watered Kentucky bluegrass, adjacent to stressed bluegrass, and subsequent increase in TWDW once the water stressed plants were removed, are strong evidence that airborne water stress signaling is occurring in both cool- and warm-season turfgrasses.

The gene expression experiments of this study show some interesting results that confirm, and may explain, some of the differences observed in the physiological studies. It appears that a major zoysiagrass response to water stress is antioxidant activity. Antioxidant levels or presence have been associated with water stress tolerance. Antioxidants may be used to identify, or select for, water stress tolerant zoysiagrass cultivars as significant variation in expression of genes encoding for antioxidants was found among the four cultivars. Another significant difference elucidated by the gene expression experiment was the species difference in expression of genes encoding for phospholipases. This difference may very well explain the marked difference in the number of days until full turgor was lost as phospholipase D is directly involved in stomatal control with higher levels of phospholipase D activity indicating enhanced abscisic acid sensitivity, stomatal control and water stress tolerance.

Maybe the most significant finding from the gene expression experiment was the almost ubiquitous increase in the expression of genes encoding for signaling related proteins including a systemin degrading enzyme and an integral membrane protein, or receptor, in well watered plants. These results, combined with the response of well watered plants measured by the water relations characteristics and preconditioning

experiments, are strong evidence of plant-to-plant communication through an airborne signal in response to water stress.

Significant cultivar difference in many water stress responses of zoysiagrass are shown in this experiment. Differences between Diamond, Cavalier, Palisades, and DALZ 8504 in leaf rolling, loss of full turgor, water release curve parameters, root characteristics and gene expression make zoysiagrass a prime candidate for further investigation into the mechanisms of water stress avoidance/tolerance. Species differences in stomatal control (affected by phospholipase D activity and abscisic acid sensitivity) may be central to improving water stress tolerance of *Z. matrella* and other turfgrasses. The apparent response of well watered plants to water stressed neighbor plants will likely be the most novel finding of this experiment. The results from this study indicate that this phenomenon is occurring and exposes a dearth in scientific understanding that must be filled. Improving water stress tolerance through breeding for parameters highlighted in this paper may very likely produce turfgrasses that can survive and maintain desired aesthetic qualities on significantly less water.

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