Osmoregulation in response to drought stress

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Introduction

Humankind is undoubtedly dependent upon plants. Our interactions are ancient and have developed gradually through eons of sharing the earth. Without their ability to transfer sunlight radiation into stored chemical energy, it is unlikely life would have evolved at all. This relationship is most clearly exemplified in modern agriculture, which produces food for our growing populations. Grasslands in particular make a significant contribution to food security by providing feed for livestock used for meat and milk production (O'Mara, 2012). Energy captured by plants can also be converted into ethanol for energy use (Tilman, Hill, & Lehman, 2006). Furthermore our largest current energy source is fossil fuels, which is actually energy stored by plants who died millions of years ago.

Given our great dependence on agriculture for food and energy, researchers have been interested in studying many species of plants in their native environments. This can inform scientists about how plants have adapted to live in their respective habitats. Another relevant consideration, in light of the shifting climate in the United States to a more arid environment, is how plants have evolved to manage drought stress. Water is very important to all plant species as it is the primary carrier of sugar and nutrients throughout plants, and itself is required for ongoing metabolic processes and growth. Therefore if a plant can survive in a very dry environment, it must have a mechanism to manage the stress and maintain metabolic processes.

Osmoregulation in plants is an important component of drought tolerance. It encompasses concerted efforts within plant cells to maintain a high water pressure so the metabolism can continue and the plant may live. It also helps to maintain the cellular integrity and minimize damage to membranes and organelles. Osmotic adjustment is part of this effort, and the first chapter of this thesis outlines a more efficient measure of osmotic adjustment suitable for high throughput analyses. These investigations were conducted using a clonal population of the switchgrass *Panicum virgatum*, a biofuel candidate.

A method similar in concept to that developed in chapter 1 was used to investigate the genetic architecture of osmoregulation in the model plant system *Arabidopsis thaliana*. In chapter 2, a powerful genetic mapping population of natural accessions of *Arabidopsis thaliana* was used to do quantitative trait loci (QTL) mapping of osmoregulation.

While these two investigations were conducted in different species, the insights gained in each about osmoregulation give the scientific community a better knowledge base about basic plant function which will be invaluable in the future.

CHAPTER 1 Osmotic adjustment in Switchgrass

Primary research mentors: Jason Bonnette, David DesMarais and Thomas Juenger

Background

To live in dry environments many plants have evolved mechanisms to deal with low soil moisture. These mechanisms classically fall into two distinct categories: drought avoidance and drought tolerance (Morgan, 1984). Some examples of avoidance include leaf rolling and shedding, changes in leaf area and anatomy, increase in stomatal and cuticular resistance, and high water potential at a given soil moisture. Avoidance also encompasses drought escape, in which plants complete their life cycle before the drying environment impedes growth or results in mortality. On the other hand, drought tolerance is often ascribed to cellular and tissue factors, such as osmoregulation and bulk elasticity adjustment, which allow for the maintenance of turgor, cellular integrity, and the continued function of metabolic pathways (Sullivan & Blum, 1986; Touchette, Iannacone, Turner, & Frank, 2007). A major component of drought tolerance is osmotic adjustment (OA, measured in MPa) (Zhang, Nguyen, & Blum, 1999).

Osmotic adjustment is the net active accumulation of solutes within plant cells in response to decreased water potential of the plant's environment (Figure 1.1). This accumulation lowers the cell's water potential, which attracts water to the cell and tends to maintain turgor pressure. A loss of turgor results in a loss of stomatal activity and processes such as cell metabolism and growth. OA therefore helps to maintain critical processes allowing survival despite decreasing water availability.

Osmotic adjustment has been characterized in many species including wheat (Živčák, Repková, Olšovská, & Brestič, 2009), rice (Blum & Nguyen, 1999), sorghum (Sullivan & Blum, 1986), barley (Blum, 1989), and indiangrass (Barker, Sullivan, & Moser, 1993). Furthermore, OA has been confirmed in switchgrass using pressure-volume (PV) analysis (Knapp, 1984) and osmotic potential (OP measured in MPa) measurements using a psychrometer (Barker et al., 1993). These two methods have been used extensively to investigate plant water relations, however both are laborious and time consuming. Creating a pressure-volume curve can take several days, and using a psychrometer to determine OP requires one to two hours equilibration time (Barker et al., 1993; Bartlett et al., 2012; Knapp, 1984). Headway has been made by Bartlett et al. (2012) to speed the collection of OP measurements by using an osmometer to measure OP of a leaf disk. These results showed a high correlation between PV and osmometer measures of OP, suggesting measuring OP through leaf disks is a faster alternative to the time consuming accepted methods (Bartlett et al., 2012).

However, measuring OP using the leaf disk method requires a 10-15 minute equilibration period in the osmometer chamber which is a rate limiting step. Our experiment proposes a more efficient method for determining osmotic potential of frozen leaf tissue that is suitable for genetic studies and rapid screening. The method was specifically tested for use with switchgrass (*Panicum virgatum*), a perennial C4 grass. In the proposed method, sap is extracted from a freeze thawed leaf using a pressure chamber, and its osmotic potential scored using a vapor pressure osmometer. These values of OP were compared to paired measurements using conventional methods to determine accuracy.

Practically, OA is the difference in OP between drought stressed and non-stressed cellular fluid at the same relative water contents (RWC) (Blum & Nguyen, 1999). For this reason, OA measurements need to distinguish osmotic adjustment from the concentration effect of simple dehydration on the sample OP (Campbell, Papendick, & Rabie, 1979). Thus it is necessary to either compare OP of rehydrated samples (the rehydration method) or use theoretical relationships to incorporate the concentration effect (Zhang et al., 1999). Since consistent RWC is essential to a reliable estimate of osmotic potential, various rehydration methods were also tested on switchgrass leaves to find a technique that accurately and consistently hydrates samples to 100% RWC and thus allow OP comparisons that minimize the concentration effect.

Our long-term goal is to identify the metabolic and biochemical responses to dehydration that drive osmotic adjustment. To this end, we also completed pilot metabolite surveys to investigate which osmolytes were accumulated and implicate specific pathways involved in osmotic adjustment. Metabolite analysis has characterized osmotic adjustment in other plants and classified osmolytes into several common categories: sugars, polyols etc. (Zhang et al., 1999). Proline has also been implicated in osmotic adjustment as both a compatible solute and reactive oxygen scavenger (Bhargava & Sawant, 2013; Delauney & Verma, 1993; Pavli, Vlachos, Kalloniati, Flemetakis, & George, 2013; Witt et al., 2012). For this investigation, metabolite content was analyzed in both stressed and non-stressed tissue through GC-mass spec. This data will both help confirm that OA is occurring in switchgrass as should be expected, and shed light on specific pathways which may be involved in the OA response of the AP13 genotype of *Panicum virgatum*.

Materials and Methods

Plant materials and growth conditions

The plant material used for this experiment was collected from thirty clones of the genotype "Alamo AP13" of *Panicum virgatum*. The Alamo genotype is from a lowland cultivar originating in Texas and widely used in genetic and genomic studies of switchgrass. Tissue and measurements were collected after 2 seasons of growth in cylinders of soil (4 ft. high, 2 ft. diameter) under a rain-out shelter on the University of Texas at Austin campus (Figure 1.2). Throughout the two prior seasons, all plants were pre-treated under a wet or dry treatment, the 15 wet treatment plants being watered twice as often as the 15 dry treatment plants. For one month prior to the experiment, wet treatment plants were watered once every 4 days and dry plants once every 8 days. Measurements and samples were collected in late June of 2013. The same two treatments were imposed on the same plants during the experimental dry down. The day before measurements began, all 30 plants were watered. For 7 days, wet treatment plants were watered every day, while dry treatment plants were deprived of water (Figure 1.3) resulting in a relatively slow and consistent drying of cylinder soil. After final dry down measures were completed on day 7 all 30 plants were once again watered. Samples harvested on day 8 are considered recovery samples following natural rehydration

Water Relations Measurements

Throughout the course of the 7 day dry-down, three random plants from each treatment were sampled from each day such that by day 7, all 30 plants had been sampled from once, and six twice. All 30 plants, 15 from each treatment, were sampled on day 7, the final day of the dry-down, and again on day 8 after re-watering. Data collected from each plant included predawn

leaf water potential (LWP) and midday leaf and stem water potential using a Scholander type pressure chamber, and relative water content (RWC) after a four hour rehydration. Stem water potential (SWP) measurements were made by covering the leaf to be sampled in a foil lined bag attached to the tiller. The leaves were allowed to equilibrate for 20 minutes, at which time the leaf was cut and processed like those for leaf water potential. In addition, a second fully emerged leaf was cut, sealed between the folds of a wet paper towel and allowed to rehydrate in dark coolers with cold packs for 4 hours. On day 7 additional leaf tissue was collected for metabolite screening.

Osmotic Potential and Osmotic Adjustment

After rehydrating for 4 hours, a 7mm hole-punch was used to collect a sample of tissue from about 2mm from the cut edge of the leaf centered over the midrib at the base of the cut leaf. This tissue would be used for the disk measurement and was stored in a micro centrifuge tube frozen in liquid nitrogen and moved to -80C if not measured immediately. The remaining leaf tissue from the same leaf was used to find OP through expressed sap. The edge of the leaf tissue was re-cut, and it was submerged in liquid nitrogen. Thawed leaftissue was pressurized in a pressure chamber (PMS Instruments, model 1000) until sap began to extrude from the exposed cut surface. 10 μL of expressed liquid sap was collected using a laboratory pipette. The osmotic potential of the sap was measured using the Vapro 5520 vapor pressure osmometer in the 10 μL chamber well (Vapro Model 5520; Wescor). The osmometer was calibrated using Wescor standards when it was out of the acceptable OP ranges. The thermocouple was cleaned as needed. To measure leaf disk OP, disks were removed from freezing and loaded into the Wescor osmometer. To decrease equilibration time, each leaf disk was punctured 20-30 times with a sharp forceps before sealing it into the osmometer chamber. Each leaf was measured successively every two minutes until it reached equilibrium, which was indicated by an increase in measurements of less than 0.01 MPa.

Calculating OA from OP

OA was estimated as the difference in OP between the wet and dry treatments on days 6, 7 and 8. Before collecting data on day 8 plants were-watered and OA was estimated with on-the-plant rehydration. OA was calculated for each method using the average OP measurement from the well watered and drought stressed samples as:

$$OA = OP_{drought} - OP_{well-watered}$$

Metabolites

Tissue was collected on day 7 of the dry-down from all replicates. It was immediately frozen in liquid nitrogen and stored at -80°C. Metabolite profiling on leaves of stressed and controlled plants was performed by gas chromatography combined with mass spectrometry (GC-MS) in collaboration with researchers at Oak Ridge National Lab.

Rehydration Analysis

Rehydration of wet and dry treatment leaves was explored using three different rehydration methods. Three leaves were cut from 6 dry treatment plants used for osmotic potential measurements. The mass of each leaf was recorded immediately after harvest, and the leaves were stored in dry plastic bags for transportation. All leaves were rehydrated with

deionized water. Method one consisted of rehydrating the leaves inside a plastic bag lined with moist paper towels. In the second method, the cut edge of the leaf was inserted directly into water until about 1 cm was submerged, and the remainder of the leaf was stood upright and covered in a plastic bag. Leaves for method 3 were allowed to float on the water-air interface in a sealed container, with only one side of the leaf directly in contact with the water. Once the rehydration began, the mass of the leaves once gently dried was recorded with decreasing frequency for up to 48 hours.

Statistics and calculations

We used linear mixed models in the R mosaic package to evaluate the extent of stress imposed by the soil drying treatment. Specifically, we fit a model examining the fixed impact of the treatment, day, and treatment by day interactions on RWC, LWP, SWP, and OP from either disk or expressed sap. The initial analyses included plant as a random term to account for the repeated nature of the sampling design and focused on days 1-7. Separate analyses were completed on day 8 data, following the re-watering of all experimental plants. Transforming the data did not significantly improve the distribution of any of the traits, so all analyses were done without transformation. Traits are normally distributed within treatment. For the models fitting RWC, LWP and SWP, the residuals are normally distributed. However there is a slight left sided tail on both the ES and disk OP residuals. For the most part, the data seems to meet the assumptions for linear modelling. Rehydration data was analyzed using a multivariate model accounting for time, plant and rehydration method.

Leaf relative water content (RWC, %) was determined according to the methods of Barrs and Weatherley (1962) based on the following equation where FM is fresh mass, DM dry mass and TM turgid mass after rehydration:

$$RWC = \frac{FM - DM}{TM - DM} \times 100\%$$

Results

Physiology Measurements

There was a significant effect of both day and treatment on RWC, predawn LWP, midday LWP, and midday SWP over the course of the experiment ($P < 0.0001$). There was not a significant interaction between day and treatment for any of the traits. Midday leaf water potential measurements over time between wet and dry treatment plants are shown in Figure 1.4B. Midday LWP decreased over time in the dry treatment and did not vary significantly in the wet. After day 3, the average dry treatment LWP was significantly lower than that of the wet treatment ($P<0.0001$). After re-watering the dry treatment, LWP on day 8 was significantly higher than on day 7 ($P<0.0001$). Stem water potential measurements are highly correlated with midday leaf water potential measurements (slope = 0.84, $R^2 = 0.92$) and show the same trends over time by treatment (Figure 1.4B, D, E).

Relative water content (RWC) over time between wet and dry treatments is shown in Figure 1.4C. RWC decreased in the dry treatment over time while wet treatment plants remained between 90-100%. In the dry treatment average RWC levels stayed high, dropping significantly on day 5 to below 80% ($P < 0.0001$) and reaching a low of under 75% on day 7 ($P < 0.039$). Dry

treatment plants were re-watered after day 7 and RWC was significantly higher on day 8 ($P < 0.0001$).

Predawn LWP (Figure 1.4A) decreased over time in the dry treatment and did not vary significantly in the wet. Predawn LWP dropped significantly after day 3 ($P < .0001$) and continued decreasing. After re-watering there was a significant increase in predawn LWP in the dry treatment ($P < 0.0001$).

Rehydration Analysis

Three rehydration methods were evaluated for well-watered and dehydrated switchgrass leaves: floating the leaf on the surface of water, submerging the cut edge in water, and folding the leaf in a damp paper towel in a bag. We fit a mixed model to the data including time, method, treatment, the time*method interaction and adding plant and leaf as random effects to account for the non-independence of the samples. There was no significant effect of method, however we noticed the floating method resulted in slightly higher rehydration. We considered this over-hydration because over time pores and air spaces will begin to fill with water, which would have been empty at 100% RWC in living tissue. Treatment was also not a significant effect. All methods show similar rehydration curves with respect to time. In the first hour, the leaves had gained over 50% and by four hours had gained 70% of their final water mass. Before 24 hours leaf mass plateaued and rehydration ceased. The time*method interaction was not significant.

Osmotic Potential compared between Leaf Disk and Expressed Sap

Osmotic potential measured by LD is plotted against paired measurements of ES for both 1 and 4 hour rehydration in Figure 1.6. Linear regressions were fit separately to the 1 and 4 hour rehydrations and to both wet and dry treatments. In the 4 hour rehydration data, ES OP measurements explain 69% of the variation in the LD measurement. The 1 hour rehydrated measurements for ES explain 68% of the variation in the LD measurement.

Figure 1.4 shows an unexpected pattern in OP development over time that is represented independently in both the LD and ES methods. The pattern shows that osmotic potential measured on days 4 and 6 in the dry treatment was lower than that measured on days 5 and 7. This trend is significant in both leaf disk ($P < 0.0068$) and expressed sap ($P < 0.0015$) and is not seen in any of the other water status measures.

Osmotic Adjustment

Osmotic adjustment was calculated using the third method outlined by Blum et al. (1999) using OP data from days 6, 7 and 8 for both LD and ES. In this method OA is calculated as the difference between OP between nonstressed and stressed leaves that are rehydrated. The data is displayed in Table 1, and displayed over time by method in Figure 1.6. OA calculated from day 6 and 7 data represents the adjustment after 6 and 7 days of drought stress. After day 7 data was collected, plants were watered across all treatments. Thus OA from day 8 is a measure of adjustment with rehydration on the plant. Each day OA estimates did not differ significantly between disk and ES methods, which suggest that either method gives good estimates of osmotic adjustment. OA was largest on day 6, and decreased the following two days, which cannot be explained through weather data collected on site.

Table 1 Osmotic Adjustment between treatments on days 6, 7 and 8

Day	Disk	ES
6	0.82 ± 0.29	1.05 ± 0.29
7	0.52 ± 0.12	0.65 ± 0.23
8	0.26 ± 0.09	0.56 ± 0.10

Metabolites

GC-MS analysis of switchgrass leaf tissue from all replicates in both stressed and controlled treatments yielded 67 identifiable metabolites. Of those, 29 were significantly altered in response to stress ($p < .0007$) and 28 of the 29 were significantly increased in the stressed treatment group. These metabolites belong to several classes, including amino acids, organic acids, polyols, and sugars. In amino acids, substantially increased levels were seen in tryptophan, phenylalanine, glutamine, proline, threonine, tyrosine, glutamic acid, glycine and GABA. In the organic acids group, significant increases were seen in malic acid, erythronic acid and maleic acid in the stressed group. In contrast, glyceric acid was significantly decreased in stressed tissue. In addition, the level of the polyols mannitol-glycoside, myoinositol, shikimic acid and quinic acid were significantly increased in the stressed tissue. And finally the sugars raffinose, glucose, fructose, galactose and mannose were also detected at elevated levels.

Metabolites that accumulated in the large quantities relative to all metabolites present within stressed tissue include the organic acids and sugars shikimic acid, malic acid, glucose, fructose, mannose.

Discussion

Osmotic adjustment is a significant trait that generally aids in combating drought stress. Relevant aspects of osmotic adjustment include the osmotic potential measurement it's derived from and an understanding of which metabolites are actually causing the change in osmotic potential. The osmotic potential measurement itself is directly dependent upon the level of hydration of the tissue (RWC), so a method like rehydration is necessary to obtain comparable estimates. To understand these aspects of osmotic adjustment in *Panicum virgatum*, a population of clonal replicates was divided into stressed and unstressed groups and subjected to declining soil moisture. Data on midday LWP, midday SWP and predawn LWP and RWC were collected to verify that the lack of irrigation effectively stressed dry treatment plants. Leaf water potential could possibly vary among leaves of the same plant. In an attempt to account for this, stem water potential measurements were collected by allowing leaves to equilibrate for 20 minutes before measurement. However stem water potential was found to be highly correlated with leaf water potential, thus a measure of leaf water potential alone will suffice for information about water status. All of these data indicated that over time the dry treatment plants showed signs of stress, while the wet treatment plants remained unstressed.

A faster method of collecting osmotic potential estimates using expressed sap compared favorably with the accepted leaf disk method (Figure 1.6). The data was highly correlated across varying states of hydration. In addition, the dry treatment from days 4 and 6 showed significantly lower average water potentials for both ES and leaf disk methods. The fact that both methods show similar unexpected patterns is further evidence that ES OP can be used as a predictor for

leaf disk OP. These patterns could be due to environmental factors such as high wind on a particular day which would increase drought stress, combined with a temperature spike. Weather data was collected daily at the experiment site (Figure 1.7). The data doesn't show a temperature spike or increase in wind on days 4 or 6, but do show that over time relative humidity and wind dropped while temperature slowly rose. Cloud cover, or a small sample size may have also contributed to unusual daily variation in OP. Additionally, the minimum measurement time for OP through the leaf disk method was about 10-15 minutes per sample while that of the ES method is only 2 minutes. This is an advantage and could make it feasible to collect osmotic potential data on large populations.

Osmotic adjustment was calculated as the difference in OP between treatments and did not vary significantly between OP measurement methods. This data is further evidence that switchgrass osmotically adjusts in response to drought stress. Osmotic adjustment values found here compare favorably with previous data in switchgrass and related species (shown in table 2). However it's difficult to directly compare osmotic adjustment values because they depend on the degree of drought stress imposed. One way to control for stress level is to compare osmotic adjustment at a given relative water content or leaf water potential.

However there are limitations to these methods of gathering OP data. The ES method is more likely to be an underestimate of OP because of the higher ratio of apoplastic fluid contained in the sap compared to a leaf disk (Campbell et al., 1979; Wardlaw, 2005). Apoplastic fluid has a very high water potential which is very close to zero (Turner, 1988). Thus the ES measurement does not give an absolute value of symplastic water potential, because it's impossible to separate it from the apoplastic fraction. However, if we assume a similar ratio of apoplastic to symplastic fluid in samples and a similar water potential for apoplastic fluid, ES measurements are an effective rank of OP in different tissues and can be compared between individuals in a well-controlled experiment. Another limitation has been suggested by Kikuta and Richter (1992) that osmotic potentials of leaf disks are lower (more negative) than parallel samples of ES because not all osmotically-active solutes are completely extracted. This trend was observed in our data and could explain the slightly higher OP estimates for ES. However again, ranks would not be affected if the ES method excludes solutes similarly across samples.

Table 2 Osmotic Adjustment comparison from previous studies. Osmotic adjustment values from this study are in bold.

Crops	**OA (MPa)**	**RWC / -LWP(MPa)**	**References**
Wheat	0.49-1.04	< 70%	(Živčák et al., 2009)
Rice	0.4-1.5	60% -70%/ 3 MPa	(Blum & Nguyen, 1999)
Sorghum	0.3-1.7	0.6- 1.2 MPa	(Sullivan & Blum, 1986)
Barley	-0.17-0.46	1.5 MPa	(Blum, 1989)
Indiangrass	0.66	0.8- 1.1 MPa	(Barker et al., 1993)
Big Bluestem	0.39	1.0-1.9 MPa	(Barker et al., 1993)
Switchgrass	0.42	1.4-1.6 MPa	(Barker et al., 1993)
Panicum virgatum	0.78	1.4	(Knapp, 1984)
AP13	0.4–0.64	65% / 3	Day 7 leaf disk
AP13	0.42–0.88	65% / 3	Day 7 expressed sap

Metabolite profiling was conducted by extracting metabolites from stressed and non-stressed tissue and analyzing it using GC-MS. Of the 67 identifiable metabolites, 28 were found

at significantly increased levels in stressed tissue, while 1 (glyceric acid) was found at increased levels in non-stressed tissues. These 28 metabolites correspond to several groups of organic compounds commonly associated with osmotic adjustment or drought stressed metabolism including amino acids, organic acids, polyols and sugars, which have been shown to be common compatible osmolytes in OA (Zhang et al., 1999). Recent metabolite studies under drought stress in maize and sorghum show very comparable results, with significantly higher metabolite content in stressed tissue from those groups listed above (Pavli et al, 2013; Witt et al., 2012). Organic acids, amino acids and sugars are also known to contribute to osmotic adjustment in Arabidopsis under drought stress (Hummel et al., 2010).

Rehydration is a common method of accounting for the concentration effect in OP measurements (Bartlett et al., 2012; Zhang et al., 1999). In general, rehydration ceased in this experiment before 24 hours. Yamasaki has suggested RWC during rehydration follows two phases, a rapid rehydration and a slow plateau (Yamasaki & Dillenburg, 1999). Catching leaves between the two phases is the ideal to avoid over-hydration in the slow plateau phase. Analysis using a mixed model showed neither method nor treatment were significant effects, which suggests using any method will not bias rehydration by treatment. However, the bag method was consistent between treatments and tended to hydrate leaves less. Of the three methods, the bag method was also simplest and allowed for convenient storage and transportation of tissue. In an effort to avoid over-hydration and gain efficiency, leaves were therefore rehydrated in bags for 4 hours.

In conclusion, the results show that determining OP from expressed sap is a reliable alternative to using leaf disks. As the expressed sap method has the potential to be much faster, this can be useful to collect OP measurements from large populations. Furthermore, the osmotic adjustment potentials calculated in this experiment are comparable to previous estimates in *Panicum virgatum* and related species. The metabolite profile of drought and non-stressed leaf tissue is also comparable to that of maize and sorghum, showing an increase in metabolites known to contribute to osmotic adjustment in other species. Thus it's likely that switchgrass osmotically adjusts in similar magnitudes and mechanisms as in related species.

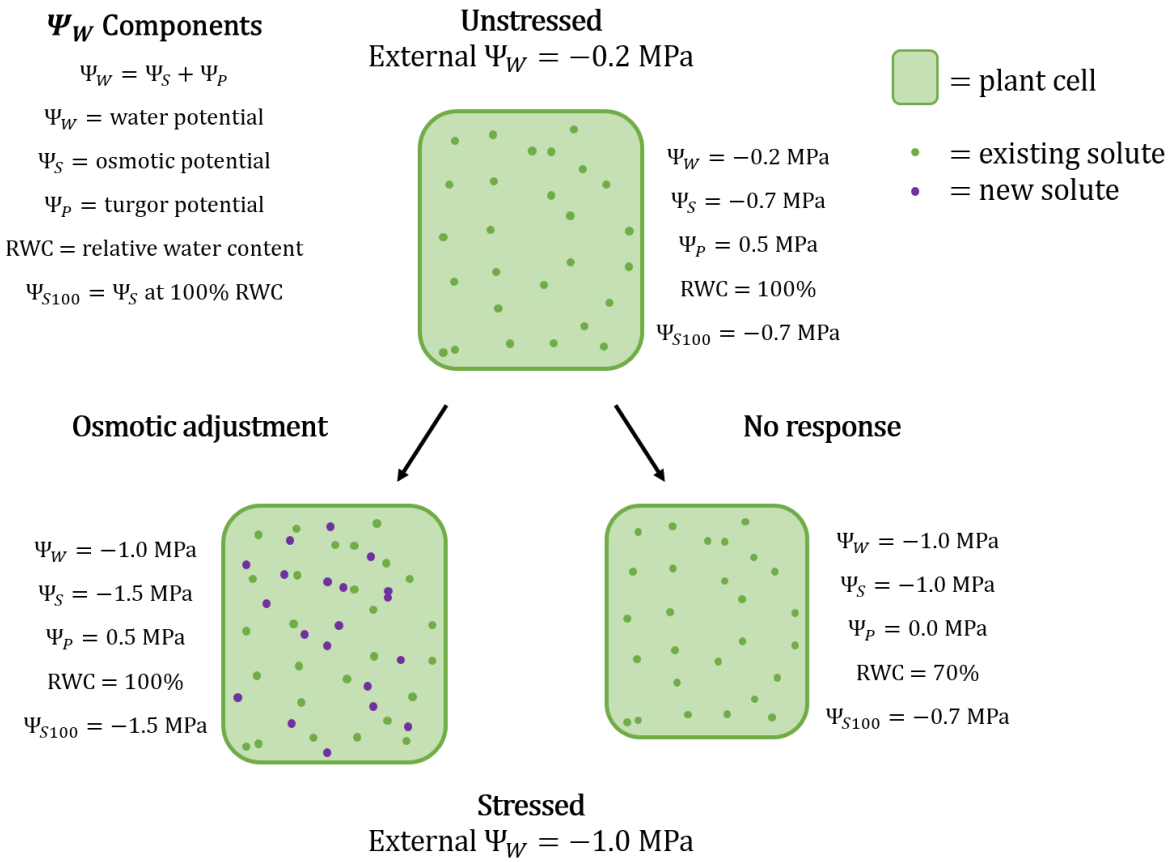

Figure 1.1 The isolated effect of osmotic adjustment in response to lowered soil moisture potential. In osmotic adjustment, cellular solute content increased by -0.8 MPa to maintain turgor and RWC. With no response, RWC and turgor pressure are lost.

Figure 1.2 Wet and dry treatment groups of the *Panicum virgatum* genotype AP13 clones under a rain-out shelter in Austin, Texas.

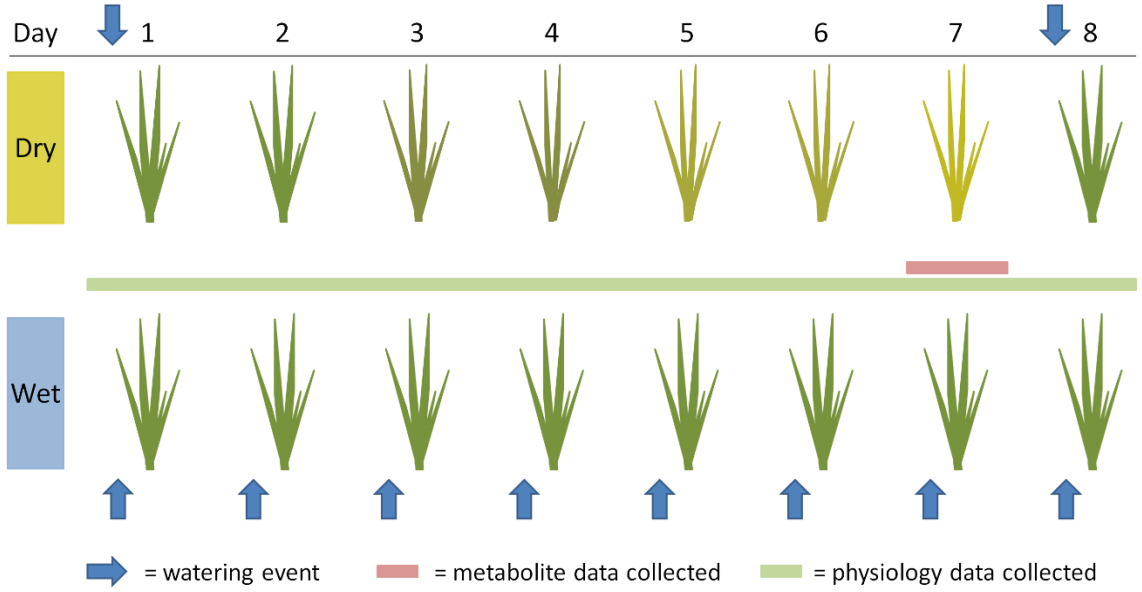

Figure 1.3 Leaf collection scheme for the experimental dry-down. Dry treatment plants were watered prior to the dry-down, then re-watered after measurements were collected on day 7. Wet treatment plants watered every day. Three different plants were sampled on days 1-6 for each treatment, and all plants were sampled on days 7 and 8.

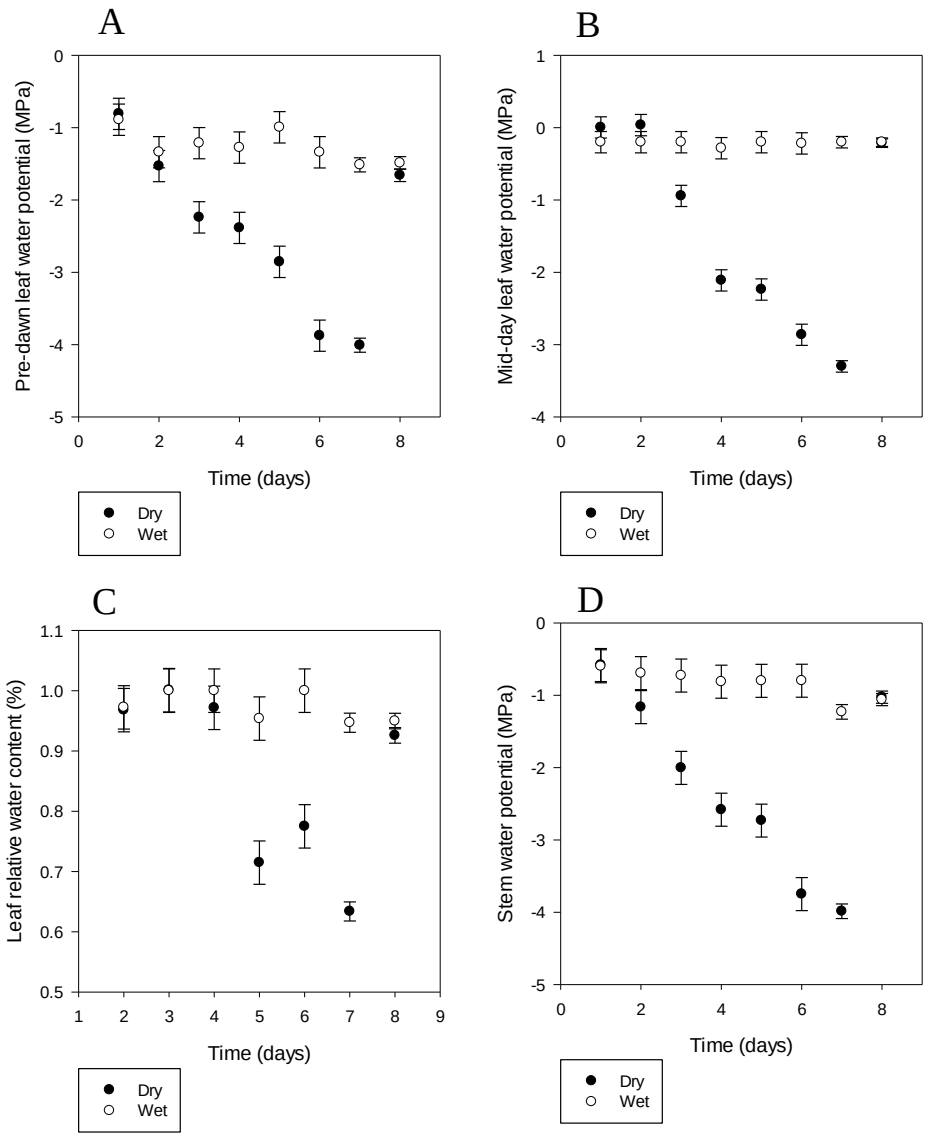

Figure 1.3 Summary of physiological treatment response over time. (A) Predawn leaf water potential (LWP) over time for wet and dry treatments (B) Mid-day leaf water potential (C) Relative water content (RWC) (D) Stem water potential over time by treatment. Error bars indicate standard error.

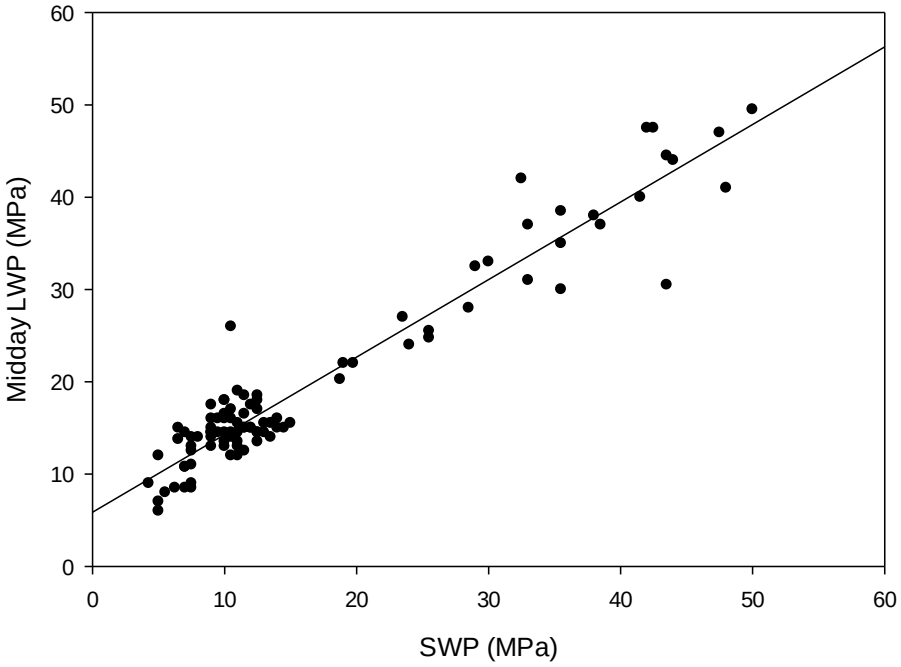

Figure 1.3E Midday leaf water potential vs. midday stem water potential.

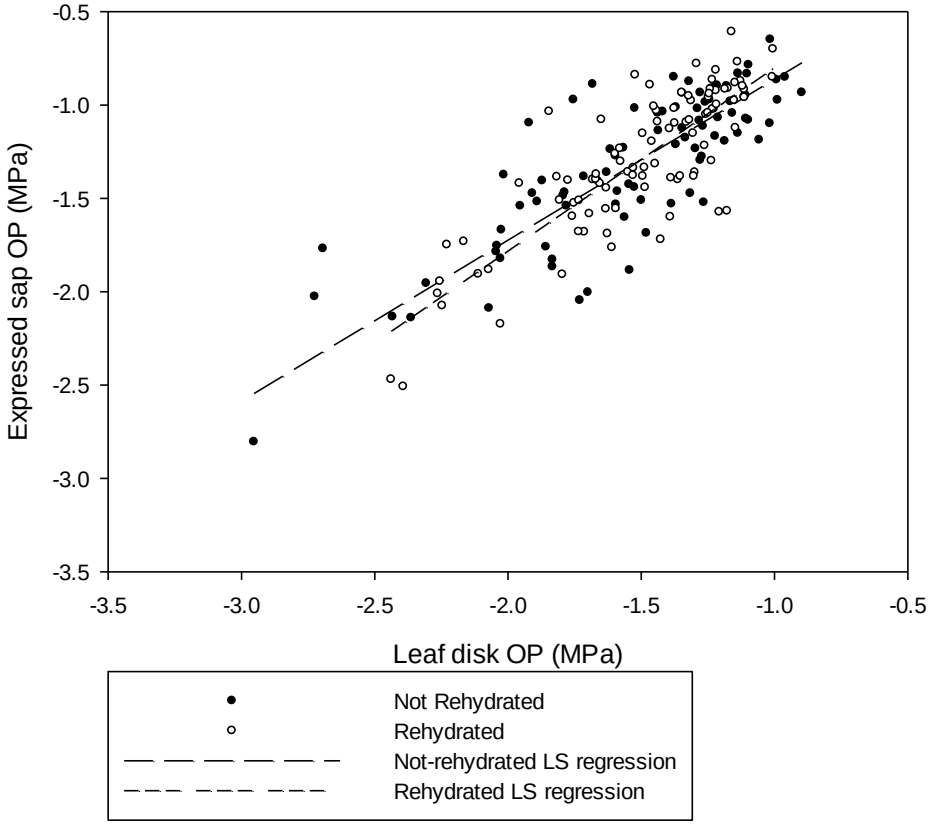

Figure 1.4 Osmotic potential over time by treatment between leaf disk (LD) and expressed sap (ES) methods. Error bars indicate standard error.

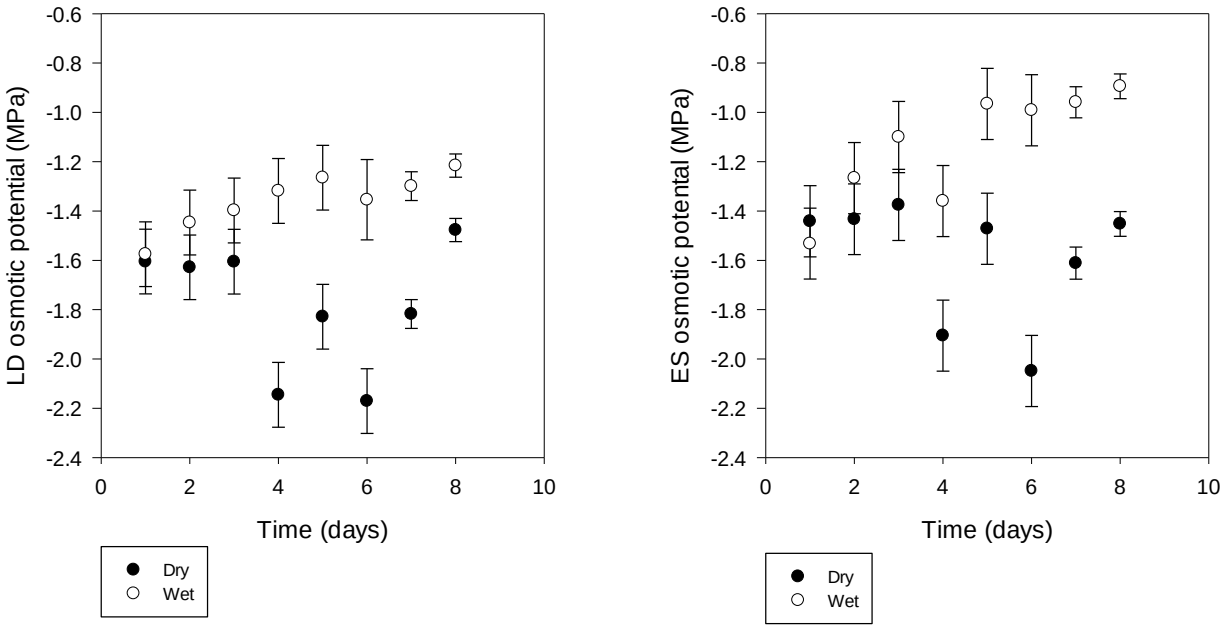

Figure 1.5 Comparison of expressed sap and leaf disk methods for measuring osmotic potential (OP). Linear regressions fit by ordinary least squares for samples that were both rehydrated and not-rehydrated prior to measuring. The non-rehydrated regression has R^2 of 0.68, and the rehydrated regression R^2 of 0.69.

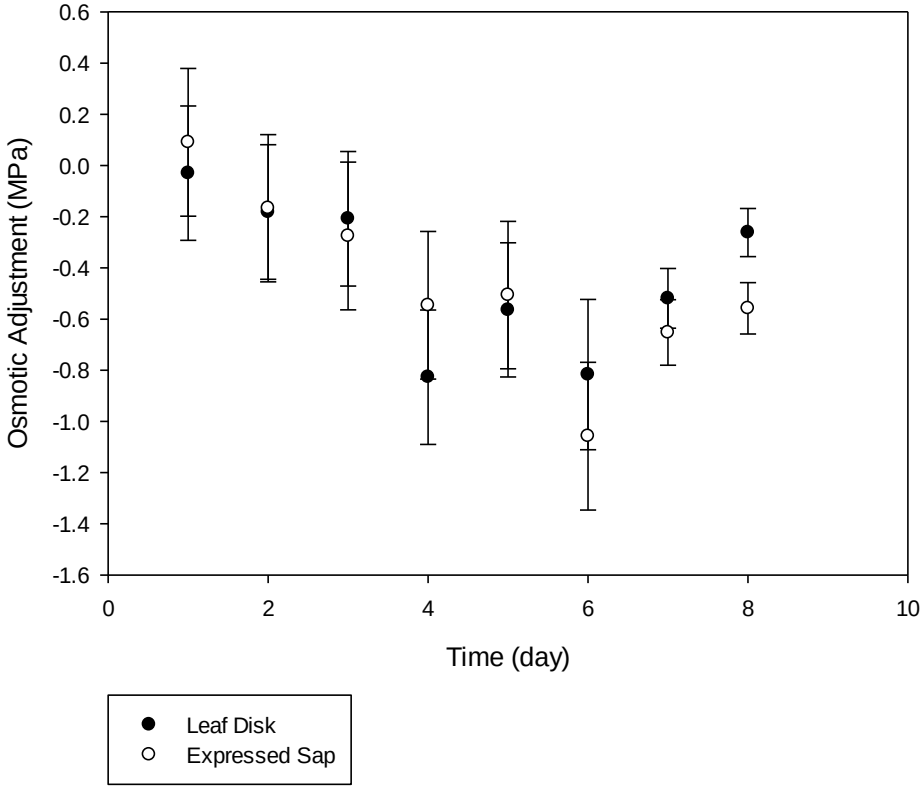

Figure 1.6 Osmotic adjustment over time by osmotic potential measurement method. Osmotic adjustment was calculated as the difference in the average osmotic potential between stressed and unstressed treatments. Error bars indicate standard error.

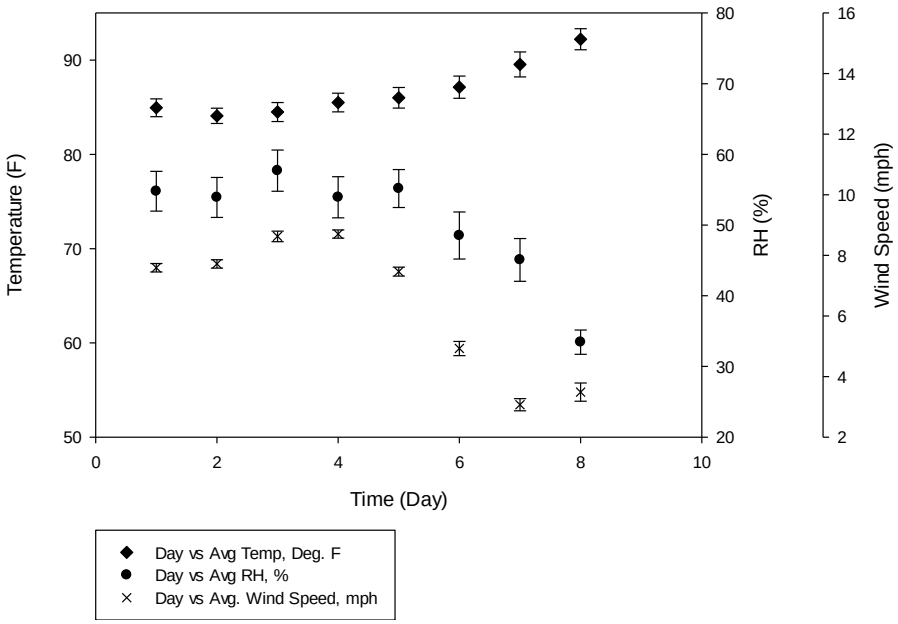

Figure 1.7 Weather data collected throughout the experimental dry-down on-site. Average temperature, Average relative humidity (RH) and average wind speed are plotted by day each with their own axis. Error bars indicate standard error.

Chapter 2

Primary research mentors: Eugene Shakirov and Thomas Juenger

Background

Efficient food production is essential in light of the ever increasing global population and changing climate. The identification and production of crops which can be productive under drought stress will help avoid food shortages in dry climates and in marginal agricultural lands. The first step in understanding the complexity of drought tolerance is to identify quantitative trait loci (QTL) which could potentially contain relevant genes. Classically, QTL mapping has been done using bi-parental breeding populations. Yet with only two parents, these populations are limited and can only map QTL to very wide genomic regions (Verbyla et al., 2014). However multi-parent advanced generation inter-cross (MAGIC) populations incorporate much more genetic diversity and with a known population structure promise to greatly increase mapping resolution for quantitative traits (Kover et al., 2009). The MAGIC design was first implemented in mice with 8 parental lines (Mott et al., 2000), but has been extended further in mice populations as well as wheat (Huang et al., 2012; Trebbi & Maccaferri, 2008) and Arabidopsis (Kover et al., 2009). A MAGIC population is made by crossing founder lines until every offspring has equal probability of inheriting an allele from every parent. The lines are then selfed for multiple generations to create recombinant inbred lines. This structure allows for much recombination, which increases the potential mapping resolution (Verbyla et al., 2014).

The osmotic potential (OP, measured in MPa) of plant tissue is an indicator of drought stress. As the osmotic potential of the soil and air decrease with drought, so must the osmotic potential of plant tissue if it can continue to extract water from the soil. Regardless of a plant's ability to uptake water from the soil, water will evaporate from the leaves. If a plant cannot adjust its osmotic potential, it will wilt, desiccate and die. One common method to adjust osmotic potential is osmotic adjustment in which specific metabolic pathways are adjusted to actively accumulate compatible solutes, like proline, into cellular fluids. This kind of accumulation will be reflected in changes of osmotic potential, as will a plant's overall reaction to the stress. The lower the osmotic potential of leaf tissue under a given soil moisture deficit, the better the plant is able to cope with decreased water availability. Thus osmotic potential can be used to score drought resistance in plant populations.

This investigation will examine the drought stress reactions of 221 MAGIC *Arabidopsis thaliana* lines in order to identify QTL underlying drought resistance. There are several reasons a MAGIC population of *A. thaliana* is expected to give insight into drought resistance. First, the species has been shown to osmotically adjust (Verslues & Bray, 2004), and has been used to successfully map quantitative traits using quantitative trait loci (QTL) mapping (Simon et al., 2008). Verslues and Bray (2004) found much variation in proline accumulation in Arabidopsis populations, and also noted unattributed variation which could be due to other solutes active in osmotic adjustment. This indicates that there is the potential in *A. thaliana* for considerable variation in drought induced osmoregulation, whether from proline or other solutes.

The MAGIC *A.* thaliana population is unique in that it encompasses the genetic variation of 19 parental ecotypes, a marked increase from the 8 parental strains used to make the mouse MAGIC population. Furthermore, all 19 natural parental accessions have had their complete genomes sequenced, assembled and annotated along with their transcriptomes (Gan et al., 2011). Gan et al. (2011) also show that QTL mapping with the MAGIC population could allow for

single gene mapping resolutions for loci of large effect. The MAGIC *A. thaliana* population has also given insight into the genetic architecture of seed size and number (Gnan, Priest, & Kover, 2014) and for germination and bolting time (Kover et al., 2009). Therefore it has proven to be a powerful genetic tool to investigate the genetic architecture of plant traits. Analysis of phenotypic associations across a portion of this MAGIC population will be used to uncover the genetic architecture of drought resistance.

Materials and Methods

Work flow for phenotype collection

Because osmotic potential cannot be scored on all 200 lines at once due to space and time constraints, seeds were split randomly into several experimental runs. Each run followed the same 13 day schedule as shown in Table 1.

Day	Procedure
0	Sterilize and plate seeds
3	Transfer plates to the growth chamber
8-9	Make PEG overlaid plates and allow them to equilibrate overnight
10	Transfer seedlings from growth plates to PEG plates
13	Collect samples

Procedures are those described by Verslues & Bray (2004) with few minor modifications.

The vapor phase seed sterilization technique is less labor intensive and was used to sterilize most MAGIC lines.

In the liquid wash seed sterilization technique, seeds were sterilized by washing with 70% ethanol followed by 80% bleach with Triton-X for 8-11 minutes with constant shaking. Seeds were then washed 3-4 times with sterile water before plating.

In vapor phase seed sterilization, *Arabidopsis* seeds were aliquotted into tubes and arranged with open lids inside a sealable plastic container. A beaker of bleach and 1M hydrochloric acid at a 25:2 ratio was sealed into the plastic container with the seeds. The chlorine gas produced in the reaction sterilized the seeds for 2-3 hours. Each tube of seeds was then rehydrated and washed with sterile water before plating.

Seeds were plated in 100mm round plates in all experimental stages. The solid nutrient media contained 6mM MES buffer and half-strength MS salts adjusted to pH 5.7 with agar at 15g/L and autoclaved before use. Squares of nylon mesh of about 4x4cm were autoclaved and fixed in the center of the plates with sterile water. Seeds were distributed evenly on the mesh, and sealed closed with micropore tape. Seeds were stored at 20°C until transferred to the growth chamber. Once in the growth chamber all seedlings were grown with the plate vertically oriented so the roots grew across the surface of the solid media rather than into it. The chambers were set at 23 C, 24 hrs. of light at maximum humidity

Polyethylene glycol (PEG) was used to lower the water potential of solid media, therefore mimicking drought stress. PEG was added to liquid growth media, which is made as solid media excluding the agar, after autoclaving. The -0.7MPa solid media osmotic potential was achieved

by adding 400g/L of PEG to liquid media, and allowing the solution to equilibrate over solidified plates overnight. Once equilibrated, seedlings on mesh were transferred from regular growth media to the PEG growth media and returned to the growth chamber.

For measuring OP, at least 100g of tissue was removed from the plates, placed in tubes with two 2mm metal beads, and frozen in liquid nitrogen. Samples were either kept in liquid nitrogen or stored in -80°C until the sap extraction. To extract sap, samples were allowed to thaw and then shaken at 6000X/min for 1 min to disrupt the cell walls, then spun in a micro-centrifuge at 15000rpm for 1 min to collect the sap. For each sample, the OP of 10uL of extracted sap was measured using a vapor pressure osmometer (Wescor 1520). Osmometer calibration was checked prior to each use and re-calibrated with Wescor standards when outside the acceptable range. The thermocouple mount was cleaned about every 100 samples.

Statistics analyses

We performed QTL analysis on mean osmotic potential per line after accounting for the effect of incubator and treating MAGIC line as a random effect in multiple regression. We found that experimental run did not have a significant effect on OP, so it was not factored into the model. Additionally, we analyzed means calculated per line irrespective of experimental run or incubator, as well as means in which only incubator was treated as a factor in a linear model and MAGIC was not treated as a random effect. These three methods of calculating mean OP measurements for each line were compared for QTL associations.

QTL analysis was completed in R using the HAPPY package as described by Kover *et al.* (2009). This analysis package was designed for multi-point QTL mapping in genetically heterogeneous animals and plants and contains standardized routines and scripts (http://mus.well.ox.ac.uk/19genomes/magic.html#) for the MAGIC *Arabidopsis* population. The HAPPY package models each individual as a mosaic of the founder haplotypes. These mosaics are estimated probabilistically using a hidden Markov model. This probability distribution is then used to test for the existence of QTL across the genome by fitting a fixed-effect linear model and evaluating the hypothesis that there are no QTL. In this analysis, the SNP marker data derived from sequencing was used in association mapping.

Heritability (H^2) was estimated as the ratio of variance among lines to total variance. Among line variance was estimated by treating MAGIC line as a random effect in a linear regression.

Results

Osmotic potential (OP) was measured on seedlings after they had been exposed to drought stress. Lines were grown in replicates of 3 distributed between 2 incubators over time in several experimental runs..

The 19 parental ecotypes were grown in one incubator at -0.7MPa stress level to determine baseline variation in OP among the parents of the MAGIC population. Variation in OP by parent is presented in Figure 2.1. OP both among parental lines and MAGIC offspring is normally distributed.

The broad sense heritability of OP was calculated to be about 0.26 among the MAGIC offspring. Thus there was variation among lines for OP, but 74% of the variation in OP cannot be explained by MAGIC line.

There were observed differences in osmotic potential between the two incubators used in the experiment, but seemingly consistent measurements of OP across experimental runs. Running a mixed model including incubator and experimental run as a factors and MAGIC as a random variable showed that incubator did have a significant effect on OP ($F = 2.326$, $p < 0.0001$), but experimental run was not a significant effect. The effect of incubator was therefore removed before QTL analysis.

The QTL analysis for OP did not identify any significant QTL segregating in the *Arabidopsis* genome. Figure 2.2 indicates that the three different estimates for least square means of OP by MAGIC line show similarly placed logP peaks, however all are consistently insignificant.

Discussion

Osmotic potential, effectively the plant cellular solute concentration, can give researchers an idea of how a particular plant is responding to drought stress. As the soil moisture decreases, it becomes harder for a plant to extract water from the soil. As water moves from high to low water potential, water will eventually be moving out of cells faster than in, resulting in a net water loss. This directly results in a loss of turgor pressure as a plant wilts and eventually dies. As plant cells lose water their osmotic potential increases. A plant which is able to cope with drought stress well will maintain a low osmotic potential (more negative). Thus a measure of OP at a certain level of drought stress across several genetic lines gives good insight into how each line comparatively reacts to drought stress.

Our study showed that OP is genetically variable across 221 MAGIC *A. thaliana* lines. Because the MAGIC Arabidopsis population is a multi-parent cross of natural accessions of *A. thaliana,* this suggests there is natural genetic variation in OP in *A. thaliana.* However, while there is some signal, there were no significant QTL mapped across any the 5 chromosomes. Three different statistical methods of calculating least square means of OP across the MAGIC lines all showed similar results. The three methods included incubator as a factor; both incubator as a factor and MAGIC as a random variable; and simply averaging OP within MAGIC lines irrespective of incubator. The fact that all estimated insignificant peaks around the same genomic regions shows that the data is relatively robust and that despite the lack of significance of any one peak, similar trends were seen in the data.

The lack of any significant genomic region is not necessarily surprising when considering evolutionarily relevant traits. The classic model of polygenic evolution proposed by Fisher is an abstraction that attributes continuous variation to a large number of mutations of infinitesimally small effect (Rockman, 2012). In that same line of reasoning, Zhang *et al*. (1999) and Verslues et al. (2006) suggest that molecular mapping of a trait like drought resistance is complicated by multiple influencing characteristics such as thickness of cuticles, stomatal opening and closing, root depth and extent and hormonal composition. These diverse traits could each be controlled by multiple genes across the genome, each contributing just a small influence on OP.

In the context of OP these genes could be associated with several drought stress induced mechanisms. One of them in switchgrass is osmotic adjustment (OA). OA is the regulation of leaf water potential at the solute level to maintain a favorable gradient for water extraction. Under osmotic adjustment, plants increase specific metabolic pathways and up-regulate gene expression in order to accumulate certain types of solutes in cellular fluids. These solutes come from a diverse makeup including amino acids, sugars, polyols, quaternary amines and ions (Zhang et al., 1999). One could easily imagine several pathways being activated at once to

accumulate various amino acids or other solutes, each having only a small effect on the overall adjustment. In studies of OA in rice, wheat and barley, Zhang et al. even noted that osmolytes may play a more complex role in conferring drought resistance than simply acting as accumulated solutes (Zhang et al., 1999). This fact shows that even the role of solutes, which is complex in OA alone, is only a portion of the overall plant drought response.

These examples show an underlying principle to the genetic analysis of quantitative traits: the mapping resolution depends on the number and effect size of contributing genes. This principle is more easily seen in a Figure 2.2, in which the components of genetic variation are partitioned between possible contributing genes. QTL analysis of a particular quantitative trait could identify few regions of large effect, like the three genes each contributing to 9% of the variation in the figure. These three genes would likely be significantly associated with a quantitative trait. However an equally possible genetic architecture looks like part B of figure 2.2 in which 27 genes each only account for 1% of variation in OP. This second example is closer to the definition of a truly quantitative trait, in which infinitely many genes only contribute a small effect on the quantitative trait. A perfect example of this second genetic architecture is human height. The heritability of height has been calculated to be very high at 80%. Yet QTL mapping of height has been unable to identify significant genes whose individual effects account for all 80% of the variation (Rockman, 2012). This suggests there are more genes with a small effect that has been indistinguishable in past studies. In order to see the effect of such genes, genetic studies need to be more powerful by increasing the population size, statistical power, and levels of recombination.

Another possibility which may account for a lack of significant QTL is epistasis. The QTL analysis used in this study ignores the possibility of gene interactions. Each loci is tested individually for whether or not it is significantly associated with OP. However it may be that several genes work together to produce an effect that is not additive. For example, say locus A and B are each di-allelic and individuals are mostly homozygous across the entire genome (like in MAGIC lines). An individual could be A or a and B or b. The possible gene combinations are AB, Ab, aB and ab. If A and B worked epistatically such that the trait in AB individuals was 16 times more efficient than individuals with aB or Ab, then the genome scan would not pick up the significant added benefit because the AB effect would be masked by the Ab and aB individuals pooled in either group.

Epigenetic influences could also account for a lack of significant genomic regions associated with OP. This component of the genetic architecture affects transcription independent of sequence data. Because QTL mapping looks for association between SNP sequence data and trait, it would ignore any sorts of histone methylation and acetylation which influences gene transcription. This could potentially be problematic because epigenetic effects can be transmitted to offspring. One could imagine a situation in which two lines have the same alleles but different epigenetic modifications. This means that there is differing levels of transcription at various loci, however the genetic code at each loci is the same.

It is important to note however that several important assumptions were made in our studies OA in Arabidopsis. In particular, we assume that a more negative osmotic potential indicates a genotype exhibiting osmotic adjustment and therefore likely more drought tolerant. As the seedlings were moved from normal nutrient agar to the water-stressed PEG agar, they were immediately osmotically stressed. This itself is not ideal for osmoregulation, as it is generally assumed a slow onset of drought stress produces the most pronounced solute adjustments. But presumably, the leaves lose water at first in the shock of the stress simply

because the water potential of the agar is lower. We assumed in our analysis that by the end of the three day treatment, plants had recovered to 100% RWC and therefore if any plants had higher osmotic potential, this was not because they were dehydrated and the result of a simple concentration effect. We also ran lines without water stress prior to this experiment and found there is little variation in osmotic potential between lines. From these principles and experiments, we assumed that it was during the drought stress period when lines varied their osmotic potentials. We also assumed the effect of apoplastic dilution (water in the tissue veins rather than inside individual cells) which would work to increase our osmotic potentials (Campbell et al., 1979), was small enough to be discounted.

However despite the lack of finding significant QTL for OP, this investigations did uncover considerable genetic variation in OP. Furthermore, we focused on screening only 221 MAGIC *A. thaliana* lines out of the larger population of over 1000. Screening more lines and increasing replication may improve power to allow detection and localization of QTL. Cloning these genes individually and looking at their effects on OP may help us better understand the mechanisms of drought responses, like how a plant senses drought, and what pathways are activated in response. Hopefully future efforts using this powerful MAGIC population will increase our knowledge of plant – environment interactions in drought.

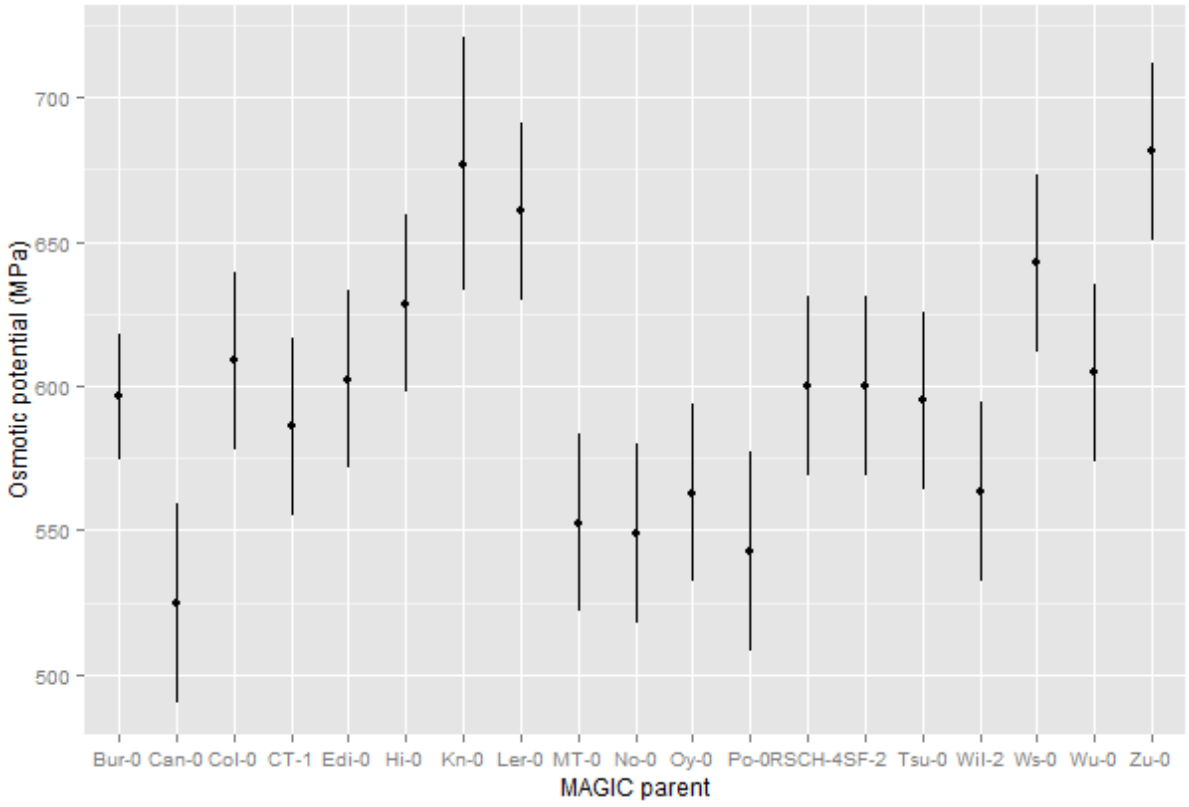

Figure 2.1 Variation in OP of 19 parental ecotypes used to create the MAGIC population. Each point represents 1-3 replicates of OP collected from stressed tissue according to these experimental methods. Error bars are standard error.

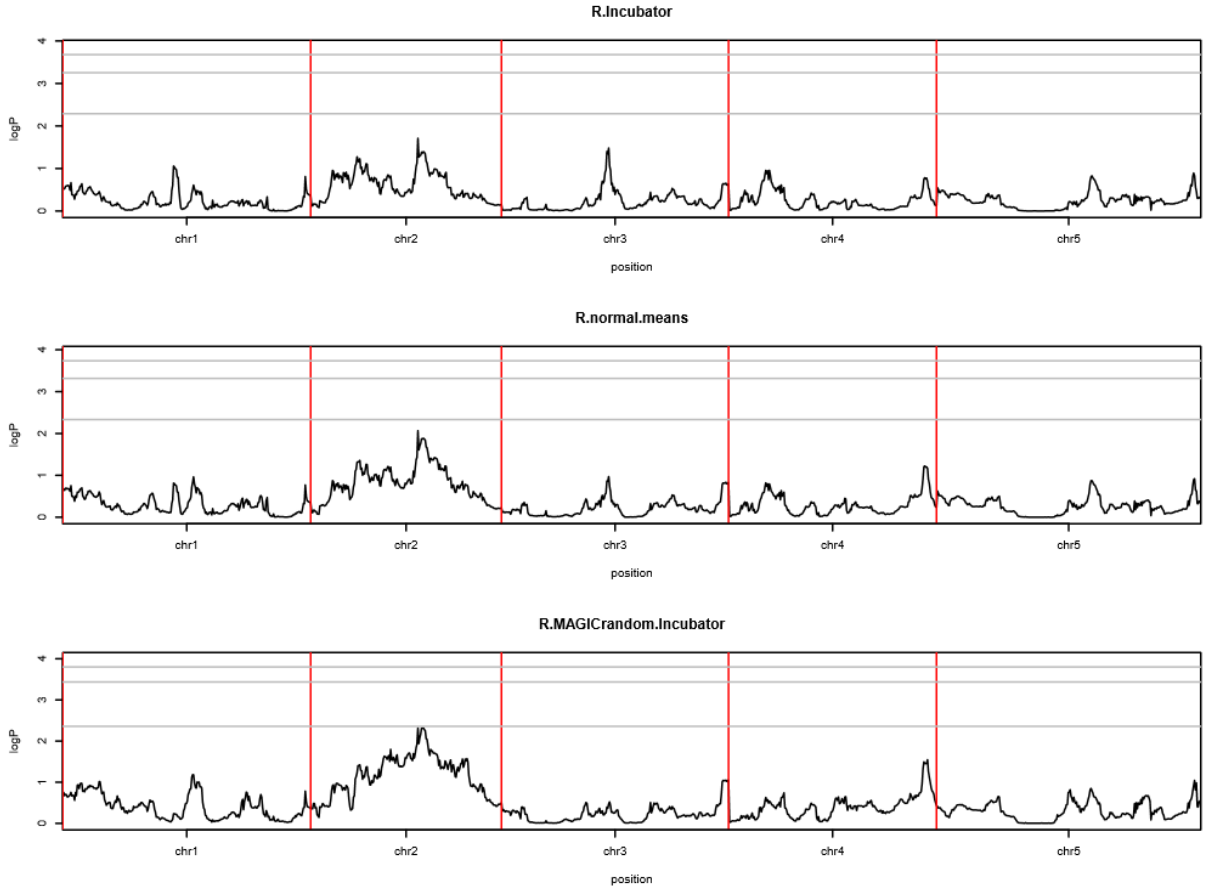

Figure 2.2 Genome wide QTL scans for association between OP and genomic SNPs in MAGIC lines of *Arabidopsis thaliana*. Mean OP per line was calculated accounting for incubator in the first scan, ignoring incubator in the second scan, and including incubator as a fixed effect and MAGIC line as a random effect in the third scan. (-LogP of 3.51 corresponds to a genome-wide p-value of <0.05).

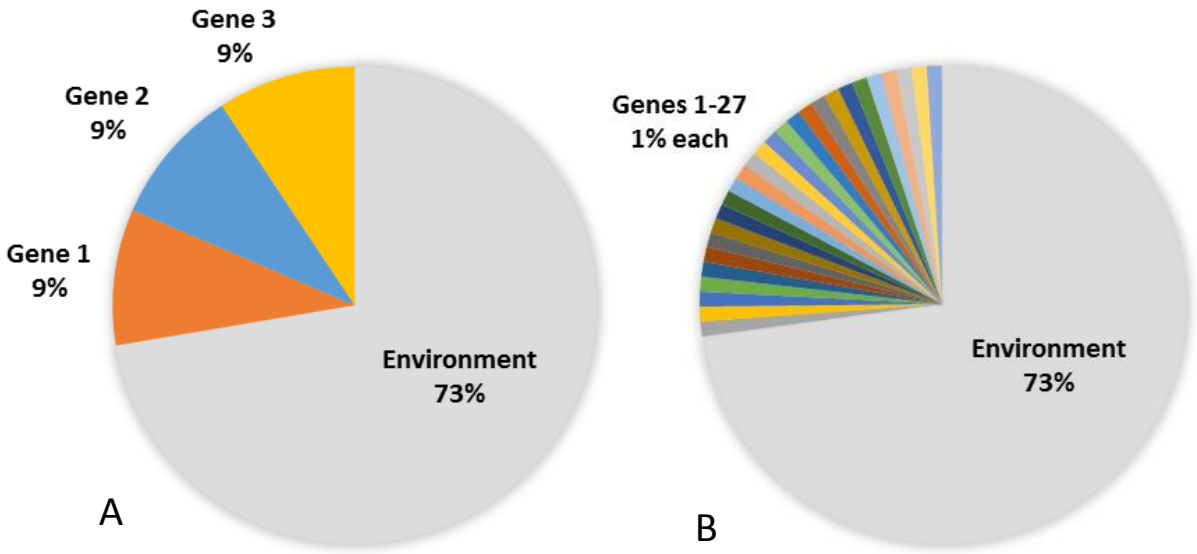

Figure 2.2 The underlying genetic architecture of a quantitative trait could fall anywhere within the spectrum of few genes each contributing large effects to many genes each contributing smaller effects. In the case of this study, 27% of the variation in OP was genetic. The proportion of genetic variation accounted for by specific genes could be large (A) or small (B) depending on the underlying genetic architecture of drought resistance in *A. thaliana*.

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