INTROGRESSION PATHWAY FOR DROUGHT TOLERANCE IN PEANUT

(*Arachis hypogaea* L.)

A Dissertation

by

JOHN MICHAEL CASON

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Chair of Committee,	Charles E. Simpson
Co-Chair of Committee	William L. Rooney
Committee Members,	Jason E. Woodward
	Peter A. Dotray
Head of Department,	David D. Baltensperger

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ABSTRACT

In this study, a hybrid of the bridge species *Arachis vallsii* Krapov. and W.C. Greg. (VSW 9902-1) and *A. dardani* Krapov. and W.C. Greg. (GK12946) was created to initiate an introgression pathway for movement of possible drought tolerance genes into the cultivated peanut (*A. hypogaea* L.). A hybrid between the two species was successfully created and confirmed based on leaf morphology, pollen counts and intermediated leaf morphology. One-hundred and seventy-five attempts were made to double the chromosome complement using 3 methods at concentrations of 0.02% and 0.03% colchicine for exposure times ranging from 6 to 24 hours. No attempt has been successful to date. In addition, a greenhouse transcriptome study with 7 day-imposed drought was conducted on *A. dardani* (12946) and the reference species *A. ipaënsis* (Krapov. and W.C. Greg.) (KGBPScS-30076) (B genome donor of the cultivated peanut). Differential gene expression analysis (EdgeR Test) of the normalized RPKM (Reads Per Kilobase Million mapped reads) values was conducted with a fold value $\geq$ abs (2) at the $p \leq 0.05$ level using CLC Genomics Workbench v8. Significant transcript levels associated with drought tolerance were found in relation to the putative drought species (*A. dardani* (12946)), which have not been reported previously. Transcripts were identified that were higher between physiological states and between species. In total, 40 genes were identified for further study.

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CHAPTER I

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an allotetraploid ($2n = 4x = 40$) that has been cultivated for thousands of years (Singh and Simpson, 1994). Today it is grown throughout the temperate and tropical part of the world and is an important international crop (Kochert *et al*. 1991; Krapovickas and Gregory, 1994). Areas of production range from subsistence farming to large scale commercial operations and are in all continents except Antarctica (ICRISAT, 2018). Total worldwide peanut production is approximately 29 million MT per year (Worldatlas, 2018), with an average yield of 1520kg/ha in 2009 (ICRISAT, 2018). Although peanut originated in South America (Hammons, 1982), currently that continent accounts for only 3% of current global production; Asia and Africa account for 56% and 40% of global production respectively (ICRISAT, 2018). Leading countries in production are China (13.4 million MT), India (7.7 million MT) and the U.S. (1.8 million MT) (Worldatlas, 2018; Holbrook, 2014).

Peanuts are used in many ways; over 50% of worldwide production is crushed for use as oil (TPF, 2015). Other uses include peanut cake and meal (TPF, 2015) and direct consumption or as an ingredient in foods. Use does vary by country; most peanuts in the U.S. are used in peanut butter, confectionary products or they are exported (NPB, 2018).

In the U.S., approximately 540,000 ha of peanuts were harvested in 2015, with an average yield of 4443 kg/ha (USDA, 2016). The estimated farm value of U.S. production

is more than one billion U.S. dollars, with peanut being listed as the 12th most valuable cash crop in the U.S. (TPF, 2015). Peanut production is concentrated in the Southern U.S., from the eastern seaboard to New Mexico. Georgia is the leading peanut producing state followed by Florida, Alabama and Texas (USDA, 2016).

Groundwater depletion and climate change have led to an increased interest in drought tolerance in many crops including peanut. Georgia alone estimated $92.5 million in production value losses in 2007 due to drought, which represents a 28% decrease in production (UGA, 2007). The increased frequency of drought events is cause for concern, because it has been estimated that up to 80% of the peanut production in the world is centered in areas that use no irrigation and are subject to unpredictable droughts (Wright and Rao, 1994). The High Plains of Texas is an excellent example of the increasing concern over drought and groundwater levels. Irrigation water coming from the Ogallala aquifer is used throughout most of the region. Chanduri and Ale (2014) reported estimates that 90% of the water pumped out of the Ogallala aquifer in Texas is for the purpose of irrigation. A further consideration is that 60% of the overall water needs in Texas are met by groundwater (TWDB, 2011). The United States Geological Survey has estimated that groundwater use in the High Plains ranges from 10.7-19.9 million Ml per year. This represents an average irrigation rate of 213.6 to 411.5 mm per year (USGS, 2012). It has been estimated that median water-levels of the Ogallala aquifer in the Texas Panhandle dropped from 25 to 67m in the 70 years since irrigated agriculture has become common (Chaudhuri and Ale, 2014). This also has been seen in other areas of the state to a lesser extent.

Recharge rates vary on several factors including type of landcover, soil type and impermeable cover (Chaudhuri and Ale, 2014). Recharge is difficult to measure; it is estimated to take from a few days to several decades in some cases, depending on the location. These facts, and a Texas population that is estimated to double by 2060 will put further pressure on an already decreasing water supply (TWDB, 2012). This has placed significant interest in the development of drought tolerance in many crops, including peanut.

Based on peanut germplasm collection data (Krapovickas and Gregory, 2007; Valls and Simpson, 2005) it is believed that there are wild species available that possess drought tolerance. However, the alleles contained in the 20 chromosome wild relatives are not readily available to cultivated peanut, due to a chromosome doubling event that left the cultigen genetically isolated. (Kocher *et al*. 1991, Kochert *et al*. 1996). To date, transfer of any genes from wild relatives has involved traditional hybridization and introgression techniques. This has occurred due to in some cases the large number of genes believed to be involved in traits such as drought, but also a lack of public acceptance of anything that is perceived as a transgenic variety because peanut is a food crop used in direct human consumption, especially in Europe (Smith, 2008).

Due to the complicated taxonomic nature of the genus, the development of introgression pathways and traditional chromosome doubling techniques are used to move alleles into cultivated peanut. The genus *Arachis* contains 9 taxonomic sections, of which section *Arachis*, as discussed below, is the largest. All of the species in section *Arachis* are cross compatible with one another (Krapovickas and Gregory, 2007). However, in many cases transferring alleles from germplasm in other sections is a long process involving

many steps. In its simplest form, a test cross is made to see if two species are compatible and if viable seed can be obtained. If a hybrid is produced, the colchicine can be used to manipulate the chromosome number of the hybrid. This allows it to possibly be hybridized with cultivated peanut (Simpson, 1991).

Based on this information, the objectives of this project were to first identify possible genes of interest for drought tolerance through the use of RNA-seq technology. Secondly, begin the development of a new gene introgression pathway, which can be used to move genes from the species *A. dardani* into the cultivated peanut.

CHAPTER II

LITERATURE REVIEW

II.1 Taxonomy and Organization of Genus *Arachis* and its Species

The genus *Arachis* contains nine taxonomic sections and eighty-two described species. It has been estimated that these sections diverged approximately 5 million years ago (Moretzsohn *et al*., 2013). After divergence, the genus moved naturally and with assistance. It has also been estimated that seed dispersal is approximately 1m/yr., due to the geocarpic nature of the genus (Krapovickas and Gregory, 2007). Other cases have been documented of water and humans carrying seeds over longer distances (Krapovickas and Gregory, 1994). The different sections tend to be clustered in different river valleys that are separated by mountain ranges, which created geographic isolation (Gregory *et al*., 1980). The center of origin for the genus is probably in what is now Southwestern Brazil or Northeastern Paraguay. The species have evolved in an area bound by a line from northeastern Brazil to the Andes in northwestern Bolivia and then south to north central Argentina, then east to the coast of Uruguay then back to northeast Brazil (Simpson personal communication). The area is south of the Amazon river and stretches from the foothills of the Andes to the Atlantic Ocean (Hammons, 1982).

Early efforts to organize the genus were based on plant morphology and cross compatibility data and led to the assignment of nine sections (Gregory and Gregory, 1979; Gregory *et al*., 1980; Krapovickas and Gregory, 2007). Extensive cross compatibility

studies have continued that further define sectional boundaries (Valls and Simpson, 2017). As data were collected, it was found that sections *Caulorrhizae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Trierectoides* and *Triseminatae* only contain species with 20 chromosomes. However, sections *Rhizomatosae* and *Arachis* contain species with both 20 chromosomes and others with 40 chromosomes (Smartt *et al.,* 1978 a and b; Stalker and Simpson, 1995; Krapovickas and Gregory, 2007). The species in section *Rhizomatosae* with 40 chromosomes (2n=4x=40) are believed to have arisen from one to several polyploidization events (Smartt and Stalker, 1982; Halward *et al*., 1991). Some 2n=4x=40 accessions in section *Rhizomatosae,* A. *glabrata* Benth. var. *glabrata* are used as forage crops for animals and as ornamental ground covers (Krapovickas and Gregory, 2007).

The 2n=4x=40 species in section *Arachis* arose from a different polyploidization event than *A. glabrata* and will be the focus of the research in this current study. Many cross-compatibility studies as well as chromosome analysis of the entire genus have been conducted (Gregory and Gregory, 1979). Early studies revealed two genomes that were designated genomes A and B (Smartt *et.al*., 1978 a and b, Husted, 1933 and 1936). As molecular biological techniques have been developed and refined, the A and B genomes have been divided further. Today some literature contains references to A, B and D (Stalker, 1991). In addition, there are some more recent references to F and K genomes, which were separated out of the original B genome group (Seijo *et al.,* 2004*;* Robledo and Seijo, 2010).

Arachis hypogaea, by taxonomic rule, is placed in section *Arachis*. It is an allotetraploid, with 40 chromosomes (2*n*=4*x*=40). Allotetraploids are a type of polyploidy

that contain two complete genomes from different species that behave as a diploid during meiosis (Fairbanks, 1999). In the case of *A. hypogaea*, Bertioli *et al*. (2011) relate that *A. hypogaea* is proposed to have formed from the fusion of unreduced gametes or a hybridization and subsequent chromosome doubling event involving two progenitor species, one from the A genome and one from the B genome (Krapovickas, 2004). This is believed to have occurred between one or only a very few individuals of two different species (Halward *et al*., 1991) and is believed to have occurred in northern Argentina or eastern Bolivia (Gregory *et al*., 1980) although, Simpson and Faries (2001), suggest a possible origin site in Peru based on archeological evidence.

There has been significant effort to determine the possible progenitor species of the cultivated peanut. Multiple species including, *A. cardenasii* Krapov. and W.C. Gregory (Smartt *et al*., 1978a), *A. helodes* Krapov. and Rigoni, *A. simpsonii* Krapov. and W.C. Gregory (Milla *et al*., 2005), *A. villosa* Benth. (Raina and Mukai, 1999; Raina *et al*., 2001) and *A. duranensis* Krapov. and W.C. Gregory (Kochert *et al*., 1996) have all been suggested as possible A genome donor parental species. Of these, the consensus is that *A. duranensis* is the most probable A genome donor (Seijo *et al*., 2004; Seijo *et al*., 2007).

For the B genome donor to cultivated peanut, *A. batizocoi* Krapov. and W.C. Gregory was the first species proposed (as cited from Smartt *et al*., 1978a), likely because it was the only known member of the B genome group for many years. As more research and additional collection work was conducted, it became apparent that *A. batizocoi* was not a progenitor species of *A. hypogaea* (Stalker and Dalmacio, 1986) and attention focused on *Arachis ipaënsis* Krapov. and W.C. Gregory (Raina and Mukai, 1999; Seijo *et al*. ,2004; Kochert *et al*., 1996). However, it has been very difficult to produce synthetic

allotetraploids between *A. duranensis* and *A. ipaënsis.* Molecular data indicated that *A. duranensis* had to be the female in the cross that formed *A. hypogaea.* However, the cross was only possible with A. *duranensis* as the male parent as reported by Fávero *et al*. (2006). Later research suggested a different accession of *A. duranensis* as the A genome donor (Grabiele *et al*., 2012) and the cross has subsequently been made successfully using that material (Simpson, 2017). It is now widely accepted that A. *duranensis* and A. *ipaënsis* are the most probable species that combined to form *A. hypogaea*.

The doubling event which formed *A. hypogaea* effectively isolated the cultivated peanut (2n=4x=40) from its wild relatives (2n=2x=20). This type of reproductive isolation created a significant genetic bottleneck that has resulted in a narrow genetic base in *A. hypogaea* (Kochert *et al*., 1991; Kochert *et al*., 1996). This left the cultigen without access to many of the alleles needed for resistance to many biotic and abiotic stresses that reside in the related germplasm (Burow *et al*., 2009).

II.2 Drought Tolerance

Drought tolerance or drought stress traits have become a major focus of research in many crops. Drought response is a complex physiological reaction where differences exist both among and within plant species. In some cases, there are tradeoffs between inherent drought tolerance and productivity. Thus, several definitions for drought tolerance have been developed. Blum (2005) reported from a physiological context, drought tolerance is best defined as ‘dehydration avoidance’ and/or ‘dehydration tolerance’. Further, it has been described as the ability of a plant to reproduce before the onset of stress is described as an escape strategy for drought (Levitt, 1972). Fleury *et al*., (2010) stated that drought

tolerance is the ability of a plant to live, grow and reproduce satisfactorily with limited water supply or under periodic conditions of water deficit. (Turner, 1979). These definitions have been used for many years and remain some of the best descriptions of drought tolerance that occur in the literature.

Economic yield is the primary concern of growers and drought tolerance is only of value if it maintains or increases yield. Unfortunately, some genotypes with exceptional drought tolerance are not responsive to yield in either drought or favorable environments (Tollefson, 2011). Consequently, breeders have traditionally selected for yield under stress which tends to produce plants with traits such as early flowering, smaller plants, small leaf area or limited tillering in cereals (Blum, 2005). This shifts the harvest index of grain or fruit/ biomass per unit, but does not necessarily increase yield potential *per se*. The selection procedure can, in some cases, increase the yield under the stress conditions.

Drought conditions are highly variable and affect a plant in many different ways. As a result, yield is not always predictable from one stress event to the next (Blum, 2005). Drought tolerance is dependent on the timing of the water availability, intensity and duration of the stress (Saint Pierre *et al*., 2012), the age and stage of development of the plants when drought stress occurs (Chimenti *et al*., 2006), as well as the organ and cell type affected during the event (Pastori and Foyer, 2002). While a drought event may not always result in the death of a plant, it can cause economic loss if it occurs at a critical period in the life cycle of a plant (Rivero *et al.*, 2007).

Drought tolerance can be categorized in three broad categories divided by the mechanisms by which they deal with the drought stress; dehydration avoidance, tolerance

and escape. Overlap occurs in each of these categories with respect to plant response to drought stress (Chaves *et al*., 2003). Chaves *et al*., (2003) explains that signaling pathways that lead to drought response can be triggered through several different biochemical pathways and have been found to be a vast interconnected network (Knight and Knight, 2001; Bohnert and Sheveleva, 1998). As a plant's response to drought is examined, it can be seen that these networks of genes are not only stress induced, but are present whether stress occurs or not and can be activated due to many different environmental cues (Blum, 1984; Passioura, 2002).

Plants selected in a drought tolerance breeding program have typically been selected in a given environment under drought stress conditions typical of that environment. Even with a specific location, these factors and their interactions can be highly variable from year to year. Because of this, when breeding for drought tolerance a breeder relies on multi-location testing under varying environments and indirectly selects for drought tolerance based on high and stable yield (Lopes *et al*., 2011). Selection of this type has generally been considered successful for a given location; however, due to high genotype by environment interaction it has not resulted in genotypes that perform well across locations (Branch and Hildebrand, 1989; Araus *et al*., 2002). This can be overcome with a long term multi-location testing program. Lopes *et al*., (2011) explain there are some examples in maize breeding that show significant increase in yield with the lower genetic gain used in a slower conventional breeding approach focusing on selection for drought adaptation (Bänziger *et al*., 2004).

II.3 Breeding Strategies

There is overlap and interaction between the drought stress mechanisms. Based on the environmental conditions a plant encounters, possible breeding strategies become evident. Chaves *et al*., (2003) reported a drought stress event can be slow developing (lasting weeks or months) or fast developing (hours or days). For example, if drought conditions develop slowly a plant can adjust by shortening its life cycle or it can optimize the resources it has available to it. In a fast-developing drought, the plant will react to minimize moisture loss (Chaves *et al*., 2003).

Since environments can vary greatly, researchers must use several environments to evaluate plots. Branch and Hildebrand (1989) suggested that selections made for pod yield *in A. hypogaea* at a single location should not be expected to perform comparably in varying environments. Therefore, multi-location, multi-year trails have been used successfully to make selections. However, this is sometimes not the fastest way to obtain drought data. As an example, Rucker *et al*., 1995 found the cultivar Florunner of *A. hypogaea* to be the highest yielding genotype in studies conducted for drought tolerance traits and concluded that years of selection under varying environmental conditions have created the excellent yielding cultivar.

Managed stress environments are another option for drought trait selection. In this situation researchers create the drought event by controlling available moisture. Researchers at CIMMYT used managed stress environments to select elite maize (*Zea mays* L.) hybrids in southern Africa (Weber *et al*., 2012). Roy *et al*., (1988) used three different drought imposed periods to evaluate *A. hypogaea* yield in Ontario, Canada and

found the growth stage of late flowering into early pod formation was the most affected by drought conditions.

An example of a managed stress environment would be a rain out shelter, which keeps rainfall off the given test or nursery. Branch and Klein, 1992 reported positive results when non-automated temporary shelters were used to screen early segregating populations of *A. hypogaea.* In these trials, artificial drought was imposed from 60 to 120 days after planting period to simulate a midseason drought. Another example of managed stress environments is controlled greenhouse experiments. This approach has been used to evaluate seedling cotton (*Gossypium hirsutum L.*) for drought tolerance traits. Basal *et al*., (2005) reported the use of controlled greenhouse trials to test for traits dealing with root architecture and found positive correlation for the use of these traits as a possible early screening technique.

Managed stress work has led researchers to look for phenotypic traits that occur in association with the desired drought tolerance under field conditions. Arunyanark *et al*., (2008) tested phenotypic traits in *A. hypogaea* that were thought to be associated with drought tolerance. They tested transpiration efficiency (TE), which is defined as the amount of biomass produced per unit of water transpired. In addition, they tested chlorophyll content and density of leaves a trait closely linked with photosynthetic capacity and the ability to maintain chlorophyll density under water deficit conditions. No genotype by environment interactions were found between chlorophyll content and density, and high correlations were found between total dry matter (TDM) and chlorophyll content and TE and chlorophyll density, which indicated those traits are useful predictors in peanut. Chen *et al*., 2013 suggested the use of leaf C isotopic composition

measurements possibly could be used as a predictor of drought tolerance in peanut. Leaf C isotopic composition involves the measurement of C isotope ratio ^{13}C:^{12}C in plant tissue, which is then correlated to intercellular CO_2 or ambient CO_2.

II.4 Genomics and Molecular Markers

Phenotypic traits can be associated with molecular genetic markers through single gene trait associations or quantitative trait loci (QTL's). If the markers are robust, meaning they reflect the trait across multiple genotypes, they then can be used in marker-assisted breeding. Fleury *et al*., 2010 stated single gene trait markers are easier to work with, but some traits such as drought tolerance involve many genes making it a very complex trait to breed for (McWilliams, 1989). Ravi *et al*. (2011) suggested that drought tolerance control in *A. hypogaea* was controlled by several main effect QTL's (M-QTL), as well as, epispastic QTL's (E-QTL). Quantitative trait loci (QTL's) are large sections of DNA that are associated with quantitative traits (Fairbanks, 1999). The sections can contain one or more genes that influence a trait of interest. Markers can be associated with these QTL's using structured populations that are related in some way, unstructured populations that span an entire genome or a combination of the two types, such as the Nested Association Mapping populations in maize (Yu *et al*., 2008) and peanut (Holbrook *et al*., 2013).

Marker technology is continually evolving. Early markers such as Restriction Fragment Length Polymorphisms (RFLP) markers where tied to nematode resistance (*Meloidogyne arenaria* (Neal) Chitwood) and *M. javanica* (Treub)Chitwood) in *A. hypogaea* (Church *et al*., 2000). This resistance is believed to be associated with a major resistance gene that is completely dominant (Burow *et al*., 1996). These markers were

expensive and time consuming to identify, as well as involved the use of a radioactive isotope to visualize the marker. Amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) represent later generations of markers that were easier to use and less expensive. The development of sequencing technology resulted in the Single Nucleotide Polymorphism (SNP) markers wherein a SNP is variation at a specific nucleotide in a genome at a specific location. A SNP marker allows researchers to distinguish different combinations of bases present in diploid genomes relatively easily. These differences can be identified and compiled into genetic maps that span the entire genome. New sequencing technology, known as high throughput sequencing, has greatly reduced the cost of compiling and assembling whole genome maps. Single Nucleotide Polymorphism markers coupled with high throughput sequencing has led to much greater resolution of the DNA sequence and the identification of large numbers of markers. This has facilitated research at the whole genome level, sometimes called genomics (Mandal, 2018).

High throughput sequencing also has allowed researchers to employ powerful new techniques, such as RNA-seq, to examine populations based on their transcriptome (transcriptomics). With this technique, RNA is extracted, and all the transcripts are sequenced that are produced in an organism at the time of collection. One of the marked advantages of the use of RNA is the ability to identify candidate genes for a given trait of interest, target specific types of RNA analysis, as well as, the ability to identify genes that are expressed more or less frequently, sometimes known as differentially expressed genes (Nagalakshmi *et al*., 2010; Bedre *et al*., 2015). From the extracted RNA, a cDNA library is produced through reverse transcription. Based on this library, genes of interest and

differentially expressed genes can be identified *de novo* or when compared to a reference genome or transcriptome. The ability to discover genes, as well as, tell if those genes are up regulated or down regulated in a particular situation has led to widespread use well beyond the genomics community (Conesa *et al*., 2016). Transcriptomic projects in peanut have successfully identified genes of interest for drought (Shen *et al*. 2015; Chopra *et al*., 2015), early embryo abortion (Chen *et al*., 2013), *Sclerotium rolfsii* (Sacc) susceptibility (Jogi *et al*. 2016) and *Ralstonia solanacearum* susceptibility (Chen, 2014).

The genome size of the cultivated peanut is approximately 3 Gb and is estimated to be about 64% repetitive content (Bertioli *et al*. 2016). The use of SNP markers in *A. hypogea* is complicated by the tetraploid nature of the species because at any one locus, four nucleotides are detected instead of two. Consequently, this makes it difficult to determine which SNP is associated with the 2 separate genomes present in *A. hypogaea* (Akhunov *et al*., 2009). These can be manually corrected when the separate genomes are examined for SNP's. (Bertioli *et al*., 2014). In addition, more recent techniques involve machine filtering with a program called SWEEP to decrease the false positive SNP calls. Research indicated that use of the tool greatly increases correct SNP identification from approximately 2-8% to 65-99% depending on coverage (Clevenger and Ozias-Akins, 2015).

As mentioned previously, the lack of allelic diversity in cultivated peanuts has increased interest in variation that is present in the wild relatives (Simpson *et al*., 1993; Nagy *et al*., 2010). This variation can be associated with novel genes of interest and serve as a guide in introgression of these new genes (Bertioli *et al*., 2014). To aid in this process The Peanut Genomic Initiative was formed to sequence and analyze the peanut genome.

The group successfully sequenced the two diploid progenitor species (*A. duranensis* and *A. ipaënsis*) of the tetraploid cultivated peanut, *A. hypogaea.* The diploid sequences were made available to U.S. and international breeders in 2014. The two diploid sequences were used as a guide in the assembly of the tetraploid genome sequence, which was released in 2017 (PGI, 2018).

II.5 Chromosome Doubling Compounds

The problems of creating fertile hybrids between tetraploid and diploid species of peanut are not insurmountable. Colchicine and oryzalin have been used for many years to induce chromosome doubling. Colchicine ($C_{22}H_{25}NO_6$) is an alkaloid derived from the bulbs of *Colchicum autumnale* L. (Lacy, 1988; Seguí-Simarro and Nuez, 2008), whereas, oryzalin (3,5-dinitro N4, N4-dipropysulfanilamide), is a dinitroaniline herbicide (Ganga and Chezhiyan, 2002). Both can be used to artificially induce chromosome doubling by decreasing the formation or persistence of mitotic spindles observed in anaphase spindle formation or an alternate mechanism involving nuclear fusion (Seguí-Simarro and Nuez, 2008; Sunderland *et al*., 1974).

Both of these compounds have been used in efforts to restore fertility to sterile hybrids of *Datura stramonium* L. (Blakeslee and Avery, 1937). They also have been used for genome duplication in many other systems including *Musa* (spp.) (Ganga and Chezhiyan, 2002), potato (*Solanum tuberosum* L.) (Kawakami and Matsubayashi, 1966), *Cosmos* (Blakeslee and Avery, 1937), triticale (*Triticale hexaploide* Lart.) (Fairbanks, 1999) and maize (*Z. mays*) (Barnabás *et al*., 1999). In *Arachis*, they have been used successfully to produce hexaploids from sterile triploids (Gregory, 1980; Garcia *et al*.,

2006) and tetraploids from sterile wide species hybrids that are diploid (Simpson, 1991; Simpson and Starr, 2001). Concentration, time of exposure and type of material treated can influence success. Norden *et al*., 1982 cited the comparison of three different application techniques of colchicine in *Arachis* hybrids. They tested the treatment of vegetative shoots, cuttings and seed and found vegetative shoot treatment to be the most successful (as cited from Spielman and Moss 1976). A different technique was used by Fávero *et al*., (2006), in which 20 cm cuttings where immersed in 0.2% colchicine for 8 h to successfully double hybrids. Simpson (1991) reported the best results by treating seeds that had just germinated. The variability of success and technique, in *Arachis* is consistent with attempts to double chromosomes in many other genomes (Ganga and Chezhiyan, 2002; Seguí-Simarro and Nuez, 2008). The process is somewhat trial and error when treating new material. The concentration or time of exposure can be adjusted, based on results from previous attempts with any given material (Simpson, 1991).

II.6 Gene Introgression

Reynolds and Tuberosa (2008) presented several ways to evaluate germplasm and gain access to the alleles that would broaden the genetic base of a species. These included: introduction of transgenic organisms, introgression from compatible genomes and interspecific or intergeneric hybridization. The use of transgenic genetically modified organisms shows great potential in development of genetic diversity to both biotic and abiotic stresses. It can be used across taxonomic groups and has shown great promise in controlled drought tolerance studies in several crops (as cited from Parry *et al*., 2005; Umezawa *et al*., 2006; Nelson *et al*., 2007). However, to date, somewhat limited public acceptance of GM products and regulatory costs limit their use. The opposition to

transgenic crops appears to be a problem of public acceptance that is not based in scientific research (AMA, 2012; WHO, 2014). Although transgenic development could be the most direct and perhaps quickest method for variety development, it is currently not an option for peanut breeders (Smith, 2008)

A second method for development of genetic diversity is to use material from a compatible genome. The wild species peanut (2n=2x=20 and 2n=4x=40) germplasm contains a large reservoir of genetic diversity that could be used to broaden the genetic base of *A. hypogaea* (Gregory and Gregory, 1979; Kochert *et al*., 1991). Further, *A. hypogaea* has been adapted to many different environments around the world. It has been estimated that domestication of the cultivated peanut began at least 3500 years ago (Singh and Simpson, 1994). The variation that developed in *A. hypogaea* is due in part to it being carried and grown extensively though South and Central America, as well the West Indian Islands. (Hammons, 1982). It has been spread by trade routes throughout the world (Higgins, 1951). While there is not always documentation to show it being intentionally introduced to new areas, it has most probably been used as an item of barter along shipping routes (Hammons, 1994). The variation has been used extensively. PI 109839 has been used as a source of resistance to early leaf spot, caused by *Cercospora arachidicola* (Hori), and early maturity. PI 203396 was used as a source of resistance to late leaf spot, caused by *Cercosporidium personatum* ((Berk. & Curt.) Deighton), southern stem rot, caused by *Sclerotium rolfsii* Sacc., as well as tomato spotted wilt virus. PI 221075 was used to successfully move *Sclerotinia minor* (Jagger) resistance genes into the commercial variety Tamspan 90 (Smith *et al*., 1991; Isleib *et al*., 2001).

A third method to develop genetic diversity is to make wide species crosses to introgress desired genes into the cultivated peanut from related wild species. This method has been used successfully in the past (Simpson, 2001; Simpson and Starr, 2001) and shows great promise for the transfer of genes that have not been accessible to *A. hypogaea* since the original chromosome doubling event occurred to form the species. To move genes in this manner a pathway must be developed that involves hybridization and artificial chromosome doubling in some order. Simpson (1991) cited at least four possible pathways with which to move genes into *A. hypogaea*, however many variations of each pathway could be used. The first pathway has been termed the hexaploid pathway. In this pathway *A. hypogaea* (2n=4x=40) is crossed with another *Arachis* spp., which is 2n=2x=20, to produce a F1 triploid, which is treated with colchicine, creating a hexaploid (2n=6x=60). The hexaploid is crossed with *A. hypogaea* followed by backcrossing or selfing several generations to lose chromosomes through the normal action of chromosome segregation and elimination (Simpson, 2001). This pathway has been used successfully in the development of both insect and disease resistance breeding lines (as cited in Moss, 1985; Singh, 1985, 1986a, b; Moss *et al*., 1989; ICRISAT, 1990), as well as in the creation of several germplasm lines (as cited from Smartt and Gregory, 1967; Stalker and Beute, 1993).

A second pathway involves crossing two *Arachis spp*. (2n=2x=20) from the A genome to produce a fertile F1 hybrid, which is then crossed with a bridge species from the B genome which is also a diploid (2n=2x=20) species. This produces a three-way hybrid (usually highly sterile) that is chromosome doubled using colchicine, to produce a fertile allotetraploid. This pathway has been used successfully to introgress high levels of early

and late leafspot resistance and root-knot nematode (*M. arenaria* and *M. javanica*) resistance into *A. hypogaea* from its wild relatives (Simpson, 2001; Simpson and Starr, 2001; Simpson *et al*., 2003).

The third pathway involves the use of colchicine to double the chromosome number of two different diploid species (2n=2x=20) individually, so that a male and a female are 2n=4x=40 (AAAA genome) and 2n=4x=40 (BBBB) or vice versa before they are hybridized. The doubled species can be hybridized and subsequently crossed with *A. hypogaea*. Another approach involves crossing two diploid species (2n=2x=20) to produce a diploid hybrid. This hybrid can be chromosome doubled and hybridized with *A. hypogaea*. These latter two pathways have been studied, but due to high levels of sterility in the hybrids, have not been successful (Simpson, 2001).

CHAPER III

MATERIALS AND METHODS

This project was conducted in 2 phases. The first objective was to identify genes of interest for drought tolerance of a proposed drought tolerant species. The second objective was to determine if the reputed drought tolerant species *A. dardani* (GK 12946) was compatible with the bridge species *A. vallsii* (accession VSW 9902-1) and begin the development of an introgression pathway to move the genes identified in objective one into cultivated peanut by creating a viable hybrid.

III.1 RNA-seq

III.1.1 Greenhouse Study

A replicated, imposed drought study was conducted during the winter of 2016 at the greenhouses of the Texas A&M AgriLife Research and Extension Center at Stephenville. The study was conducted in an IBG greenhouse operating on a Wadsworth Step-50 temperature control system. The system operated where the heaters cycle on if the temperature drops below 21^{o}C and the cooling system cycles on if the temperature exceeds 32 ^{o}C.

The study contained 4 biological replications for two species, *A. dardani* and *A. ipaënsis,* at two physiological states (Chopra *et al*., 2014). *Arachis ipaënsis* was included because it is the B genome progenitor of *A. hypogaea* and represents the best match of the

two reference sequences available through the Peanut Genomic Initiative (peanutbase.org). The use of *A. ipaënsis* allowed the study to be aligned with the published reference genome as well as to search for genes in related species. Similar transcriptomics studies have been conducted and assembled *de novo* (Burow personal communication), as well as, using the reference sequence for *A. duranensis* (Clevenger *et al*., 2016). Only a few of the most recent studies have begun to take advantage of the peanut reference species, but to date no studies have used the B genome sequence as a reference for alignment and to our knowledge none using *A. dardani*. For this study, drought was defined as a greater than 10% reduction in relative water content (RWC), which is a measure of water deficit in the leaf of a plant relative to its fully turgid state and serves as an indicator of hydration status (Barr and Weatherly, 1962).

Plants were grown in 24 cm plastic pots in a Winthorst fine sandy loam soil. Collection of leaf and root tissue occurred at 75 days after planting (DAP) at which time drought was imposed for 7 days and a minimum % RWC of less than 80% was obtained before sampling. Visual signs of drought, such as leaflet closing, leaflet curl, main stem curl and loss of turgidity were used as indicators of drought stress. Imposed drought was started on 8 February 2017. Collection of the shoot and root tissue of the well-watered control began on 14 February 2017 at 9:00 a.m. Tissue samples were taken from the unexpanded tetrafoliate leaves and apical meristems of the lateral branches of each of the biological replicates. Subsequently, the entire root system of each biological replicate was harvested and kept separate. To minimize differences in gene expression due to time of day collection of stressed tissue occurred one day later 15 February 2017 at 9:00 a.m. It took approximately 3 hours to harvest leaf and root tissue on both days.

Preliminary drought stress testing was conducted on alternate plants to gauge the rate and degree of drought stress. It was determined that *A. ipaënsis* showed signs of drought stress before *A. dardani*. Based on this it was determined to sample when the *A. ipaënsis* plants exhibited drought stress.

Relative water content samples were taken of all plants immediately before tissue samples were collected to serve as an indication of physiological state for the study. The fourth expanded tetrafoliate was removed at the stipule from each of the plants in both control and stressed plants for both species. If the fourth expanded tetrafoliate was not satisfactory after visual inspection the fifth expanded tetrafoliate was sampled. Likewise, if the fifth expanded tetrafoliate was not satisfactory the third expanded tetrafoliate was selected. Samples were then immediately weighed for fresh weight on a Fisher Scientific XA-200Ds analytical balance. After weighing, the samples were immersed in water for 24 hours and reweighed to obtain the turgid weight. Once turgid weights were obtained samples were placed in a Blue M (General Signal, Garland Texas) dryer at 37ºC for 7 days. After the drying period samples were immediately weighed to obtain the dry weight. The three weights were then used to calculate the % RWC (Barr and Weatherly, 1962)

$$\%RWC = [(FW-DW)/(TW-DW)] *100$$

Where FW is the fresh weight, DW is the dry weight and TW is the turgid weight. Sample RWC values were calculated and subjected to analysis of variance (ANOVA) and least significant difference (LSD) analysis in JMP Pro 12 (JMP, Cary, NC).

III.1.2 RNA Extraction and Sequencing

All tissue samples were flash frozen immediately upon collection with liquid nitrogen (LN_2) and stored in a -80°C SO-LOW (So-Low Environmental Equipment Co., Cincinnati, OH) ultra-low freezer until extraction. The shoot and root tissue RNA was extracted at the Texas A&M AgriLife Research and Extension Center at Stephenville. Ribonucleic Acid was extracted using an Qiagen RNeasy kit according to manufacturer's instructions. In this procedure, tissue was first ground in liquid nitrogen using a mortar and pestle and subsequently lysed using denaturing buffers. Samples were then centrifuged using a QIAshredder homogenizer to remove insoluble material and reduce viscosity of the lysate. Ethanol is then added to promote binding to a silica-based membrane which is applied to a RNeasy Mini spin column. High-salt buffers allow RNA longer than 200 bases to bind to the silica membrane and contaminates are washed away. Supernatant containing RNA was transferred to fresh tubes for storage until sequencing.

One microgram of total RNA from each of 32 samples (four biological replicates of two species in two physiological states) were sent to Texas A&M AgriLife Genomics and Bioinformatics Services in College Station, TX, where RNA quality was accessed using a Fragment analyzer (Advanced Analytical Technologies, Inc., Ankeny, Ohio). An RNA quality number (RQN) of 5.7 minimum was used as criteria for sequencing. Complimentary DNA libraries were prepared for each sample according to manufacturer's instructions using the Illumina TruSeqTM RNA Sample Preparation Kit. Libraries were sequenced using eight lanes of Illumina HiSeq 2500 with barcoding to multiplex biological replicates. Read counts averaged 25,000,000 single end reads per sample and had an average read length of 50 base pairs (bp). Real time Sequence cluster identification,

quality control prefiltering, base calling and uncertainty assessment were done using default settings of Illumina's HCS 2.2.68 and RTA 1.18.66.3 software. Base call files (Sequencer.bcl) were demultiplexed and formatted as FASTQ files using bclefastq 2.17.14 script configureBcltoFastq.pl.

The *A. ipaënsis* (K30076.gnm) (B genome donor) reference genome was used and annotated with the associated .GFF annotation file from Legume Information System website (legumeinfo.org, Ames, IA). Sequence reads were aligned to 41,801 gene models using CLC Genomics Workbench version 8.1 (CLC Inc., Aarhus, Denmark). Default settings of minimum length fraction requirement of 0.9 and a minimum similarity fraction of 0.8 were used to align the samples with the reference genome. Reads Per Kilobase per Million mapped reads (RPKM) were used instead of actual read counts for comparing gene coverage. Principal component analysis (PCA) of the RPKM normalized data was used for quality control of RNA-seq data.

Normalized RPKM counts were used for differential gene expression (DGE) analysis. Differential gene expression analysis was conducted using the EdgeR package which is available with CLC Genomics Workbench. EdgeR uses several statistical methodologies simultaneously and can be applied to genomic count data. Fold change between samples and FDR-adjusted P-values were used to identify genes that are significantly up or down regulated. CLC Genomics® workbench default settings were used (total count filter cutoff = 5 reads) for DGE analysis. A RPKM fold changes values of ≥ 2 and an FDR-adjusted p-value ≤ 0.05 were used as minimum values to be considered as possible genes of interest. These levels were selected based on previously published data (McKinley *et al*., 2016).

Comparisons were made for all combinations between species and physiological state. For clarity, comparisons were assigned a number (Comp #). A description of each comparison follows with indication of species and physiological state. Each DGE comparison was conducted on both shoot tissue and root tissue separately. Comparisons included: *A. dardani* well-watered versus *A. dardani* stressed (Comp 1), all *A. ipaënsis* versus all *A. dardani* (Comp 2), *A. ipaënsis* stressed versus *A. dardani* stressed (Comp 3), *A. ipaënsis* well-watered versus *A. dardani* stressed (Comp 4), *A. ipaënsis* well-watered versus *A. ipaënsis* stressed (Comp 5), all species well-watered versus all species stressed (Comp 6), *A. ipaënsis* well-watered versus *A. dardani* well-watered (Comp 7) and *A. dardani* well-watered versus *A. ipaënsis* stressed (Comp 8). Microsoft Excel 2016 was used to filter and process the results.

III.2 Crossing

The wild species *A. vallsii* (accession VSW 9902-1), was used as the female of the cross. This species was chosen because of its ability to cross with many of the described sections (Custidio, Valls and Simpson (manuscript in preparation)). *Arachis dardani*, accession GK 12946 was used as the male in the cross. The species was selected for its potential to contain drought tolerance. It has been defined as adapted to extreme environmental conditions (Krapovickas and Gregory, 2007).

Seeds of the male and female parents were wrapped in germination towels and placed into a Stults germinator for four days. The germinator operated on a 12 hour photoperiod at a light temperature of 29^{o}C and a dark temperature of 21 ^{o}C. Plants to be used as females were planted in 36.2 cm diameter baskets (figure 1) and plants to be used

as males were planted in 12 cm clay pots. Baskets and pots were filled with Winthorst fine sandy loam soil. The crossing programs for the project also were conducted in an IBG greenhouse operating on a Wadsworth Step-50 temperature control system.

Crossing programs were conducted in both the spring and fall of 2013-2017, except

Figure 1. A picture showing the crossing block layout with an *A. vallsii* female plant in a 36.2 cm basket with marked pollinations and hybridization isolation pots.

for fall of 2014 (table 1; table A3). During each crossing block, female plants were assigned crossing numbers based on the overall number of the cross within the Texas A&M AgriLife crossing program in Stephenville. During each crossing block one

additional male and two additional female backup plants were maintained in a 12-cm clay pot as reserves for each of the species represented in the crossing program. If needed these plants were used to replace plants of the original crosses. The new plants were assigned new crossing numbers that followed the system mentioned above.

Due to the low percentage of successful pollinations, a target of 20-30 pegs were sought for each crossing block. The crossing procedure is a variation of the method described by Norden (1980). Adaptation of the method allows for much higher percentage of successful pollinations (Simpson, personal communication). It is a two-step process consisting of emasculation and pollination. To be eligible for pollination

Table 1. A table showing crossing block information with the male and female parents, planting dates, first flower dates and flower color of 9 crossing blocks.

Crossing Block	Female	Male	Planting date	First Flower date	First Flower color
13X	9902-1	12946	3/26/2013	4/16/2013	orange
13FX	9902-1	12946	8/8/2013	8/28/2013	orange
14X	9902-1	12946	4/21/2014	5/19/2014	orange
14FX	-	-	-	-	-
15X	9902-1	12946	2/16/2015	3/20/2015	orange
15FX	9902-1	12946	8/8/2015	8/28/2015	orange
16X	9902-1	12946	3/25/2016	4/18/2016	orange
16FX	9902-1	7215	9/15/2016	10/19/2016	orange
17X	9902-1	7215-1	3/20/2017	4/16/2017	orange
17FX	9902-1	7215	8/16/2017	9/17/2017	orange

the male and female plants were inspected to ensure that both would be flowering the following morning. One exception was made; in some cases, male flowers were picked a few days early and stored (up to a week) in a 5 ºC refrigerator (Simpson, 1996). Flower emasculations were conducted between 1500 and 1900 h CDT the night before pollination

was to occur (a complete flower diagram can be found in Peanuts Culture and Uses pp. 57) (Gregory *et al.,* 1973). Buds to be emasculated were held individually between the thumb and index finger to ensure stability. A set of forceps was used to remove the calyx. The braccaylx was then folded away from the remainder of the flower, the standard petal was opened gently and folded out of the way. The wing petals were hooked behind the bottom of the standard petal to reveal the fused keel petals, which were pulled downward from the base with the forceps. It was then hooked behind the wing petal to expose the anthers and stigma. The pollen grains on the anthers were immature, so there was little danger of the pollen grains shedding. The anthers were removed as close to the filament base as possible before maturation to prevent self-pollination. The keel petal and the standard petal were then closed to ensure the stigma did not desiccate before pollination. In addition, a moist paper towel was draped over the flower to maintain a humid microenvironment and further prevent desiccation. A 70% ethyl alcohol solution was used to sterilize the forceps between emasculations.

The modification of the Norden technique used occurs during pollination (Norden, 1980). Between 0700 and 0900 h CDT, the morning following emasculation, the flower being used as the pollen source was dissected by removing the standard and wing petals to allow the removal the keel petal with the anthers still inside with scissors. It is important to note that only a fully-opened flower can be used as a pollen source otherwise the pollen is immature, sheds poorly and is difficult to get to adhere to the stigma (Norden, 1980). A second set of forceps was then used to re-open the female flower as in the emasculation process to expose the stigma. The keel petal containing the anthers of the male flower was then slipped over the stigma of the female flower. Once in place the keel was gently

squeezed to burst the pollen sacs. After the keel was placed on the stigma, the flower was marked and dated. It was re-covered with a moist paper towel. A 70% ethyl alcohol solution was used to sterilize the forceps between each pollination. Pollinations were monitored for emergence of pegs each morning. Pollinations were verified as successful if the peg emerged with the desiccated, pollinated flower still attached to the peg tip, to allow positive identification. If verified, a marking string was tied around the peg and attached to a small wooden stake with the date, male pollen source used as well as pollination method used.

Due to the long lateral branches that are characteristic of the female of the cross (VSW 9902-1), the pollinations were most often conducted outside the diameter of the basket in which the plant was growing. To allow for the maximum number of pegs the branches were allowed to run along the benchtops and when a peg emerged a 12-cm clay catch pot was placed under the branch. The pots were filled with Winthorst fine sandy loam soil. The marked pegs entered the soil of the pot and matured. In some cases, multiple pegs were allowed in a single catch pot. Seeds were left to mature until the above ground portion of the peg showed visual indication of maturity. At that time, the peg was clipped and the catch pot was sifted for the pod produced from the pollination. At the time of harvest the stake, marking stick, peg and pod were placed in a paper sack and allowed to air dry. Seeds rested for a minimum of 4 months to avoid dormancy issues. After drying, pods were examined for presence of viable seed. Seed were scored according to an adaptation of the Gregory system based on potential viability and then stored until use. The Gregory system uses a classification of category 1 seed, characterized as a large plump seed to a category 4 seed which is characterized at a shriveled sliver that possibly would

not germinate (Simpson, personal communication). Seeds were treated with ethylene before planting to ensure that dormancy was not an issue. Selected seed were planted and used to confirm hybridization. Criteria used to determine hybridization were pod and seed morphology flower morphology leaf morphology and fertility (López-Caamal and Tovar-Sánchez, 2014).

CHAPTER IV

RESULTS AND DISCUSSION

IV.1 Relative Water Content

Relative water content (RWC) was calculated after 7 days of imposed drought stress for each biological replicate of the stress and well-watered *A. dardani* and *A. ipaënsis* plants, using the formula described by Barr and Weatherly (1962) (Table A1) (tables with A designation found in the appendix). One value corresponded to both shoot and root tissue samples of the RNA-seq study. Analysis using ANOVA and LSD (Table 2) were conducted on these RWC samples.

Table 2. LSD results for 4 replications of relative water content (RWC) data collected for two species of interest in two physiological states. Relative water content is a measure of water deficit in the leaf of a plant relative to its fully turgid state and serves as an indicator of hydration status.

Species	Water Status	Mean Relative Water Content
A. dardani	well-watered	89.88 a
A. ipaënsis	well-watered	89.27 a
A. dardani	drought	77.78 b
A. ipaënsis	drought	41.93 c

Analysis for RWC was highly significant ($p < .0001$**). There was no statistical difference in RWC observed with respect to LSD between the species in their well-watered state. However mean RWC of the stressed *A. dardani*, was significantly greater than that of the stressed *A. ipaënsis* (table 2).

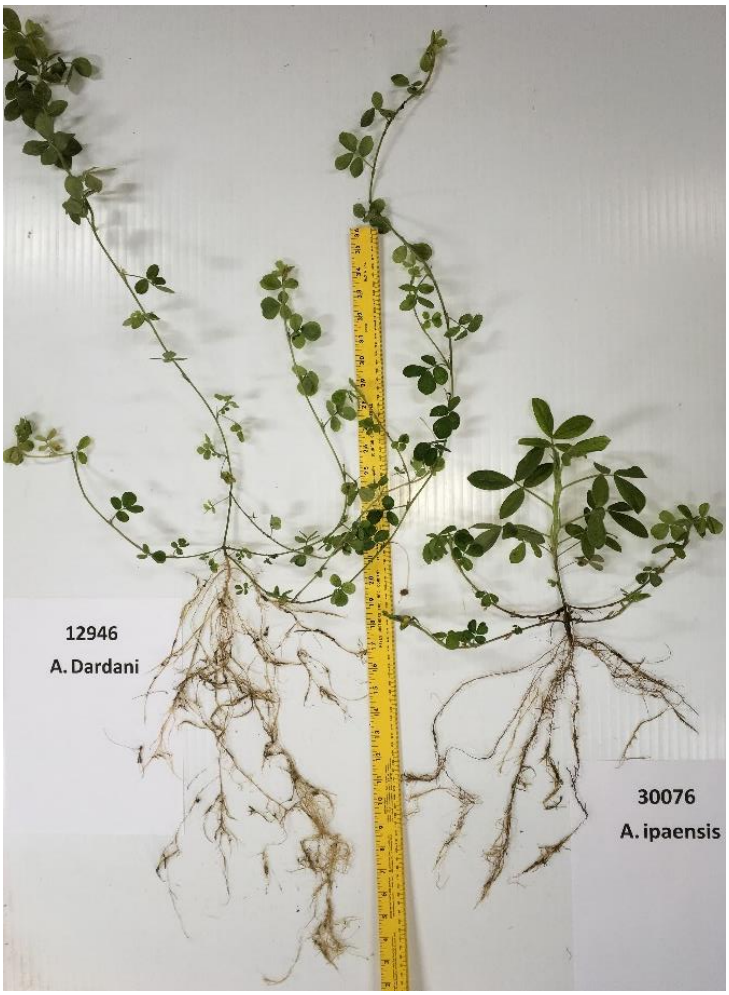

Figure 2. A picture showing the difference in the root systems of *A. dardani* and *A. ipaënsis* at 75 days after planting (DAP).

There was a significant difference in the RWC between the well-watered and stressed samples after the 7 day stressed samples, indicating that plants were experiencing drought stress conditions. In addition, finding of differences between the two species is

not surprising given the environment for which *A. dardani* is adapted when compared to the environment in which *A. ipaënsis* is adapted. One possible morphological explanation

Figure 3. A picture documenting the presence of plant hairs and leaf angle adjustment in *A. dardani*.

was observed at harvest of the root system for RNA collection samples. It was obvious that the *A. dardani* plants had a much more extensive root system than *A. ipaënsis*, which allows it to extract more moisture from the soil to maintain a greater RWC (figure 2). Other morphological characteristics that could allow *A. dardani* to maintain higher RWC are plant hairs for light deflection (Ning *et al*., 2016) and leaf angle adjustment (paraheliotropism) to lower the exposure to sunlight (Pastenes *et al*., 2004) (figure 3).

IV.2 Differential Gene Expression Analysis

Differential gene expression analysis levels were set at fold change ≥2-fold change and the false discover rate (FDR) corrected p-value of ≤ .05. This was based on previously published studies involving transcriptomics in several crops (Kebrom and Mullet, 2016; McKinley *et al*., 2016; Uli *et al*., 2017). Zandkarimi *et al*. (2015) reported master

regulators genes involved in drought in grape (*Vitis vinifera* L.) below the 4-fold level in both leaf and root tissue. These levels can be attributed to the type of genes that were trying to be identified. Control genes in a pathway, sometimes called master regulators, occupy the top of the regulatory chain and by definition should not be under another genes control. In many cases they exhibit only small fold changes that in turn causes larges changes in genes further down the regulatory cascade (Chan and Kyba, 2013). In order to maximize the impact of the genes to pursue further it was decided to focus on the genes exhibiting these small fold changes. In some cases, genes with much greater fold changes were considered, and in all cases considered, gene ontology suggested these genes were associated with proteins that occur later in the drought response pathway. In Comp 1-6 a total of 28,549 genes were identified with active transcripts (figure 4). Many genes occurred in one or more of the comparisons. One anomaly that was identified was that the genes in comparisons 7 and 8 seemed to be somewhat isolated. While there were some similarities in the genes identified, they did not have as many genes in common as the other comparisons. This is hypothesized to be because of the difference in genomes of the two species and the environments in which they evolved and represents an area of possible study in the future.

In addition to the 8 shoot tissue comparisons, identical experiments were conducted on the root tissue that was sampled separately. Here again, 8 DGE analysis experiments were conducted on all possible combinations of root samples based on both physiological state and species at fold change ≥ 2 at the FDR corrected p-value of $\leq .05$. In Comp 1-6, DGE analysis of identified 31, 441 genes with significant transcript numbers (figure 5).

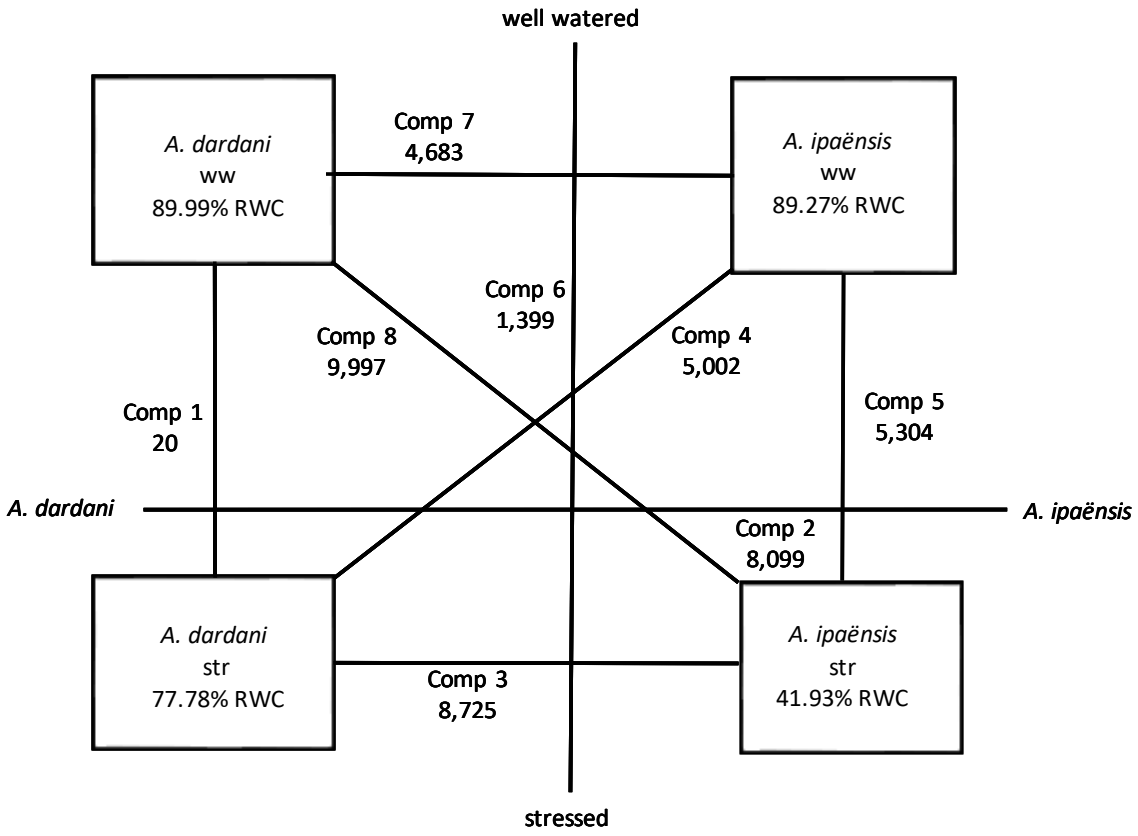

Figure 4. A figure showing 8 shoot tissue DGE comparisons and the number of genes significantly up or down regulated 2 fold at an FDR-corrected p-value $\leq .05$

Again, Comp 7 and 8 did not contain useful information and as such, they were not used any further. They do represent an area of potential further study.

When all the DGE comparisons are taken as a whole there are numerically more genes active in the root comparisons than the shoot comparisons. While this finding does not indicate a correlation between importance of the root system to drought tolerance and

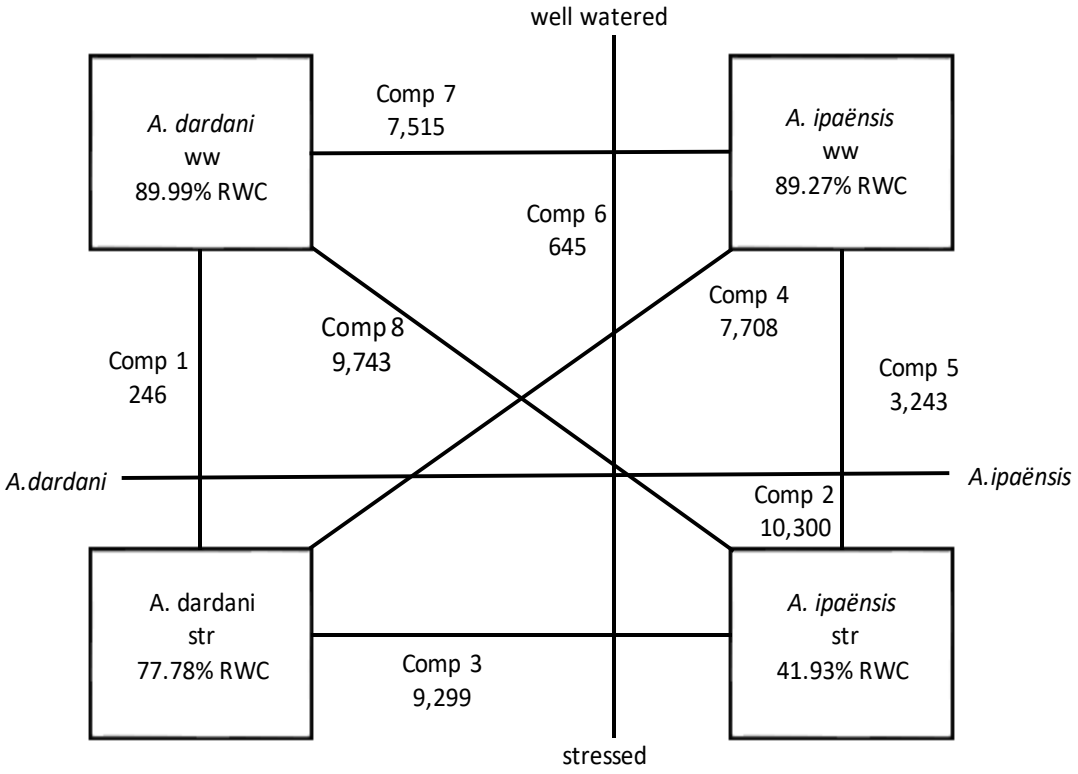

Figure 5. A figure showing 8 root tissue DGE comparisons and the number of genes significantly up or down regulated 2 fold at an FDR-corrected p-value ≤ .05

the amount and genes that are actively transcribing while under drought stress, it does represent a specific area that warrants further study.

The number of genes involved, and the complexity of the drought response necessitated a choice on where to focus the current research. The list presented is by no means exhaustive and it is likely that additional genes and useful information can be elucidated from this dataset. Genes in Comp 1, *A. dardani* well-watered versus *A. dardani* stressed seemed like an obvious choice due to the ability to maintain a greater RWC under

drought stress. However, upon closer examination it was found that an argument could be made for most of the 8 comparisons being associated with drought tolerance. For example, Comp 5 could be important for two reasons. First because it represents genes that were associated with drought tolerance that could be used for breeding in current cultivated material, due to its place as the B genome donor to the cultivated peanut. Second, when combined with the three comparisons involving *A. ipaënsis* to *A. dardani* stressed (Comp 2, 3 and 4), genes also could be determined that were associated with drought tolerance in *A. ipaënsis* and up or downregulated in *A. dardani* when compared to *A. ipaënsis*.

To identify master regulator genes, genes that were expressed ≥2-fold level were considered. As mentioned previously, master regulator genes also known as transcription factors (TF) control many downstream genes in a pathway with only small up or downregulation of their transcription (Zandkarimi *et al*. 2015) and represented the genes that offered the most potential impact on drought response.

In addition, the presence of a gene in multiple comparisons was used as a determining factor for further study. While a shift in gene expression that appeared in one comparison could be valid, gene expression shifts that occurred in multiple comparisons have higher likelihood of being associated with drought response. For example, some genes, such as Araip.Q9N4T and Araip.6HW1F, were up or downregulated in Comp 2, 3, or 4 that were not found in the Comp 1. However, the genes were found to have produced products associated to drought stress response in the current literature.

While there were unique genes found in this comparison it was hypothesized that because *A. ipaënsis* was at the lower limit of RWC as it related to plant recovery, some of

the genes in *A. ipaënsis* that were active earlier in its drought response mechanism would not be as active later in its physiological state of drought response. This study was more interested in finding genes that explain why *A. dardani* could maintain higher RWC under similar imposed drought conditions. Because of this, *A. ipaënsis* well-watered versus *A. dardani* stressed comparisons also were used as a comparison of interest, due to its similarity in RWC. It was determined that when these three comparisons between species (Comp 2, 3, and 4) were taken in conjunction with one another, we could further narrow down the total number of drought-responsive candidate genes. The final comparison that was used was the all well-watered versus all stressed comparison (Comp 6) across both species. Some genes were identified in this comparison, but due to the overall differences of the species this comparison did not reveal as many genes active at a significant level. However, there were many genes found in this comparison (Comp 6) common to other comparisons (Comp 1,2,3,4 and 5).

A list of candidate genes that occurred in multiple comparisons of shoot tissue was compiled. Genes were included in which there was some indication of gene function from the current literature. This study was not designed to determine all genes involved in drought response, but rather to identify a set of genes for marker development. In total, 11 genes in shoot tissue occurred in at least two of the six comparisons of interest and could be associated with drought tolerance (table 3). Based on gene function and ontology, several of these genes occur late in the drought response gene cascade. This first group consisted of Araip.CD04I, Araip.9I2KK and Araip.KW34A which produce chitinase A and benzyl alcohol O-benzoyltransferase proteins, which have been associated with response to stress (Veluthakkal and Dasgupta, 2012; Yu *et al*., 1998; Ahmad *et al*., 2017).

Araip.HUQ4W produces a lipoxygenase protein, which is a known cell signaling agent during stress periods (Lim *et al*., 2015; Kottapalli *et al*., 2009). Araip.8C4ZH and Araip.BLF65 produce proteinase inhibitors related to drought response and senescence related processes (Downing *et al*., 1992; Simova *et al*., 2009). Finally, Araip.X36HH was identified as a protein associated with the sieve tubes mechanism, which have been shown to remain functional during oxidative stress such as drought stress in pumpkin and cucumber (Walz et al., 2002). A second group occurs earlier in the drought response gene cascade. Araip.NU3NI and Araip.6HW1F produce a binding protein involved in ABA signaling and stomatal closure and represent the only genes on this list that were downregulated (Seiler *et al*., 2014; Lee *et al*., 2010). Araip.Q9N4T and Araip.8H742 are involved in protein homeostasis (Othman *et al*., 2014) and cell functions at elevated temperature (Park and Seo, 2015).

Similarly, 12 genes in root tissue were associated with drought response (Table 4). Araip.BJ3QY, Araip.5V3AJ, Araip7JJ4S and Araip.TSX3Z produce peroxidases (Koussevitzky *et al*., 2008; Veljovic-Jovanovic *et al*., 2006), CAP (Catabolite Activator Proteins) proteins (Daszkowska-Golec *et al*., 2017) and a senescence-associated protein (Seo *et al*., 2011). Araip.7JJ4S is one of three root genes that were down regulated.

All are proteins confirmed to be involved in the drought response cascade. Araip.ED5JD, and Araip.TEP2W produce proteins in cell wall extension (Zhao *et al*., 2011) and root growth (Basset *et al*., 2014) that have been associated with drought response in other species. Interestingly, there was some variation of up and down regulation between physiological state and species for Araip.ED5JD. In comparisons

Table 3. A table describing shoot genes fold changes at the FDR corrected p ≤.05 level and the protein that is produced.

Gene	Chromosome	Differential Expression analysis Fold Change at the FDR correct p<.05						Protien
		Comp 1 dww vs dstr	Comp 2 i vs d	Comp 3 is vs ds	Comp 4 iww vs dstr	Comp 5 iww vs i str	Comp 6 ww vs str	
Araip.BLF65	Chr2	2.37	5.76	9.18	7.03			cysteine proteinase inhibitor
Araip.CD04I	Chr9	3.39	7.7	7.61	23.01	2.82		chitinase A
Araip.9I2KK	1071	3.24	1026.26	1009.26	3044.4			chitinase A
Araip.8C4ZH	Chr5	4.44	109.19	87.48	2495.57	26.86		kunitz trypsin inhibitor 1
Araip.KW34A	Chr6	2.32	2.65	5.05	2.88			benzyl alcohol O-benzoyltranserase like [Glycine max]
Araip.8H742	Chr9	2.2	3.04	3.35	2.35			Bowman birk trypsin inhibitor
AraipX36HH	Chr9	35.3	23.25	22.89	293.92	13.22		sieve element occlusion protein
Araip.HUQ4W	Chr2		1028.29	1047.48	1049.44			lipoxygenase 1
Araip.Q9N4T	Chr8		532.12	574.12	1107.48			chaperone for a heat shock protien
Araip.B85X3	Chr3		2.13	3.88				Drought-repsonsive element-binding transcription factor (DREB)
Araip.6HW1F	Chr1		-1.96	-1.64	-2.58			DWD(DDB1-binding WD40 protein) hypersensitive to ABA2
Araip.NU3NI	Chr2	-6.21			-16.71	-17.5	-11.76	abscisic acid receptor PYL4-like [Glycine max]

Comp 1 (dww vs dstr)= *A. dardani* well-watered vs. *A. dardani* stressed

Comp 2 (I vs d)=*A. ipaënsis* vs *A. dardani*

Comp 3 (is vs ds)= *A. ipaënsis* stressed vs *A. dardani* stressed

Comp 4 (iww vs dstr)=*A. ipaënsis* well-watered vs *A. dardani* stressed

Comp 5 (iww vs istr)= *A. ipaënsis* well-watered vs *A. ipaënsis* stressed

Comp 6 (ww vs str)= well-watered vs stressed

Table 4. A table describing root genes fold changes at the FDR corrected p ≤.05 level and the protein that is produced.

Gene	Chromosome	Differential Expression analysis Fold Change at the FDR correct p<.05						Protien
		Comp 1 dww vs dstr	Comp 2 i vs d	Comp 3 is vs ds	Comp 4 iww vs dstr	Comp 5 iww vs istr	Comp 6 ww vs str	
Araip.ED5JD	Chr1	58.98	-73.92	-75.1	-35.77	2639.25	1687.6	expansin-like B1
Araip.N4WPE	Chr9	3.5	2.33	4.29	3.09			mavicyanin-like [Glycine max]
Araip.HEJ11	Chr6	13.26	18.15	87.31	20.73		11.99	Glutaredoxin family protein
Araip.55BM4	Chr6	10.13	46.09	69.76	104.46			probable galactinol--sucrose galactosyltrnsferase 2 like isoform X2 [Glycine max]
Araip.SHF6J	Chr9	8.99	35.2	92.73	48.37			receptor lectin kinase
Araip.VKB3S	Chr6	5.85	2.89	4.29	6.63			glycine-rich RNA-binding protein 2
Araip.86EDZ	Chr1	3.79	6.78	15.83	8.05			laccase 10
Araip.BJ3QY	Chr5	4.45	2.87	4.27	5.11			Peroxidase superfamily protein
Araip.5V3AJ	Chr7	3.03	2.35	3.98	3.16			Peroxidase superfamily protein
Araip.7JJ4S	Chr10	-44.77	-219.98	-23.4	-895.25	-4.17		CAP (Cystein-rich secretory proteins, Anigen 5 and Pathogenesis-related 1 protein) superfamily protein
Araip.W7ACI	Chr8	-69.86	-4.96	-174.12	-170.21			CAP (Cystein-rich secretory proteins, Anigen 5 and Pathogenesis-related 1 protein) superfamily protein
Araip.Q9N4T	Chr8		1678.16	574.12	1753.22			chaperone for a heat shock protien
Araip.TSX3Z	Chr7		2339.25	1592.99	1201			senescence-associated protein
Araip.TEP2W	Chr3		1671.11	5385.32	1037.63			metallothionein 3

Comp 1 (dww vs dstr)= *A. dardani* well-watered vs. *A. dardani* stressed

Comp 2 (I vs d)=*A. ipaënsis* vs *A. dardani*

Comp 3 (is vs ds)= *A. ipaënsis* stressed vs *A. dardani* stressed

Comp 4 (iww vs dstr)=*A. ipaënsis* well-watered vs *A. dardani* stressed

Comp 5 (iww vs istr)= *A. ipaënsis* well-watered vs *A. ipaënsis* stressed

Comp 6 (ww vs str)= well-watered vs stressed

between well-watered vs. stressed state the gene was upregulated but across species it was down regulated in *A. dardani,* indicating a possible trait that is unique to the species and represents an area of possible further study. Araip.55BM4, Araip.N4WPE are involved in cellular metabolism (Sengupta *et al*., 2015) and cell viability (Coa *et al*., 2015). Araip.HEJ11 and Araip.86EDZ are involved in drought stress regulation (Guo *et al*., 2010) and ROS signaling (Cho *et al*., 2014). Araip.SHF6J and Araip.VKB3S function in stomatal density (Ouyang *et al*., 2010) and protein binding (Yang *et al*., 2013). One final gene which was identified as a heat shock protein was Araip.W7ACI which was downregulated and discussed later

All of these genes are associated with drought response, however large fold changes that are occurring in some transcripts indicate a position late in the drought response gene cascade. Transcription factors (TF) often occur early in biochemical pathways and they bind to promoter regions to up or downregulate many genes in a given pathway (figure 6) (Lata and Prasad, 2011). For this reason, they represent a set of candidates to target for possible marker development. Many transcription factors have been identified in other crops that are associated with drought response. A list of genes encoding common transcription factors, including NAC (No apical meristem, Arabidopsis transcription activation factor, cup shaped cotyledon), bZIP (basic leucine zipper), Alfin like, CAMTA (Calmodulin-Binding Transcription Activator), AP2/ERF (APETALa2/ethylene-responsive element-binding), DREB (dehydration-responsive element-binging), AREB/ABF (ABA-responsive element binding/ARED-binding factor) and MYB (myeloblastosis) TFs, were obtained from peanutbase.org.

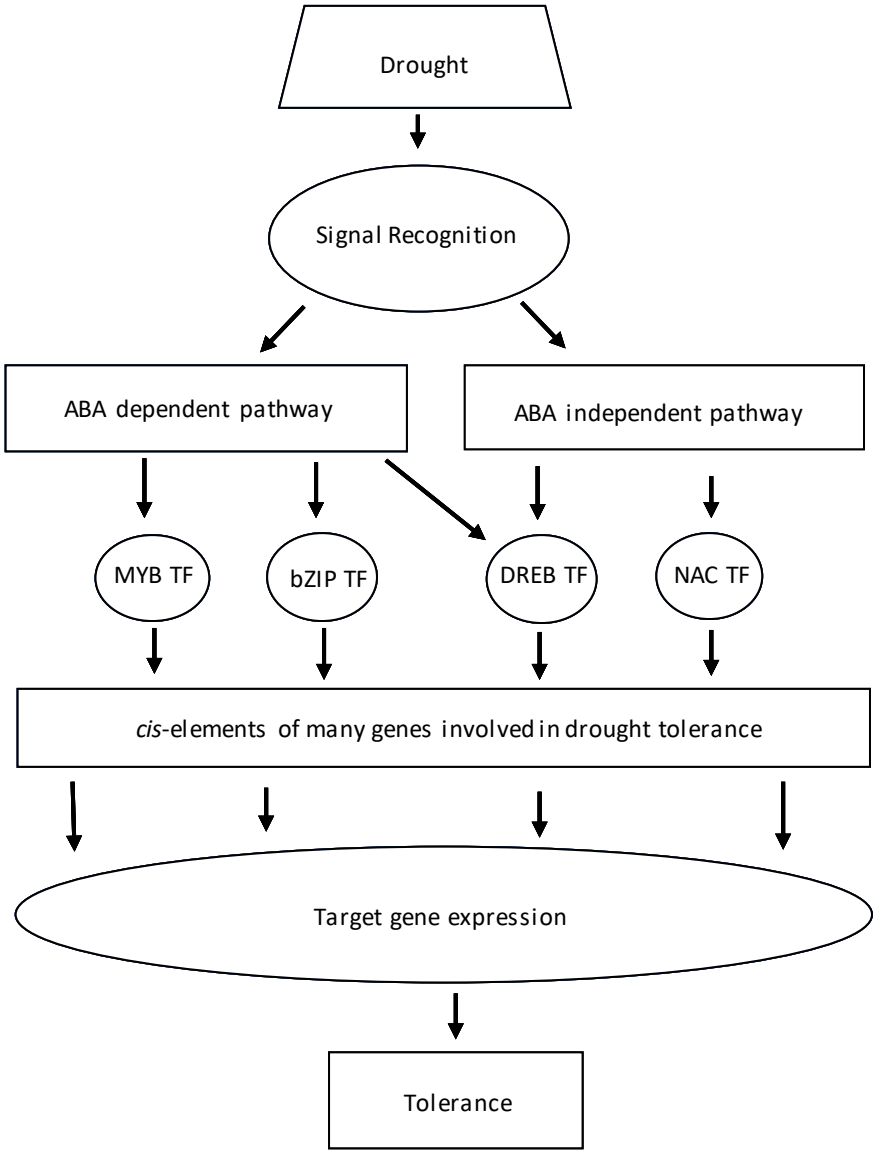

Figure 6. A figure depicting various transcription factors and their role in drought response (*reprinted from Lata and Prasad, 2011).

The database search of the TF list identified NAC, bZIP, MYB Alfin-like and AP2/ERF transcription factors with 174, 214, 745, 15 and 2 gene annotations, respectively. The DGE comparisons were examined for the presence of the transcription factors

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obtained from peanutbase.org list. A total of 71 genes were found in at least one comparison (table A2). As previously stated, genes occurring in multiple comparisons were examined for gene function and ontology. A total of 14 genes encoding transcription factors associated with drought in other crops were identified with a fold change ≥ 2 and a FDR corrected p-value $\leq .05$ (table 5) that also were found in multiple DGE comparisons. Genes involved in drought response can be divided into two broad categories. Those involved in protection of cells during stress and those that act as upstream regulators of the drought response. Many of the genes found in the initial analysis fall into the first group. These genes are likely involved in drought response, because in many cases, they have been shown to impart drought tolerance. Some of the genes found in the initial analysis fit into the second group. These regulator genes of the second group represent a group of genes which, if markers could be developed, hold the potential to make a greater impact on drought tolerance. Transcription factors also fall into the second group and are known to operate in both the abscisic acid (ABA) dependent and ABA independent pathways. Abscisic Acid is a plant hormone that is involved in abiotic stress response. Transcription factors encoding DREB, NAC, MYB, bZIP and Hs (heat shock) proteins that operate in both pathways were found differentially expressed in this study.

Araip.B85X3 is a gene encoding a TF known as a DREB protein. The DREB proteins belong to a larger protein family known as AP2/ERF TFs. They represent some of the most studied groups of TFs' in current literature. Dehydration responsive element Binding TFs function by binding to promoter regions in drought responsive genes and have been documented to occur in both the ABA dependent and ABA independent pathways (Lata and Prasad, 2011). There are two groups of DREB proteins, the first is a group that

Table 5. A table indcating up or down reguated genes encoding transcription factors known to affect drought tolerance at a ≥ 2 fold change at an FDR corrected p-value of ≤.05.

			Comp 1 dww vs dstr	Comp 2 i vs d	Comp 3 is vs ds	Comp 4 iww vs dstr	Comp 5 iww vs istr	Comp 6 ww vs str	
Araip.B85X3	chr3	leaf		x	x				DREB
Araip.310T2	chr7	leaf		x	x	x	x	x	NAC protein domain
Araip.M7SF9	chr4	leaf		x	x	x	x		MYB transcription factor
Araip.333QY	chr3	root		x	x	x	x	x	NAC domain protein 3
Araip.DL86S	chr8	root		x	x	x	x	x	NAC domain protein 19
Araip.KM0ZG	chr3	both	x (r only)	x	x	x			NAC protein domain
Araip.YL288	chr4	root		x	x	x	x		NAC domain protein
Araip.23BBS	chr10	root		x	x	x	x		bZIP transcription factor
Araip.01FEX	chr10	root		x	x	x	x		bZIP transcription factor
Araip.LQ8RU	chr3	root		x	x	x	x		MYB transcription factor MYB64
Araip.U6PZK	chr5	root		x	x	x	x		MYB transcription factor
Araip.U7ZVD	chr6	root		x	x	x	x		MYB transcription factor
Araip.29KNU	chr2	root		x	x	x			heat shock transcription factor
Araip.25NFE	chr6	root		x	x				heat shock transcription factor

Comp 1 (dww vs dstr)= *A. dardani* well-watered vs. *A. dardani* stressed
Comp 2 (I vs d)=*A. ipaënsis* vs *A. dardani*
Comp 3 (is vs ds)= *A. ipaënsis* stressed vs *A. dardani* stressed
Comp 4 (iww vs dstr)=*A. ipaënsis* well-watered vs *A. dardani* stressed
Comp 5 (iww vs istr)= *A. ipaënsis* well-watered vs *A. ipaënsis* stressed
Comp 6 (ww vs str)= well-watered vs stressed

is induced in response to cold (DREB1) and a second group was identified as induced by drought (DREB2), although there is some overlap in response by the two groups. There have been DREB proteins identified in corn (Liu *et al*., 2013), soybean (Ha et al., 2015), rice (Sakum *et al*., 2006; Wang *et al*., 2008; Srivastav *et al*., 2010), chickpea (Molina et al. 2008), rapeseed (Liu et al., 2015), Arabidopsis (Nakashima *et al*., 2014), pine (Lorenzo *et al*., 2011) and poplar (Cohen *et al*., 2010).

Araip.310TS, Araip.333QY, Araip.DL86S, Araip.KM0ZG and Araip.YL288 were all genes expressed that encode an NAC TFs. The TFs in this family operate in the ABA independent signaling network. The NAC family proteins encode TFs that regulate downstream gene transcription of drought induced genes, such as EARLY RESPONSE TO DEHYDRATION (ERD1) with the proper recognition sequence (Rohit *et al*., 2016). This family of TFs in Arabidopsis have been linked to play a role in control of root architecture and drought tolerance. They have been identified by DGE in the roots of soybean (Le *et al*., 2011), cotton (Ranjan and Sawant, 2014) and rice (Moumeni, 2015). In studies overexpression of NAC TFs from other species caused the transgenic lines under moderate drought to exhibit increased lateral root growth (Janiak *et al*., 2016).

Araip.23BBS and Araip.01FEX encode bZIP TFs. These proteins are a part of the larger group known as AREB/ABF, which are a part of the ABA dependent signaling network that is involved in plant development (Rohit *et al*., 2016), They are known to be active in guard cells (Kim, 2006) and have been documented in studies involving *Phaseolus acutifloius* and *P. vulgaris* (Rodriquez-Uribe and O'Connell, 2006). They have been found to be involved in the regulation of genes encoding downstream including: late

embryogenesis abundant proteins (LEA), response to dehydration (RD) proteins and CAP proteins.

Myeloblastosis TFs are expressed by Araip.LQ8RU, Araip.U6PZK and U7ZVD. These proteins are associated with many processes in plants such as development and metabolism. Transcriptome analysis under drought stress has previously associated them with regulation of transpiration rate and stomatal opening in an ABA-dependent manner (Rohit et al., 2016). They have been identified in Arabidopsis (Seo et al., 2009) and rapeseed (Liu et al., 2015). Finally, Araip.W7ACI, Araip.29KNU and Araip.25NFE encode Hs TFs. These TFs are thought to operate downstream of other TFs and have been linked to influence from DREB2 signaling leading to thermotolerance and plant growth (Ulrike, 2013). Taken together these TFs represent excellent potential candidates for marker development. However, studies involving the validation of the genes identified using q-PCR need to be conducted. Additionally, experiments must be designed to determine the extent of the variation present in each species. The bridge species used in the introgression program also should be sequenced to determine gene presence as well as copy number variations present.

IV.3 Crossing

The wild species *A. vallsii*, (VSW 9902-1) was used as the female of the cross. This species is found in the Pantanal of Mato Grosso do Sul of Brazil in periodically flooded grasslands (Krapovickas and Gregory, 2007). In its native environment, the lateral branches grow along the top of tall native grasses and can produce pegs that will descend approximately 1 m to reach the soil surface (Simpson personal communication). *Arachis*

vallsii was chosen because of its ability to cross with many of the described sections. Previous studies have successfully crossed *A. vallsii* with sections *Caulorrhizae, Arachis*, *Procumbentes* and *Erectoides* (Custidio, Valls and Simpson (manuscript in preparation)).

Arachis dardani, accession GK 12946 was used as the male in the cross. It is a member of section *Heteranthae*. The species has been defined as adapted to extreme environmental conditions (Krapovickas and Gregory, 2007). *Arachis dardani* is found in the northeast region of Brazil where it typically grows in wooded Caatinga shrublands. The Caatinga has a shallow stony soil and only two defined seasons per year, a wet and dry season (Krapovickas and Gregory, 2007). The area is considered a dry forest region and receives less than 250 mm of annual precipitation (McGinley, 2018). *Arachis dardani* is an annual or biannual plant that usually grows vegetatively in its first season with prodigious seed production in its second year. Natives in the region describe heavy grazing of *A. dardani* (Simpson personal communication).

As indicated earlier, *A. dardani* has several characteristics associated with other drought mechanisms, including trichomes and leaf angle deflection, which were each observed during this research (figure 3). These represent additional genes that could be targeted at a later date for Marker Assisted Selection (MAS) to increase a plants ability to manage exposure to drought. Additionally, it also was observed during our research that *A. dardani* pegs emerged on average about 3 days after flowering. This is most likely an adaptation to its arid environment where the ability to set and mature seed in a short wet-season is imperative. This trait represents a characteristic that could be considered an escape mechanism with regards to drought tolerance. This mechanism not only represents a mechanism to allow plants to survive in water limited conditions but could possibly be

used as a means of breeding for early maturity, which is a desirable characteristic in the Southwestern U.S. growing region.

Table 6. A table with the production of 9 crossing blocks of *A. vallsii* x A. *dardani* with LSD grouping for seed produced.

Crossing block	Male	Pollinations	Pegs	Seed
13X	12946	136	5	3 b
13FX	12946	83	6	0 b
14X	12946	251	36	8 b
14FX	12946	-	-	-
15X	12946	133	13	6 b
15FX	12946	212	9	2 b
16X	12946	194	21	0 b
16FX	7215	106	24	21 a
17X	7215-1	53	0	0 b
17FX	7215	170	36	30 a

The ability to create a viable hybrid is the first step in the introgression process. With this cross, as with many crosses involving distantly related *Arachis* species there is a high failure rate in both successfully obtaining a peg and furthermore successfully obtaining a viable seed. Because of this the use of seed from additional crossing blocks were used to obtain enough material as the introgression process was continued (table 6).

Validating the creation of a hybrid of *A. vallsii* x *A. dardani* was a primary objective of this project. Hybridization was confirmed, based on pollen counts indicating 100% sterility, flower morphology equal to *A. dardani* (the male of the cross), intermediate

leaf, pod and seed morphology between the two parents (figure 7). This is the first report of a successful hybridization Section *Arachis* with section *Heteranthae*.

Although it was not in the defined objectives of this research, additional information on cross-compatibility was obtained between *A. vallsii* and *A. dardani*. An additional accession of *A. dardani* (V-7215) was included after the spring 2016 crossing block. This accession was used when *A. dardani* (12946) plants did not flower when needed. Plant

Figure 7. Pictures contrasting the leaf morphology of (clockwise) *A. vallsii*, *A. dardani* as compared to the intermediate morphology of a *A. vallsii x A. dardani* hybrid and the flower morphology of the hybrid.

morphology of the two accessions were evaluated and the only visible difference was the size of the flower.

Table 7. A table showing the attempts to double the chromosome number of the *A. vallsii* x *A. dardani* hybrid.

Tissue type	**Concentration**	**Treatment duration**									
Cuttings (103)		6hrs	7hrs	7.5hrs	7.75hrs	8hrs	8.25hrs	9hrs	10hrs	18hrs	24hrs
	.02% Col.		12			89					
	.03% Col.	2									
Submersion apical meristem (62)											
	.02% Col.					18		5	1	15	20
	.03% Col.	2				3					
Submersion of seed (10)											
	.02% Col.			1	1	6	1				
	.03% Col.	1									

Arachis dardani (7215) was collected further inland, approximately 1500 km west of the location for *A. dardani* (12946). Analysis of variance of the number of seed produced from each crossing block showed a significant difference ($p < .0011$*) between crossing blocks containing *A. dardani* (12946) and *A. dardani* (7215 and 7215-1) (Table 6). The average successful pollination to seed percentage was 2.45% for *A. dardani* (12946) and 17.9% for *A. dardani* (7215). Furthermore, it was apparent that *A. dardani* (7215) was more cross-compatible during the crossing blocks involving that accession. Pegs involving *A. dardani* (7215) emerged in approximately 7 days. During earlier crossing blocks involving *A. dardani* (12946) it took 21-30 days for pegs to emerge. The 7-day time period was similar to self-pollinations of *A. vallsii* or *A. dardani* which take an average about 3 to 7 days for pegs emergence.

Figure 8. A picture of a hybrid seed following colchicine treatment that is showing some promise of chromosome doubling.

Multiple attempts were made to continue development of the introgression pathway to move genes from *A. dardani* to cultivated peanut (*A. hypogaea*). This includes attempted chromosome doubling using colchicine treatment of seed and stem tissue according to previously published literature (Faveró, 2006, Simpson, 1991). In all, 10 attempts have been made with seed, 108 attempts with stem cuttings and 62 attempts of soaking the apical meristem of lateral branches (Table 7) (Table A4). Concentrations of 0.02% and 0.03% colchicine for time periods between 6 hours to 24 hours have been attempted. A starting point 0.02% for 8 hours was used and new attempts were adjusted at various 15-minute increments based on previous treatment results. The process is slow and based on availability of each of the tissue types. Treatment of the seed shows the greatest promise while treatment of cuttings and apical meristem have not shown strong response in any treatment (figure 8). To date no attempt has been successful.

CHAPTER V

CONCLUSIONS

In conclusion, RNA-Seq is a powerful new transcriptomics tool that researchers are starting to use on a large scale (Wang *et al*., 2009). It gives researchers the ability to design an experiment that can isolate a specific question that is being asked and take a snapshot of an organism at that specific time. This can provide great insight into the genetic underpinnings of a given species and provide direction on areas of future research. One consideration that needs to be accounted for is the fact that you are only looking at a plant at a specific time under very specific conditions. While this allows a researcher to use experimental designs that answer very specific questions when dealing with a complex quantitative trait such as drought, it necessitates the use of multiple experiments to identify genes under different types of stress and at different physiological ages. In addition, great care should be taken to design an experiment that partitions as much variation as possible to isolate the question of interest. Currently, the cost of the level of sequencing required for complex experiments is a limiting factor. However, as cost per sample is reduced, accounting for variation will be more feasible and will make transcriptomic experiments a widely used tool in a plant breeder's toolbox.

The initial hypothesis that *A. dardani* (12946) contains novel variation that is currently unavailable to the cultivated peanut was confirmed. Several genes associated late in the drought response gene cascade in other species were identified both up and down regulated at statistically significant levels in *A. dardani*. Furthermore, genes encoding transcription factors known to occur earlier in the drought response cascade were

identified. In order to breed for the greatest amount of drought tolerance these transcription factors represent a valuable way to affect as many genes as possible that are associated with drought tolerance. Additionally, many transcription factors are *cis* acting and therefore occur on the same gene as the genes that they are influencing. During recombination some individuals inherit whole chromosomes or large chucks of chromosomes. If these individuals can be identified that contain many of these genes of interest, great strides in genetic gain could be made very quickly.

In conjunction with the elevated transcript levels identified in *A. dardani*, genes were identified as associated with drought that were conserved across both species. The genes and transcription levels identified in *A. ipaënsis* (B genome donor) should be similar to the genes in *A. duranensis* (A genome donor), as well as *A. hypogaea*. Although conformation is required that genes are present in elite material, the genes could be targeted for the development of SNP and insertion/deletion markers for use in current Marker Assisted Breeding (MAB) programs trying to breed for drought tolerance.

Traditional gene introgression is currently the only acceptable method available for movement of genes between wild and cultivated peanut. This research represents the first report of the use of *A. vallsii* as a bridge species used in crosses involving species of the *Heteranthae* section of the genus *Arachis*. This opens up new pathways in which genes can potentially be moved into *A. hypogaea*. Development of the current pathway represents the most direct route to move the genes identified in this research into cultivated peanut.

Immediate future research developing from this project will involve validation of the genes identified in the RNA-seq study using QPCR, development of SNP and INDEL markers for use in MAS and continued development of the introgression pathway. If successful, the development of populations with drought tolerance genes that can be used in the Texas A&M AgriLife breeding program will be possible. This species also represents a possible candidate for transfer of drought tolerance genes using the emerging technology of CRISPR Cas 9. In this technology genomes can be edited in a very precise way at a low cost. Currently public acceptance of this technology is still somewhat unknown; however, it does represent an efficient way to unlock many of the genes that are currently not accessible to peanut breeders. All research conducted in this project fits into the long-term goals including development and release of varieties with traits introgressed from *A. dardani.*

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APPENDIX

Table A1. A table with RWC data of each biological replicate of the transcriptomics study.

RWC %	Species	Treatment
90.69653	*A. ipaënsis*	well watered
92.12439	*A.dardani*	well watered
93.75199	*A. ipaënsis*	well watered
88.12936	*A.dardani*	well watered
84.52716	*A. ipaënsis*	well watered
89.50666	*A.dardani*	well watered
88.11136	*A. ipaënsis*	well watered
89.79298	*A.dardani*	well watered
36.81130	*A. ipaënsis*	7 day drought stress
85.04595	*A.dardani*	7 day drought stress
36.84500	*A. ipaënsis*	7 day drought stress
67.48548	*A.dardani*	7 day drought stress
37.58737	*A. ipaënsis*	7 day drought stress
73.48383	*A.dardani*	7 day drought stress
56.46286	*A. ipaënsis*	7 day drought stress
85.10484	*A.dardani*	7 day drought stress

Table A2. A list of genes transcribing know transcription factors associated with drought repsonse identified from peanutbase.org.

Gene	TF family	Tissue type	Gene	TF family	Tissue type
Araip.9BR1Z	NAC	leaf	Araip.T9C3C	MYB	leaf
Araip.T6ICI	NAC	leaf	Araip.U3SIL	MYB	leaf
Araip.KM0ZG	NAC	leaf	Araip.VAD3A	MYB	leaf
Araip.AVV74	NAC	leaf	Araip.W7TQM	MYB	leaf
Araip.YL288	NAC	leaf	Araip.X5L3I	MYB	leaf
Araip.64GCN	NAC	leaf	Araip.YW29A	MYB	leaf
Araip.310T2	NAC	leaf	Araip.Z0JT3	MYB	leaf
Araip.I6LH9	NAC	leaf	Araip.ZMP4R	MYB	leaf
Araip.714GL	NAC	leaf	Araip.333QY	NAC	root
Araip.333QY	NAC	leaf	Araip.8NR3H	NAC	root
Araip.DL86S	NAC	leaf	Araip.DL86S	NAC	root
Araip.M5DKY	NAC	leaf	Araip.KM0ZG	NAC	root
Araip.GU9EZ	NAC	leaf	Araip.YL288	NAC	root
Araip.C5IZ7	bZIP	leaf	Araip.23BBS	bZIP	root
Araip.7LB5G	bZIP	leaf	Araip.01FEX	bZIP	root
Araip.SS9JQ	bZIP	leaf	Araip.7LB5G	bZIP	root
Araip.RX4PW	bZIP	leaf	Araip.H4YUS	bZIP	root
Araip.19A3Z	MYB	leaf	Araip.SS9JQ	bZIP	root
Araip.21G20	MYB	leaf	Araip.H0JCT	bZIP	root
Araip.35TTL	MYB	leaf	Araip.RX4PW	bZIP	root
Araip.45253	MYB	leaf	Araip.A9GHS	bZIP	root
Araip.6Q4TC	MYB	leaf	Araip.A0U1L	bZIP	root
Araip.AH7CK	MYB	leaf	Araip.24TKU	MYB	root
Araip.DHK4N	MYB	leaf	Araip.2L6E3	MYB	root
Araip.EGQ9J	MYB	leaf	Araip.62YF9	MYB	root
Araip.F3V4B	MYB	leaf	Araip.EX7BP	MYB	root
Araip.GU31N	MYB	leaf	Araip.I60S5	MYB	root
Araip.I4CAM	MYB	leaf	Araip.L0YAL	MYB	root
Araip.I4P6Q	MYB	leaf	Araip.LQ8RU	MYB	root
Araip.IE1YD	MYB	leaf	Araip.M7SF9	MYB	root
Araip.KEX5D	MYB	leaf	Araip.P4ZBN	MYB	root
Araip.L60K4	MYB	leaf	Araip.QF82S	MYB	root
Araip.LQ8RU	MYB	leaf	Araip.R4WKP	MYB	root
Araip.M7SF9	MYB	leaf	Araip.U6PZK	MYB	root
Araip.PJS9A	MYB	leaf	Araip.U7ZVD	MYB	root
Araip.QF82S	MYB	leaf	Araip.C2J94	Alfin	root
Araip.R9J0M	MYB	leaf			

Table A3. Crossing log showing pollination date for Spring 2013- Fall 2017.

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				29-Apr	30-Apr	2-May	7-May	8-May	9-May	14-May	16-May	17-May
2013	Spring	13X-1	9902-1 X 12946				1	1		2		2
2013	Spring	13X-2	9902-1 X 12946	1	3		2	1	2	2	2	5
2013	Spring	13X-3	9902-1 X 12946	2		2	2	1		1	4	
2013	Spring	13X-4	9902-1 X 12946	1				2	1	3	3	1
2013	Spring	13X-5	9902-1 X 12946									
2013	Spring	13X-6	9902-1 X 12946									
2013	Spring	13X-9	9902-1 X 12946									

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				21-May	22-May	23-May	24-May	28-May	29-May	30-May	5-Jun	6-Jun
2013	Spring	13X-1	9902-1 X 12946		6	4	3				3	
2013	Spring	13x-2	9902-1 X 12946	2	5	3	3	6				
2013	Spring	13x-3	9902-1 X 12946			2			5			
2013	Spring	13X-4	9902-1 X 12946	5								
2013	Spring	13X-5	9902-1 X 12946	2	2	1	1		2	6	3	8
2013	Spring	13X-6	9902-1 X 12946	3	2		2		1	1		1
2013	Spring	13X-9	9902-1 X 12946		1		1		2		1	2

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				18-Sep	19-Sep	20-Sep	23-Sep	24-Sep	25-Sep	26-Sep	30-Sep	1-Oct
2013	Fall	13FX-1	9902-1 X 12946	1	1	1	1			1		
2013	Fall	13FX-2	9902-1 X 12946	1	1		1	1	1	2	1	1
2013	Fall	13FX-3	9902-1 X 12946			2			1	1		1
2013	Fall	13FX-4	9902-1 X 12946	1		2				1	1	1
2013	Fall	13FX-5	9902-1 X 12946	1		1					1	

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				4-Oct	14-Oct	15-Oct	16-Oct	17-Oct	18-Oct	27-Oct	30-Oct	31-Oct
2013	Fall	13FX-1	9902-1 X 12946		1			1				
2013	Fall	13FX-2	9902-1 X 12946	1	3	4		3	1	2	2	1
2013	Fall	13FX-3	9902-1 X 12946	1	3	2		1		3	1	
2013	Fall	13FX-4	9902-1 X 12946			3	2	1	1	1	2	2
2013	Fall	13FX-5	9902-1 X 12946	1		1	1	1	1			2

Year	Season	Cross	Parents	Date	Date
				5-Nov	6-Nov
2013	Fall	13FX-1	9902-1 X 12946		
2013	Fall	13FX-2	9902-1 X 12946		
2013	Fall	13FX-3	9902-1 X 12946		1
2013	Fall	13FX-4	9902-1 X 12946	3	
2013	Fall	13FX-5	9902-1 X 12946	2	

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				22-Apr	28-Apr	29-Apr	1-May	2-May	8-May	12-May	13-May	14-May
2014	Spring	14X-11	9902-1 X 12946									
2014	Spring	14X-12	9902-1 X 12946									
2014	Spring	14X-13	9902-1 X 12946	1			1					
2014	Spring	14X-14	9902-1 X 12946						1	1		
2014	Spring	14X-15	9902-1 X 12946									
2014	Spring	14X-16	9902-1 X 12946								2	
2014	Spring	14X-17	9902-1 X 12946							1		
2014	Spring	14X-18	9902-1 X 12946		1	2		1			2	
2014	Spring	14X-19	9902-1 X 12946									1
2014	Spring	14X-20	9902-1 X 12946									
2014	Spring	14X-21	9902-1 X 12946									1
2014	Spring	14X-22	9902-1 X 12946									

Table A3. Continued

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				16-May	18-May	20-May	21-May	22-May	23-May	24-May	24-May	27-May
2014	Spring	14X-11	9902-1 X 12946				1					1
2014	Spring	14X-12	9902-1 X 12946		2	3		2	2	4	3	2
2014	Spring	14X-13	9902-1 X 12946				2					
2014	Spring	14X-14	9902-1 X 12946		1							2
2014	Spring	14X-15	9902-1 X 12946		1					1		1
2014	Spring	14X-16	9902-1 X 12946		1				1		2	2
2014	Spring	14X-17	9902-1 X 12946	1		1		1	1	1	3	1
2014	Spring	14X-18	9902-1 X 12946	1		2		2	1	2	1	
2014	Spring	14X-19	9902-1 X 12946	2								
2014	Spring	14X-20	9902-1 X 12946					1			2	
2014	Spring	14X-21	9902-1 X 12946				1				1	
2014	Spring	14X-22	9902-1 X 12946									

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				28-May	29-May	30-May	31-May	2-Jun	4-Jun	5-Jun	6-Jun	9-Jun
2014	Spring	14X-11	9902-1 X 12946				1					6
2014	Spring	14X-12	9902-1 X 12946	2	1		4	3	2	2	3	
2014	Spring	14X-13	9902-1 X 12946									
2014	Spring	14X-14	9902-1 X 12946			1		1	1	1		1
2014	Spring	14X-15	9902-1 X 12946		1	2		1	3			1
2014	Spring	14X-16	9902-1 X 12946	1	1	1	1	2	1			3
2014	Spring	14X-17	9902-1 X 12946	1	1	2	3	1	2	2		2
2014	Spring	14X-18	9902-1 X 12946		1		1	1				3
2014	Spring	14X-19	9902-1 X 12946						1	1		
2014	Spring	14X-20	9902-1 X 12946				1		1			
2014	Spring	14X-21	9902-1 X 12946		1	1						
2014	Spring	14X-22	9902-1 X 12946			1		1				

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				10-Jun	13-Jun	16-Jun	17-Jun	18-Jun	19-Jun	20-Jun	23-Jun	24-Jun
2014	Spring	14X-11	9902-1 X 12946						1			1
2014	Spring	14X-12	9902-1 X 12946	3	2	3	2	4		2	3	4
2014	Spring	14X-13	9902-1 X 12946									
2014	Spring	14X-14	9902-1 X 12946									
2014	Spring	14X-15	9902-1 X 12946	1		1		3				
2014	Spring	14X-16	9902-1 X 12946			1	1				1	1
2014	Spring	14X-17	9902-1 X 12946	1	2		1			1	3	1
2014	Spring	14X-18	9902-1 X 12946				2				2	
2014	Spring	14X-19	9902-1 X 12946			1	1	1			2	1
2014	Spring	14X-20	9902-1 X 12946	1		3						
2014	Spring	14X-21	9902-1 X 12946		1		1	1	3			2
2014	Spring	14X-22	9902-1 X 12946	1				1				

Year	Season	Cross	Parents	Date	Date	Date	Date	Date
				25-Jun	26-Jun	27-Jun	30-Jun	3-Jul
2014	Spring	14X-11	9902-1 X 12946	1				1
2014	Spring	14X-12	9902-1 X 12946	4	1	4	3	
2014	Spring	14X-13	9902-1 X 12946					
2014	Spring	14X-14	9902-1 X 12946					
2014	Spring	14X-15	9902-1 X 12946		1	2	1	3
2014	Spring	14X-16	9902-1 X 12946	2	2		1	1
2014	Spring	14X-17	9902-1 X 12946	2	1	2	2	
2014	Spring	14X-18	9902-1 X 12946					
2014	Spring	14X-19	9902-1 X 12946	1				
2014	Spring	14X-20	9902-1 X 12946					
2014	Spring	14X-21	9902-1 X 12946					
2014	Spring	14X-22	9902-1 X 12946					

Table A3. Contiued

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				14-Apr	20-Apr	21-Apr	24-Apr	27-Apr	6-May	8-May	11-May	13-May
2015	Spring	15X-2	9902-1 X 12946	2	3	2	4					1
2015	Spring	15X-3	9902-1 X 12946		2	6	2	1				3
2015	Spring	15X-4	9902-1 X 12946				3	3	4	4	3	1
2015	Spring	15X-5	9902-1 X 12946				1		5	6	4	

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				15-May	18-May	19-May	20-May	21-May	25-May	26-May	1-Jun	2-Jun
2015	Spring	15X-2	9902-1 X 12946				4	2			6	2
2015	Spring	15X-3	9902-1 X 12946	7	2							
2015	Spring	15X-4	9902-1 X 12946	1	5		1	4	7	8	4	1
2015	Spring	15X-5	9902-1 X 12946		1	5		4	2	2	2	3

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				21-Sep	22-Sep	23-Sep	24-Sep	25-Sep	28-Sep	29-Sep	30-Sep	1-Oct
2015	Fall	15FX-1	9902-1 X 12946	1		2	2	3		1	2	2
2015	Fall	15FX-2	9902-1 X 12946	2	4		4	5		1	2	9
2015	Fall	15FX-3	9902-1 X 12946	2	4	2	1	3	4	4	2	
2015	Fall	15FX-4	9902-1 X 12946	2	1		2		2	4	3	

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				2-Oct	5-Oct	6-Oct	7-Oct	8-Oct	9-Oct	12-Oct	13-Oct	23-Oct
2015	Fall	15FX-1	9902-1 X 12946	3	3	4			1	1	1	4
2015	Fall	15FX-2	9902-1 X 12946		2	3	7		3	3	1	2
2015	Fall	15FX-3	9902-1 X 12946	5	4	2	2	4	2	1		1
2015	Fall	15FX-4	9902-1 X 12946	3	2	2	1	6	4			1

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				24-Oct	25-Oct	26-Oct	31-Oct	1-Nov	5-Nov	6-Nov	10-Nov	17-Nov
2015	Fall	15FX-1	9902-1 X 12946	2	4				1	4	3	3
2015	Fall	15FX-2	9902-1 X 12946	2	1			4	2	3	2	1
2015	Fall	15FX-3	9902-1 X 12946		2			4	3			
2015	Fall	15FX-4	9902-1 X 12946		2	3	5	5	2			

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				13-May	14-May	16-May	17-Jun	18-May	19-May	20-May	21-May	23-May
2016	Spring	16X-3	9902-1 X 12946	2	1	4	1	4	2	2		3
2016	Spring	16X-4	9902-1 X 12946	1		2	1	1		1		1
2016	Spring	16X-5	9902-1 X 12946									
2016	Spring	16X-6	9902-1 X 12946	2		2	4	1	2	2	4	5
2016	Spring	16X-7	9902-1 X 12946	1								
2016	Spring	16X-8	9902-1 X 12946	2			1		1		1	1
2016	Spring	16X-9	9902-1 X 12946		2	1	2				1	
2016	Spring	16X-10	9902-1 X 12946							1		1

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				24-May	25-May	27-May	31-May	1-Jun	2-Jun	3-Jun	8-Jun	9-Jun
2016	Spring	16X-3	9902-1 X 12946	2	4	4		2	2	1		
2016	Spring	16X-4	9902-1 X 12946	1		1						
2016	Spring	16X-5	9902-1 X 12946									1
2016	Spring	16X-6	9902-1 X 12946	5	3	3		16	3	6	2	4
2016	Spring	16X-7	9902-1 X 12946					2		1	1	2
2016	Spring	16X-8	9902-1 X 12946			1					1	1
2016	Spring	16X-9	9902-1 X 12946			1	1		3			1
2016	Spring	16X-10	9902-1 X 12946	2					1			

Table A3. Continued

Year	Season	Cross	Parents	Date	Date	Date	Date
				10-Jun	11-Jun	13-Jun	15-Jun
2016	Spring	16X-3	9902-1 X 12946			3	
2016	Spring	16X-4	9902-1 X 12946				
2016	Spring	16X-5	9902-1 X 12946				
2016	Spring	16X-6	9902-1 X 12946	13	8	10	8
2016	Spring	16X-7	9902-1 X 12946	2	1		1
2016	Spring	16X-8	9902-1 X 12946	4			1
2016	Spring	16X-9	9902-1 X 12946	1	1		
2016	Spring	16X-10	9902-1 X 12946				

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				5-Nov	7-Nov	10-Nov	11-Nov	14-Nov	15-Nov	28-Nov	29-Nov	30-Nov
2016	Fall	16FX-11	9902-1 X 7215	4	2							
2016	Fall	16FX-12	9902-1 X 7215			1	3	4	2	4	1	1
2016	Fall	16FX-13	9902-1 X 7215						1	3		
2016	Fall	16FX-14	9902-1 X 7215			1	1		1	2		2
2016	Fall	16FX-15	9902-1 X 7215					1				

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				1-Dec	2-Dec	3-Dec	5-Dec	6-Dec	7-Dec	9-Dec	10-Dec	12-Dec
2016	Fall	16FX-11	9902-1 X 7215									
2016	Fall	16FX-12	9902-1 X 7215		7		3	4	2	2	3	2
2016	Fall	16FX-13	9902-1 X 7215	2	2				2		2	
2016	Fall	16FX-14	9902-1 X 7215	2	1	2	1	1	2	4	2	
2016	Fall	16FX-15	9902-1 X 7215	2	1				1		1	

Year	Season	Cross	Parents	Date	Date	Date	Date
				15-Dec	19-Dec	21-Dec	22-Dec
2016	Fall	16FX-11	9902-1 X 7215				
2016	Fall	16FX-12	9902-1 X 7215	3	1	3	4
2016	Fall	16FX-13	9902-1 X 7215				
2016	Fall	16FX-14	9902-1 X 7215	4	2	2	1
2016	Fall	16FX-15	9902-1 X 7215	1			

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				24-Apr	25-Apr	26-Apr	28-Apr	5-May	8-May	9-May	10-May	11-May
2017	Spring	17X-19	9902-1 X 7215-1	1	1		2			1	2	2
2017	Spring	17X-20	9902-1 X 7215-1		2	1	1	1	1		4	6
2017	Spring	17X-21	9902-1 X 7215-1								1	1
2017	Spring	17X-22	9902-1 X 7215-1									
2017	Spring	17X-23	9902-1 X 7215-1	1								

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				12-May	16-May	17-May	18-May	19-May	22-May	23-May	25-May	7-Jun
2017	Spring	17X-19	9902-1 X 7215-1	1				1		3		
2017	Spring	17X-20	9902-1 X 7215-1	3		4	3	2	2		2	2
2017	Spring	17X-21	9902-1 X 7215-1							1		1
2017	Spring	17X-22	9902-1 X 7215-1									
2017	Spring	17X-23	9902-1 X 7215-1									

Year	Season	Cross	Parents	Date	Date	Date	Date	Date	Date	Date	Date	Date
				6-Oct	10-Oct	12-Oct	13-Oct	19-Oct	23-Oct	24-Oct	25-Oct	26-Oct
2017	Fall	17FX-24	9902-1 X 7215									
2017	Fall	17FX-25	9902-1 X 7215	1	3		1					2
2017	Fall	17FX-26	9902-1 X 7215					1				
2017	Fall	17FX-27	9902-1 X 7215									
2017	Fall	17FX-28	9902-1 X 7215	2	1	4	1		1	4	3	4
2017	Fall	17FX-29	9902-1 X 7215		2							
2017	Fall	17FX-35	9902-1 X 7215					3	10	9	6	5

Table A3. Continued

Year	Season	Cross	Parents	Date 30-Oct	Date 31-Oct	Date 1-Nov	Date 2-Nov	Date 3-Nov	Date 6-Nov	Date 7-Nov	Date 8-Nov	Date 9-Nov
2017	Fall	17FX-24	9902-1 X 7215									
2017	Fall	17FX-25	9902-1 X 7215		1		1					
2017	Fall	17FX-26	9902-1 X 7215									
2017	Fall	17FX-27	9902-1 X 7215									
2017	Fall	17FX-28	9902-1 X 7215	4	3	4	8	3	2		2	
2017	Fall	17FX-29	9902-1 X 7215									
2017	Fall	17FX-35	9902-1 X 7215	5	5	4	5	3	7	10	5	6

Year	Season	Cross	Parents	Date 10-Nov	Date 15-Nov	Date 20-Nov	Date 21-Nov
2017	Fall	17FX-24	9902-1 X 7215				
2017	Fall	17FX-25	9902-1 X 7215				
2017	Fall	17FX-26	9902-1 X 7215				
2017	Fall	17FX-27	9902-1 X 7215				
2017	Fall	17FX-28	9902-1 X 7215				
2017	Fall	17FX-29	9902-1 X 7215				
2017	Fall	17FX-35	9902-1 X 7215	11	10	3	5

Table A4. A chart summerizing the date, tissue type, concentration, time of exposure and number of attempts of colchicine treatments.

Tissue type	Concentration	Treatment duration									
Cuttings (103)		6hrs	7hrs	7.5hrs	7.75hrs	8hrs	8.25hrs	9hrs	10hrs	18hrs	24hrs
	.02% Col.		(5) 8/23/13			(4) 6/15/16					
			(7) 10/31/13			(5) 6/23/13					
						(5) 7/12/13					
						(5) 8/23/13					
						(10) 10/31/13					
						(20) 4/7/14					
						(40) 9/24/14					
	.03% Col.	(1) 2/15/15									
		(1) 6/3/15									
Submersion apical meristem (62)											
	.02% Col.					(5) 12/19/13		(3) 9/2/15	(1) 9/5/15	(5) 5/27/14	(10) 9/24/14
						(7) 1/13/14		(2) 9/28/15		(5) 9/25/14	(10) 9/29/14
						(6) 4/7/14				(5) 12/15/14	
	.03% Col.					(1) 2/15/15					
						(2) 9/28/15					
Submersion of seed (10)											
	.02% Col.			(1) 6/4/17	(1) 6/4/17	(1) 9/10/16	(1) 9/10/16				
	.03% Col.	(1) 7/7/15				(1) 2/18/15					
						(2) 7/15/15					

Table A5. A Glossary of abbreviations with definitions.

ABA- Abscisic Acid- A plant hormone associated with developmental processes and stress response.
AFLP- Amplified Fragment length polymorphism- A Polymerase Chain Reaction (PCR) based molecular marker that used restriction enzyme digestion and adaptor ligation, followed by PCR amplification to determine presence or absence of a polymorphism.
Alfin-like- A nucleic acid binding protein that functions as a transcription factor and has been associated to plant stress response.
AMA- American Medical Association- A professional organization publishing a peer reviewed journal of current medical research.
ANOVA- Analysis of Variance- A statistical procedure to separate variance in categories for a set of observations.
AP2/ERF- APETALA2/ Ethylene Responsive Factor- A large group of transcription factors associated with plant stress response.
AREB/ABF- ABA-responsive element binding/ARED-binding factor-
Bp- Base Pair- A pair of complimentary bases in DNA, consisting of a purine base and a pyrimidine base.
bZIP- Basic Leucine Zipper- A family of transcription factors involved in numerous fundamental cellular processes.
CAMTA- Calmodulin-Binding Transcription Activator- A family of transcription factors associated with plant stress response.
CAP- Catabolite Activator Protein- A protein associated with plant stress response and thought to be involved in cell signaling and homeostasis.
cDNA- complementary DNA- DNA that is reverse transcribed from RNA transcripts.
CDT- Central Daylight Time- Coordinated Universal Time - 5:00

Table A5. Continued

CIMMYT- Centro Internacional de Mejoramiento de Maíz y Trigo- A international non-profit research and training center in Mexico focusing on Maize and Wheat.
CLC Genomics Workbench- A QUIAGEN Bioinformatics Inc. software package for analyzing genomics data.
CRISPR Cas9- Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated Protein 9- A prokaryotic immune response system where a endonuclease cleaves DNA at a specific short repetitive sequence which can be modified to edit genomes in eukaryotes.
DAP- Days After Planting- An ascending number that represents the number of days an action was taken after planting
DGE- Differential Gene Expression- A statistical procedure used to discover differences in expression levels of experimental groups.
DNA- deoxyribonucleic acid- A self-replication material present in almost all living organisms, also known as the carrier of genetic information.
DREB- Dehydration Responsive Element Binding- A transcription factor involved in abiotic and biotic stress tolerance in plants.
DW- Dry Weight- A measure of plant tissue after 7 days drying at 37^{o}C.
EdgeR Test- Statistical test developed for differential gene expression analysis.
E-QTL-Epistatic Quantitative Trait Loci- A Quantitative Trait Loci associated with a particular locus that interacts with other loci in a vast interconnected network.
ERD1- Early Response to Dehydration 1- A transcription factor found in the plant stress signaling pathway.
FDR- False Discovery Rate- a method of conceptualizing type 1 error rates.
FW- Fresh Weight- A measure of plant tissue immediately upon collection.
Gb- Gigabase- 1,000,000,000 base pairs
h- hours

Table A5. Continued

ha- hectacre-10^4 m^2
Hs- Heat Shock- A transcription factor in the plant stress response pathway involved in cell homeostasis at elevated temperature.
IBG- Ickes-Braun Glasshouses Inc.- A greenhouse manufacture.
ICRISAT- International Crops Research Institute for the Semi-Arid Tropics- A international non-profit research and training center in India focusing on Peanut (Groundnut), Chickpea, Pigeonpea, Pearl millet, Sorghum, Finger millet and small millets.
INDEL- Insertion/Deletion- A type of mutation where an insertion or deletion of one or more bases occurs.
JMP Pro 12- Statistical Software owned by SAS Inc.
kg- kilogram- 1000 grams
KOAc- Potassium acetate
LEA- Late Embryogenesis Abundant Protein- A protein that serves as a protector of other proteins during plant stress response.
LSD- Least Significant Difference- A statistical probability when exceeded indicated statistically a significant difference.
MAB- Marker Assisted Breeding- Breeding schemes that incorporate the use of molecular markers as selection criteria.
MAS- Marker Assisted Selection- A type of indirect selection where selection is based on the presence or absence of a marker linked to a trait of interest.
Ml-Megaliter- 1,000,000 liters
mm- millimeter- .001 meters
m-meter- The basic unit of measure in the metric system.

Table A5. Continued

M-QTL- Main Effect Quantitative Trait Loci- A Quantitative Trait Loci associated to a locus known to control a large portion of variation.
MT- Metric ton- 1,000,000 grams
MYB- myeloblastosis- A family of transcription factors that is a DNA binding domain associated with stress response in plants
NAC- No apical meristem, Arabidopsis transcription activation factor, cup shaped cotyledon- A family of transcription factors regulating plant growth and stress response.
NPB- National Peanut Board- A non-profit organization that promotes the peanut industry
PCA- Principle Components Analysis- A statistical procedure to visually represent a set of possible correlated variables.
PGI- Peanut Genomic Initiative- An international collaboration between research and industry to sequence the cultivated peanut and its progenitor species.
QPCR- Quantitative Polymerase Chain Reaction- A type of PCR that monitors target DNA amplification in real-time and can be used to quantify gene expression.
QTL- Quantitative Trait Loci- A section of DNA with a locus that correlates to a phenotypic trait.
RD- Response to dehydration- A protein associated with plant stress response.
RFLP- Restriction Fragment Length Polymorphism- A molecular marker based on a variation in the length of DNA based on cutting the DNA at a specific site using a specific restriction enzyme. Radioactive isotopes are bonded to the site to aid in identification.
RNA- Ribonucleic acid- a messenger material in all living organisms used to carry genetic instructions.
RNase A- Ribonuclease- An enzyme that catalyzes the degradation of RNA into smaller components.

Table A5. Continued

RNA-seq- RNA sequencing- A sequencing technique (also known as Transcriptomics) that reveals the presence and quantity of RNA transcripts that are actively being transcribed in a sample at the time of sampling.

ROS- Reactive Oxygen Species- In biology: A chemically reactive compounds formed as a natural byproduct of oxygen metabolism associated with oxidative stress. It is known to be associated with cell signaling.
RPKM- Reads Per Kilobase Millions-a method of quantifying and normalizing RNA-seq data.
RQN- RNA Quality Number- A measure of RNA quality following extraction. RWC- Relative Water Content- An estimate of the current water content of a sampled leaf tissue relative to the maximum water content it can hold.
SNP- Single Nucleotide Polymorphism- A class of molecular markers that occur in DNA at the single nucleotide level.
SSR- Simple Sequence Repeat- A class of molecular markers that identify 2-6 base pair sequences that are repeated in DNA.
SWEEP- Sliding Window Extraction of Explicit Polymorphisms- Software used to filter SNP in polyploids for high-quality SNP discovery.
TDM- Total Dry Matter-A measurement of mass after all moisture is removed.
TE- Transpiration Efficiency- Total biomass produced per unit of water transpired.
TF- Transcription Factor- A protein that initiate and regulate the transcription of genes.
TPF- The Peanut Foundation- A non-profit foundation associated with the American Peanut Council that supports peanut research.
TWDB- Texas Water Development Board- A state of Texas entity that manages the state's water resources.
TW- Turgid Weight- A measure of plant tissue after 24 hours of submersion in reverse-osmosis water.

Table A5. Continued

UGA- University of Georgia at Athens- A U.S. public university involved in peanut research.
USDA- United States Department of Agriculture- U.S. government agency tasked with oversight of the U.S. farm programs.
USGS- United States Geological Survey- A United States government agency in charge of the study of U.S. landscape and natural resources.
WHO- World Health Organization- A specialized agency of the United Nations concerned with international public health.