Integration of physiological and molecular approaches for selecting peanut genotypes with superior drought tolerant and nitrogen fixation traits

by

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Abstract

The vulnerability of peanut to drought varies depending on physiological characteristics, crop growth stages, and environmental conditions. This study examined the effects of drought stress on symbiotic nitrogen fixation (SNF), carbon isotope discrimination and transcriptional profiles of various peanut genotypes. Two parental lines (Tifrunner and C76-16) and 14 recombinant inbred lines were evaluated under three irrigation regimes (irrigated control, middle-season drought and late-season drought) in rainout shelters. A greater reduction of the percentages of shoot N derived from the atmosphere (%Ndfa) and carbon isotope discrimination (Δ) occurred under middle-season drought than late-season drought. Under middle-season drought, both %Ndfa and Δ were higher in drought tolerant lines than drought susceptible lines, and a positive correlation between %Ndfa and Δ was observed under both drought treatments. Genes responding to drought stress were examined in four genotypes (drought tolerant: C76-16 and AU-587; drought susceptible: Tifrunner and AU-506) under irrigated control and middle-season drought. Whole-transcriptome sequencing analysis identified 7,780 differentially expressed genes (DEGs) in Tifrunner, 9,767 in AU-506, 12,348 in AU-587 and 13,005 in C76-16. Of the identified DEGs, 2,457 DEGs were shared by all four genotypes. Functional analysis of the shared DEGs identified a total of 139 enriched gene ontology (GO) terms and 43 enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways. This research expands our current understanding of the mechanisms that facilitate peanut drought tolerance and shed light on breeding advanced peanut lines to combat drought stress.

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Chapter One

Review of Literature

Cultivated peanut or groundnut (*Arachis hypogaea* L.) is an annual legume being cultivated primarily for its high-quality edible oil (43-55%) and easily digestible protein (25-28%) in its seeds (Devi 2010; Reddy et al., 2003; Upadhyaya, 2005). It belongs to the *Fabaceae* family and the genus *Arachis.* The species name, *hypogaea*, means under the earth. The crop is grown in over 100 countries including tropical, subtropical, and warm temperate regions with about 25.4 million hectares (ha) worldwide (Upadhyaya, 2005; Balota, 2012; Boriss and Kreith 2006; FAO, 2013).

With 1.89 million tons (Tg) production, the U.S. becomes the world's fourth largest peanut producer (behind China, India and Nigeria) and the world's leading exporter (FAO, 2013). Peanuts contribute over $4 billion each year to the country's economy in the U.S. (Kambiranda, 2011). Peanut cultivation in U.S. is mainly concentrated in Alabama, Arkansas, Florida, Georgia, Mississippi, New Mexico, North Carolina, Oklahoma, South Carolina, Texas, and Virginia, which is leading by Georgia with 53% of U.S. production (USDA, 2018).

In the U.S., four types of peanuts are mainly cultivated (Grise et al., 1982; Shin et al., 2010):

1. Runner group: These peanuts were introduced in the 1970s, and known for their uniform, medium-sized kernel. It is the dominate variety grown in the U.S. now, which account for 80% of total production across the country. The runner type is most commonly for making peanut butter, candies and snack nuts and typically grown in Georgia, Alabama, Texas and Florida, South Carolina and Oklahoma.

2. Virginia group: It is the largest peanut among all varieties and accounts for 15% of total peanut production. This group is primarily grown in Virginia, North Carolina, South Carolina and West Texas, and often used in gourmet snacks
3. Spanish group: It has the smallest kernels of all four types and known for its red skins. Due to the higher oil content of the Spanish type, they are mostly in peanut oil, as well as in candies and salted peanuts. This group is mainly grown in Texas, Oklahoma, and only account for four percent of peanut production in the U.S.
4. Valencia group: This variety contains three or more kernels per pod and known for its sweet flavor. They are commonly used in roasted peanuts and excellent for boiled peanuts. It accountokl;l for about 1% of U.S. production, and almost exclusively cultivated in New Mexico.

1.1 Growth stages of peanut

During peanut development, physiological and genetic characteristics of peanut change over the growing season, including plant responses to environmental stress. Research related to the growth stages and life cycles of peanut plant has been extensively conducted by different groups (Pattee, 1974; Schenk, 1961; Williams, 1978; Williams, 1981). One widely accepted model for describing peanut growth stage was developed by Boote (1982), where the growth phase descriptions were developed based on the visually observable vegetative (V) and reproductive (R) events. The vegetative growth stage is determined by the number of developed nodes on the main axis of the peanut plant. The vegetative growth stage is beginning with emergence, which is defined as VE. During the VE stage, the cotyledons are near the soil surface and showing some part of the plant visible. There would be a vegetative stage "V_0" when cotyledons are fully open at or slightly below the soil surface. After "V_0" stage, the node would

be counted as developed when the tetrafoliolate has fully developed and the leaflets are sufficiently unfolded. Vegetative stage "V_1" is defined as one developed node with one fully unfolded tetrafoliolate leaf. Subsequent vegetative stages up to "V_n" are defined by the number of the nodes on the main stem of peanut. The amount of nodes developed is largely depended on the environmental conditions, such as air, soil temperature, soil moisture, and plant maturity. The determination of reproductive stages is also based upon visually observation events such as flowering, pegging, fruit development, and seed maturation. The reproductive stages are defined into nine stages, R_1 (beginning bloom), R_2 (beginning peg), R_3 (beginning pod), R_4 (full pod), R_5 (beginning seed), R_6 (full seed), R_7 (beginning maturity), R_8 (harvest maturity), and R_9 (over mature pod). The classification of reproductive stages is similar to those developed for soybean by Fehr and Caviness (1977). However, the specific dates and durations of each V and R stage are largely depended on the crop and the environmental conditions. Reproductive stage R1, beginning bloom, is defined as the date that 50% of peanut plants have at least one open flower at any node on the plant. After R_1 stage, peg elongation is beginning to occur, which is denoted as R2 stage. Stage R_3, beginning pod, is defined as the date when 50% of plants have at least one peg in the soil with a swollen ovary at least twice the width of the peg. At the duration of R_3 stage (around 51 days after planting), peanut plants have reached the maximum rate of dry matter, although the canopy may not be fully closed and the maximum leaf area may not be achieved (McCloud, 1974; McGraw, 1977). At R_4 reproductive stage, 50% of peanut plants would have at least one fully-expand fruit and beginning to add significant pod numbers and pod weight. However, the vegetative accumulation of peanut remains at the maximum of the plants. Shortly after the R_4 stage, seed cotyledon starts to develop, and the growth stage reaches R_5, when half of the plants showing visible cotyledon growth. The R_6 reproductive stage, full seed, requires about

half of plants have at least one pod which is fully occupied by seed. The R_7 stage occurs when 50% of the plants have one or more pods that show inner pericarp coloration. Although R_7 stage is defined as "beginning maturity", the crop as a whole really reaches the highest activity on seed fill phase and seed maturation at that time. When half of population shows 66% to 75% of fully developed pods demonstrated by the testa color change or inner pericarp coloration, the growth stage reaches R_8, harvest maturity. The exact fraction of the fruits at harvest maturity is largely determined by the cultivar type and the environmental conditions. After R_8 stage, some pods may show an orange-tan coloration on the testa, which indicate the achievement of the over mature stage, R_9.

1.2 Plant growth-promoting bacteria

Soil is replenished with microorganisms including bacteria, fungi, actinobacteria, protozoa, and algae (Glick, 2012). Of the different microscopic life forms, bacteria are by far the most common group (often around 10^8 to 10^9 cells per gram of soil) in the soil, with only about 1% of the total number of cells are culturable (Schoenborn, 2004). Soil conditions including temperature, moisture, and the presence of salt and other chemicals as well as the number and types of plants found in those soils have tremendous influences on both the plant host and the quantity of bacteria in the soil (Glick et al., 1999). For example, the number of culturable bacteria could be as low as 10^4 cells per gram of soil in the environmentally stressed soils, such as drought (Timmusk, 2011). Bacteria are generally not evenly distributed in soil, and its diversity and richness is typically much higher around the plant roots (i.e. rhizosphere) than that in the rest of the soil due to the selection driven by plant root exudate metabolites (Badri, 2009a; Badri, 2009b; Bais, 2006; Canarini et al., 2019; Compant et al., 2019). Soil bacteria could affect plants in one of the three ways, which are beneficial, harmful or neutral (Lynch, 1990).

Environmental conditions may also change the interactions between a bacterium and a plant (Glick, 1999). As an example, the nitrogen fixing bacteria can facilitate plant growth by providing fixed nitrogen when the nutrients are in a limited amount in the soil, however they are unlikely to provide any benefit to plants when significant amounts of chemical fertilizer present in soil.

The term of plant growth-promoting bacteria (PGPB) describes all the bacteria that can promote plant growth. Even though the physiological mechanisms are different based on the host-bacteria interactions, PGPB still affect plants in a similar manner by direct or indirect interactions (Glick, 1999; Gamalero and Glick, 2011). In direct interaction, PGPB promote plant growth by facilitating nutrients acquisition or modulating plant hormone levels. In indirect interaction, PGPB can benefit plant development by decreasing the inhibitory effects of various phytopathogenic agents, acting as a biocontrol agent (Gamalero and Glick, 2011). As a self-pollinating annual legume, peanut extensively interacts with the soil bacteria in all different aspects at all different growth phases, both positive and negative. Peanut can form symbiotic relationship with bacteria such as *Rhizobium* spp. and *Bradyrhizobium* spp. (Sinclair et al., 1995; Dinh et al., 2013). 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase containing bacteria substantially increase drought resistance and productivity in peanut (Dey et al., 2004).

1.2.1 ACC deaminase-containing bacteria

1.2.1.1 Roles of ethylene in plants

Modulating phytohormone levels is one of the effective mechanisms that PGPB promote plant development. During plant growth and development, plants frequently subject to the growth limiting environmental conditions. When plants subjected to the nonlethal environmental stress, hormones play key roles in response to their environment (Davies et al., 2006). Plants

often attempt to adjust their endogenous phytohormones levels in order to alleviate or eliminate the negative effects of the environmental stressors (Salamone et al., 2005). Along with adjustments made by plant itself, microorganisms in rhizosphere may also facilitate plant by modulating phytohormones under in vitro conditions (Salamone et al., 2005). Therefore, many PGPB can alter phytohormone levels and further affect the plant's hormonal balance and also the response to stress (Glick et al., 2007).

Ethylene is an important phytohormone that has a wide range of regulatory functions in every stage of plant life (Ma et al., 2002a). It regulates plant growth in numerous ways at several phases of plant development including promoting root initiation, inhibiting root elongation, promoting fruit ripening, promoting flower wilting, stimulating seed germination, promoting leaf abscission and inhibiting mycorrhizae-plant interaction (Abeles et al., 1992). Due to the simplicity of ethylene in chemical structure, there are many compounds that could be converted to ethylene through various metabolism pathways. In higher plants, methionine serves as the major precursor of ethylene (Yang et al., 1984; Lieberman et al., 1965; Lieberman et al., 1966), and the conversion of methionine to ethylene represents the major pathway of ethylene biosynthesis (Ma et al., 2002a). In ethylene biosynthesis, methionine would be converted to S-adenosylmethionine (SAM) by SAM synthetase at the present of ATP. Then, 1-Aminocyclopropanecarboxylic acid (ACC) would be synthesized by ϒ-elimination reaction using SAM as substrate at the present of ACC synthase. Finally, ACC oxidase would metabolize ACC to ethylene, carbon dioxide and cyanide. The rate-limiting steps in the ethylene biosynthetic pathway are catalyzed by ACC synthase and ACC oxidase, both of which are encoded by multiple genes and are differentially expressed upon external and internal stimuli (Fluhr et al., 1996).

The regulatory effects of ethylene could be either positive or negative. “Stress ethylene” is synthesized as a response to various environmental stresses including extremes of temperature, high light, flooding, drought, the presence of toxic metals and organic pollutants, radiation, wounding, insect predation, high salt, and various pathogens including viruses, bacteria, and fungi (Abeles et al., 1973; Morgan and Drew, 1997). An increased level of ethylene that is formed in response to environmental stresses can either exacerbate symptoms of stress or lead to responses that enhance plant survival under adverse conditions, depending upon the plant species, its age and the nature of the stress (van Loon and Glick, 2004). This contradictory effect of stress ethylene on plants could be explained by a two-phase model proposed by Glick et al. (2007). When plants are exposed to stress, an initial small amount of ethylene would be produced quickly as a response to the stress. The initial peak of ethylene production is suggested to deplete the existing ACC pool within plant tissues, initiate the protective response of plants such as transcription of pathogenesis-related genes and induction of acquired resistance (van Loon and Glick, 2004). If the stress is chronic and intense, as a consequence, the transcription of ACC synthase genes would be substantially increased (Yang and Hoffman., 1984), which in turn produced a larger amount of ethylene. This second large ethylene peak will induce the senescence and chlorosis process of the plants, which will further interrupt the defensive mechanism of the plant, inhibit plant survival, and lead to reduced crop performance. Since the second peak of ethylene production resulted by the increased transcription of ACC synthase genes, the inhibitors of ethylene synthesis would obviously decrease stress effects in plants (Yang and Hoffman., 1984).

As a phytohormone, ethylene plays roles particularly in the symbiotic relationship between rhizobia and the host plant. It affects nodule number and development as well as the

location of nodule primordia; it also involves in nodule formation and senescence (Ma et al., 2002a, b; Glick, 2014, Nascimento et al., 2016; López et al., 2018). Grobbelaar (1971) indicated that application of exogenous ethylene had an adverse effect on the nodulation and nodule functions. A reduction in the biosynthesis of endogenous ethylene is associated with an increase in nodulation (Guinel et al., 2015; Nascimento et al., 2018; Khalid et al., 2017).

1.2.1.2 ACC deaminase-containing plant growth-promoting bacteria

Although the nodulation process is mainly regulated by the host legume, ACC deaminase-containing rhizobacteria can promote nodulation and nitrogen fixation efficiency by reducing the ethylene level in the roots even under drought stress. ACC deaminase was initially identified and isolated from the yeast *Cyberlindnera saturnus* and *Pseudomonas* sp. strain ACP (Honma and Shimomura, 1978). Further studies have been done extensively on ACC deaminase which indicated the presence of the enzyme in a wide range of different bacteria (*Azospirillum, Agrobacterium, Achromobacter, Burkholderia, Ralstonia, Pseudomonas, and Enterobacter*) and fungi (Sheehy et al., 1991; Honma, 1993; Jacobson et al., 1994; Glick et al., 1995; Campbell and Thomson, 1996; Mayak et al., 2004; Babalola et al., 2003; Blaha et al. 2006). ACC deaminase activity is also detected in *Rhizobium* spp. Of the 233 isolated *Rhizobium* spp. from various sites in southern and central Saskatchewan, 27 species possessed the ACC deaminase activity (Duan et al., 2006).

1.2.1.2.1 ACC deaminase and its structure

ACC deaminase is an enzyme that catalyzes the cleavage of ACC to NH_3 and α-ketobutyrate resulting into decrease in ethylene level in plants which in turn resumes root growth. Several studies addressed the detailed biochemical properties of ACC deaminase and the important reaction mechanism (Honma and Shimomura, 1978; Honma et al., 1993; Ose et al.,

2003; Jacobson et al., 1994; Zhao et al., 2003; Hontzeas et al., 2004a). Data obtained in these studies showed that ACC deaminase is a multimeric enzyme (homodimer or homotrimer) with a subunit molecular mass of approximately 35-42 kDa and one molecule of essential co-factor pyridoxal phosphate (PLP) tightly bound to each subunit. To gain further insight into the function of this PLP-dependent enzyme, the crystal structures of ACC deaminase and an ACC deaminase homologue without this activity (from *Pyrococcus horikoshii*) have been determined by different groups (Yao et al., 2000, Ose et al., 2003; Karthikeyan et al., 2004a; Fujino et al., 2004). The crystal structures, along with site-specific mutagenesis studies, have allowed to identify the essential amino acid residues for substrate recognition and reaction. These studies have indicated that ACC deaminase folds to form two domains, each of which has an open twisted α/β structure similar to the β-subunit of the PLP-dependent enzyme tryptophan synthetase.

1.2.1.2.2 Genetics of ACC deaminase

As indicated before, ACC deaminase activity has been observed in a large number of different soil microorganisms; especially, it is more frequently found in the rhizosphere soils (Glick et al., 1995; Blaha et al., 2006). Interestingly, even within a same genus and species of microorganism, some strains displayed ACC deaminase activity, however, other strains may not. For example, Holguin and Glick (2001) indicated that some of *Azospirillum* strains do not contain ACC deaminase, while Blaha *et al*. (2006) found some particular *Azospirillum* strain contain *acdS* gene. Phylogenetic analysis of known ACC deaminase genes performed by Hontzeas *et al.* (2005) suggested that ACC deaminase genes may not always locate on the chromosomal DNA, instead, they may likely locate on the plasmid. In 2006, Young *et al.,* proposed a putative *acdS* gene located on the plasmid pRL10 in *R. leguminosarum* bv. Viciae

strain 3841. Likewise, *R. leguminosarum* bv. *Trifolli strain* NZP514 has an *acdS* gene on plasmid pRtr514a with an *acdR* located upstream of *acdS.* In addition, *acdS* gene has also been reported to be located on an accessory plasmid, pSmeSM11a. A putative regulatory protein, encoded by *acdR*, is located upstream of the deduced *acdS* in one strain of *Sinorhizobium meliloti* (Stiens et al., 2006), while another *S. meliloti* strain does not contain *acdS* gene at all (Ma et al., 2004).

In *B. diazoefficiens* USDA 110 (strain: USDA 110, old-name: *B. japonicum* U1SDA 110), the putative ACC deaminase gene blr0241 has been identified in the genome of *B. japonicum* (Kaneko et al., 2002). Moreover, Hoa et al., (2004) displayed the differently expressed proteins of *B. japonicum* USDA 110 in the symbiotic and non-symbiotic state using two-dimensional gel electrophoresis. The ACC deaminase gene was significantly upregulated at the symbiotic state, which indicated that the ACC deaminase involved in nodulation process by degrades the precursor (ACC) of the plant hormone ethylene and acts to lower the ethylene concentration around bacteroids. This would result in the stimulation of nodule formation since ethylene act as inhibitor for nodulation. In addition, *S. meliloti* Rm1021, a rhizobial strain that lacks ACC deaminase, is capable of nodulating its host legume, alfalfa, more efficiently and competitively when it is transformed with the structural and regulatory ACC deaminase genes from *R. leguminosarum* bv. *viciae* 128C53K (Ma et al. 2002b). Other reports demonstrated that *acdS* gene in rhizobia is under transcriptional control of the *nifA* promoter, which regulates the transcription of *nif* genes (Nascimento et al., 2012; Nukui et al., 2006; Sullivan et al., 2002). Moreover, the ACC deaminase activity has been associated with nitrogen fixation and the delay of nodule senescence (Tittabutr et al., 2015). Therefore, the decrease in nitrogen fixation might lead to ethylene synthesis and nodule senescence (López et al., 2018). On the other hand,

delayed nodule senescence may associate with the increase in ACC deaminase activity. Consequently, ACC deaminase might be a biological strategy to improve nodulation and to delay nodule senescence (Glick, 2012).

1.2.1.2.3 The relationship between ACC deaminase-containing bacteria and plants

ACC deaminase-containing PGPB can promote plant growth and development by decreasing ethylene levels, which further induce the salt tolerance and reduce drought stress in plants (Nadeem et al., 2007; Zahir et al., 2008). A model that describes the association between ACC deaminase-containing bacteria and plants was proposed by Glick et al. (1998). In this model, PGPB colonize and bind the seeds or roots in response to tryptophan and other small molecules in the plant exudates (Penrose and Glick, 2001). In return, the bacteria would synthesize and secrete indole-3-acetic acid (IAA), which would stimulate cell proliferation, root elongation and ACC synthase transcription along with endogenous IAA in plant. Some of the ACC synthesized by ACC synthase would be exuded from the seeds and roots along with other small molecules, then it would be taken up by PGPB. Therefore, ACC would be substantial cleaved by the enzyme, ACC deaminase, by converting it to NH_3 and α-ketobutyrate. In this model, PGPB serves as a sink for lowering IAA-induced ACC, and further limiting ethylene levels in plants. Thus, plants colonized by ACC deaminase-containing PGPB would have longer roots, longer shoots and higher resistant to growth inhibition by a variety of ethylene-inducing stresses. However, the amount of ethylene could never be completely depleted in the plant since ACC oxidase has a much higher affinity for ACC than ACC deaminase (Glick et al. 1998). Thus, ethylene levels in plants ultimately depend upon the ratio of ACC oxidase to ACC deaminase, when ACC deaminase-containing bacteria exist in the rhizosphere.

1.2.2 Biological nitrogen fixation

Facilitating resource acquisition is one of the best-studied mechanisms of bacterial plant growth promotion include providing plants with resources/nutrients that they lack such as fixed nitrogen, iron, and phosphorus. Many agricultural soils lack a sufficient amount of one or more of these elements so that plant growth is suboptimal. To obviate this problem and obtain higher plant yields, farmers have become increasingly dependent on chemical sources of nitrogen and phosphorus. Besides being costly, the production of chemical fertilizers depletes nonrenewable resources. The oil and natural gas used to produce these fertilizers poses human and environmental hazards. It would obviously be advantageous if efficient biological means of providing nitrogen and phosphorus to plants could be used to substitute for at least a portion of the chemical nitrogen and phosphorus that is currently used.

Biological nitrogen fixation (BNF) is the process that occurs when diazotrophs, nitrogen-fixing organisms, convert the nearly inert atmospheric nitrogen (N_2) into ammonia (NH_3), which can then be incorporated into organic components (Russelle, 2008; Unkovich et al., 2008; Vitousek et al., 1997). With 40% of land surface was covered by agriculture worldwide including 3.4 billion ha of pastures, 1.4 billion ha of arable land with 250 million ha legumes and 136 million ha of permanent crops, BNF made tremendous contribution to agricultural production. Tons of consumable nitrogen were provided to plants, and then finally used as feed for livestock or for human consumption (Unkovich et al., 2008). Due to the vast amount of organic N on Earth is derived from BNF, BNF is considered as the second most important biologically mediated process on earth, which is right after photosynthesis (Unkovich et al., 2008).

Notwithstanding the BNF had a substantial contribution to agricultural production and natural ecosystem, the role of BNF has been diminished during last decades due to the increased

utilization of synthetic fertilizer, which is almost indispensable for modern agricultural practices (Bohlool et al., 1992; Roger, 1995; McCown, 1996; Smil, 1997). Nevertheless, continuously use of synthetic fertilizer might substantially contribute to environmental pollution, accelerate depletion of the stocks of non-renewable energy resource used in fertilizer production, and reduce crop yields (Plucknett and Smith, 1986; Barker and Chapman, 1988; Byerlee, 1987; Odum, 1989; Pingali et al., 1990). As an oilseed legume plant, peanut fix N through the symbiosis process by interacting with the slow-growing rhizobia, *Bradyrhizobium* spp. (Taurian et al., 2006). Peoples and Craswell (1992) reported the proportion of experimental peanut N derived from N_2 (%Ndfa) ranges from 0.22-0.92, with an average of 0.68. Peoples et al. (1995) further reported a %Ndfa average of 0.58 peanut cultivated on farmers' fields. With the large %Ndfa of peanut, BNF substantially supported to accomplish the relatively high yield of peanut and considerable one of the resources of crop's nutrient supply for poor and small-scale farmers who cannot afford to use costly chemical fertilizer (Wunna et al., 2009). Thus, BNF could serve as a perspective alternative since unabated use of synthetic N fertilizer in modern agriculture practice has led to a global concern (Bohlool et al., 1992).

1.2.2.1 Symbiotic relationships between plant hosts and rhizobia

In addition to *Rhizobia* spp., a wide diversity of diazotrophs has been identified, which are all prokaryotes (*Bacteria* and *Archaea*). Some N_2-fixing prokaryotes can fix N_2 as a free-living organism, for example *Azospirillum* spp. (Bashan and Levanony, 1990), while others grow in association with various plants or fungal species (Soltis et al., 1995). The relationships between diazotrophs and plants ranges from the loose association of heterotrophic bacteria growing surround plant roots, to endophytic bacteria residing in the vascular tissues of tropical grasses, to highly evolved symbiotic association which could benefit both parties, microbe and

plant (Peoples et al., 1995).

Among all the different BNF mechanisms, the symbiotic relationship between legumes and rhizobia are responsible for the major contribution to the farming system (Ledgard et al., 1995; Herridge et al., 2008). Herridge (2008) reported estimated amount of N fixed by legume plant is around 2.95 Tg for the pulses and 18.5 Tg for the oilseed legumes, representing 31% to 43% of global BNF production. In California, up to 75% of the protein in dairy products is contributed by symbiotic nitrogen fixation by alfalfa (Phillips, 1999). The symbiotic relationship between *Rhizobium* (the fast-growing rhizobia) and *Bradyrhizobium* (the slow-growing rhizobia) spp. and leguminous plants roots introduces a model to study the complex mechanisms that control plant cell division and nodule development (Caetano-Anolles and Gresshoff, 1991). Considerable progress has been made during the past few decades in characterizing the processes that initiate infection and control nodule formation in physiological, biochemical, and molecular biological perspectives in plant and the bacteria (Caetano-Anolles and Gresshoff, 1991; Lopez et al., 2018; Nascimento et al., 2016; 2018; Qiao et al., 2016; Yuan et al., 2017).

1.2.2.1.1 Legume-symbiotic bacteria interactions

The development of symbiotic relationship is usually highly specific between bacterial symbiont and legume, which means the bacterial symbiont could only inoculate a restricted number of hosts, and each host could only be nodulated by a restricted number of microsymbionts. Before nodulation formation, host plant secretes some substances, including flavonoids and some other small molecules, which act as chemo-attractants for *Rhizobium* and *Bradyrhizobium* spp. The active chemotaxis could induce gene expressions in bacteria and drive *Rhizobia* moving towards to defined root regions and form a firm attachment with the root system (Ames, 1981; Bergman, 1988; Caetano-Anolles, 1988; Gresshoff, 1990; Kape, 1991;

Parke, 1985; Aguilar et al., 1988; Armitage et al., 1988; Caetano-Anolles et al., 1988; Peters and Verma; 1990; Nascimento et al., 2018; Yuan et al., 2017). During the formation of bacterial attachment, plant lectins bind to bacterial polysaccharide receptors and mediate the attachment of rhizobia to the root hair regions (Dazzo, 1983; Halverson and Stacey, 1986). In 1989, Diaz et al. isolated the lectin glycoproteins gene from pea and transformed it into the genome of clover roots using *Agrobacterium rhizogenes* transformation. Then, the clover roots were nodulated by both clover and the pea microsymbionts, suggesting that the lectin glycoproteins could play a crucial role in determining the symbiosis specificity.

After bacterial attachment, the root hairs would be induced to branch, deform, and curl at the presence of bacterial exudates (Bhuvaneswari and Solheim, 1985; Yao and Vincent, 1969). Only the young root could be curled sufficiently to entrap bacterial cells in a pocket of host-cell walls. In soybean, the initial infection process would occur within 12 h after the contact with nitrogen fixing bacteria. This process is then followed with the generation of tubular structures. The infection threads penetrate through the root hair cell and grow into the root cortex where they release the bacteria within the host cytoplasm enclosed in a peribacteroid host-derived membrane (Newcomb. 1981). At this point, the bacteria released within the host cytoplasm would differentiate into bacteroids, which is functionally able to convert the atmospheric N_2 into NH_3 in exchange for the fixed carbon sources by plant photosynthesis (Goodchild and Bergerson, 1966; Rolfe and Gresshoff, 1988).

In some other reports, a morphological event which is root cortical cell division is suggested as one of the prerequisite for thread infection (Hadri et al., 1998; Gage et al., 2004; Madsen et al., 2010; Kawaharada et al., 2017). In soybean, the first divisions occur in the hypodermal layer and the plant would develop spherical (determinate) nodules (Calvert, 1984).

In temperate legumes, such as pea and alfalfa, the earliest divisions occur in the inner cortex (Dudley, 1987; Libbenga and Harkes, 1973) and it would develop cylindrical or indeterminate nodules. In some cases, bacteria can enter the plant through cracks or middle lamellae when the cell divisions are induced by bacteria (Hadri et al., 1998). In others, they take advantage of already divided cells or use wounds produced during the emergence of a lateral root to enter the host. Such is the case with *Arachis, Sesbania, Stylosanthes, Neptunia* (Boogerd et al., 1997). Promiscuous nodulation of *Arachis* has been attributed to the mode of infection via cracks in the epidermis. The intercellular penetration of *Bradyrhizobium* would also lead to a (hyper) sensitive response by the peanut (Boogerd et al., 1997). After entry, *Bradyrhizobium* cells occupy the space between epidermal and cortical cells. Beneath some of the axillary root hairs, basal cells become enlarged. The enlarged basal cells are the first to become infected by some of the invading bradyrhizobial cells, while others continue to spread intercellularly. Then, the invaded large basal cells would divide and become smaller and further incorporated in the nodule tissue. As the entire nodule tissue originates from the infected cell, the rod-shaped bradyrhizobial cells would differentiate into large, spherical bacteroids. After crack entry, intercellular spreading, and nodule organogenesis, finally the mature active nodule is established (Boogerd et al., 1997).

Even though plant species respond differently to the nodulation infection, there are similarities among different plant species. Nodulation responses in legume species start from the initial induction of cell division in the cortical region. Then, it follows with the induction of pericycle cell division, which in turn results in the formation of a nodule with tissues from both origins, the cortex, which leads to the infected zone and part of the inner nodule parenchyma, and the pericycle derived tissues. The presence of two regions of cell growth results in the characteristic peripheral vascular tissue arrangement of the legume nodule.

1.2.2.1.2 Bacterial genes related to nodulation

In the past few decades, our knowledge of the nodulation process and the related bacterial genes has been substantially expanded (Long, 1989; Rolfe and Gresshoff, 1988). The nodulation-related genes are significantly different in the fast-growing and slow-growing rhizobia species. In the fast-growing *Rhizobium* species, the nodulation-related genes usually carried out on the large plasmids, about 200 to 1500 kb. These genes are denoted as *nod, nol, syr, exo, lps, ndv, nif and fix.* However, *Bradyrhizobium* spp., the slow-growing species, carry the symbiotic genes mainly on chromosome. Several biochemical studies were conducted to investigate the potential roles of these nodulation-related genes, which pointed out the possible involvement of genes in hormone metabolism, membrane transport, and lipid and polysaccharide synthesis. In the fast-growing *Rhizobium* spp., nodulation genes are generally organized into two clusters. The first cluster is the common *nodABC* genes which are responsible for both root hair curling and the initiation of cell division. They are usually interchangeable among *Rhizobium* spp. and followed immediately downstream by *nodIJ.* In addition, those common *nodABC* are preceded by divergently transcribed regulatory *nod* gene (Debelle, 1986; Djordjevic, 1985; Downie, 1985; Egelhoff, 1985). The second cluster is the host-specific nodulation genes, which are required for bacteria-legume specificity. They are mainly responsible for the correct induction of root hair curling and cell division in the specific legume host. The locations of those genes are dependent on *Rhizobium* spp. For example, they locate immediately downstream of *nodABC* in *R. meliloti* (*nodH, nodFEG,* and *nodPQ*). However, in *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* (*nodFELMN* and *nodO*), they locate downstream of *nodD* (Cervantes et al., 1989; Debelle et al., 1986; Debelle and Shanna, 1986; Djordjevic et al., 1986). In slow-growing bacteria like *B. japonicum,* the organization of the *nod* genes differs significantly from those fast-

growing bacteria (Hennecke et al., 1988). *Bradyrhizobium japonicum* carries a set of common *nodABC* genes preceded by an open reading frame, *nodY,* and a conserved sequence, which are responsible for *nodABC* transcription (Banfalvi et al., 1988; Hahn and Hennecke, 1988; Lamb and Hennecke, 1986). In addition, three DNA regions have been identified in *B. japonicum,* which are responsible for encoding host-specificity functions in soybean, cowpea and mung bean (Hahn and Hennecke, 1988; Gatlfert et al., 1990; Niewkoop et al., 1987). Besides those genes, a soybean specific nodulation gene, *nolA*, was also identified (Sadowsky et al., 1991). However, not many literatures were found on peanut-specific nodulation. Another group of genes, which is accountable for the synthesis of surface and extracellular polysaccharides was also identified and sequenced. They are believed to be involved in root nodulation entry during the infection process in many different aspects, such as synthesis of extracellular heteropolysaccharides, capsular polysaccharide, and lipopolysaccharides (Halverson and Stacey, 1986).

After the formation of nodule structure, the bacteria would differentiate into bacteroids and start synthesizing nitrogenase to convert N_2 into NH_3. In regarding of nitrogenase structure, it consists of two components, one part is homodimeric Fe protein which is encoded by *nifH* and the other component is the tetrameric molybdenum-iron (MoFe) protein with MoFe cifactir, which is encoded by *nifD* and *nifK* (Mylona et al., 1995). In the process of nodule formation, a large number of nitrogenase (*nif*) genes are involved for nitrogen fixation, which include some of structural genes, genes that are responsible for Fe protein activation, iron molybdenum cofactor biosynthesis, and some of the regulatory genes that are required for enzyme synthesis and functioning. The *nif* genes are typically organized in a cluster around 20-24 kb which includes seven operons encoding 20 different proteins (Glick, 2012).

Because the complexity of nitrogen fixation system, the improvement of nitrogen fixation

through genetic engineering strategies is exceptionally hard. Despite the complexity, some of the studies succeeded in improving the nitrogenase activity by gene transformation to improve respiratory rate at lower oxygen requirement. Following transformation of *R. etli* with a *Vitreoscilla* sp. hemoglobin gene, the transformed rhizobia had two or three-fold of respiration rate than the untransformed strain. The bean plants that inoculated with the genetically engineered *R. etli* had 68% increase in nitrogenase activity than plants inoculated with wild-type *R. etli.* The nitrogen content in leaf and resultant seeds increased 25-30% and 16%, respectively (Ramirez et al., 1999).

1.3 Drought

Drought stress has been the major environmental factor causes reduced agricultural productivity and food safety in field crops worldwide and its effect is economically devastating when it occurs at critical growth stages of plants (Chen et al., 2013; Kambiranda, 2011; Pimratch, 2008; Rivero et al., 2007). The American Peanut Council has identified drought damage along with the preharvest aflatoxin contamination as the most serious challenge facing the U.S. peanut industry. Drought could be perceived by the plant from the surrounding environment varies spatially and temporally at several different scales (Kambiranda, 2011). It was highly documented that water deficient could affect peanut production at several different aspects including membrane lipids and photosynthetic activity (Lauriano et al., 2000), mineral nutrition adsorption, metabolism, growth and yield (Soler, 2012; Suthar & Patel, 1992), nitrogen fixation and its related traits (Sinclair et al., 1995; Pimratch, 2008) and increasing susceptibility to fungal invasion (Blankenship et al., 1984; Cole et al., 1989).

Patel and Golakiya (1988) reported the total productivity of groundnut still remained constant even the total cultivation area has been increased worldwide since rainfall is a critical

factor in many groundnut-production countries. For example, the peanut productivity ranges from 0.7 to 0.8 Mg ha^{-1} under drought, which is relatively low when compare with cultivation under a commercial system, where its productivity ranges 2.0 to 4.0 Mg ha^{-1} (Upadhyaya, 2005). In the world, over half of the production area, which accounts for 70% of the peanut growing area fall under arid and semi-arid regions, where rainfall is generally erratic and insufficient, frequently causing unpredictable drought stresses at different duration and intensities, which is the most important constraint for peanut commercial production (Reddy et al., 2003; Songsri et al., 2008). More than 6.7 million metric tons loss in annual world peanut production, which equivalent to over US$520 million, was contributed by water deficits (Subbarao et al., 1995). Majority of the peanut production area in the U.S is under rain-fed conditions and only limited acreage could be irrigated. However, water access is practically difficult for improving since water is a scarce resource (Songsri et al., 2008, Chen et al, 2013). Therefore, more efficient agricultural strategies must be adopted urgently, to ensure future food-supply for the ever-growing population, which must do so under the limited irrigation water.

1.3.1 Effects of drought stress on peanut plant

It has been well demonstrated that drought stress has adverse effects on peanut plants in an array of biochemical and physiological processes at molecular, cellular, and whole-plant levels, including water relations (Babu and Rao, 1983), photosynthesis (Bhagsari et al., 1976), mineral nutrition uptake (Kulkarni et al., 1988), nitrogen fixation (Reddi and Reddy, 1995; Lenka and Mishra, 1973), metabolism (Pandey et al., 1984; Reddi and Reddy, 1995) and growth, and yield of groundnut (Suthar and Patel, 1992). Drought conditions also influence peanut production indirectly by influencing the growth of weeds, agronomic management and the intensity of insects, pests and disease. For instance, the preharvest aflatoxin contamination is

believed to be related with late season drought stress.

Parameters including relative water content (RWC), osmotic potential, transpiration rate, stomatal conductance, leaf water potential, could influence water relations in peanut imposed with moisture stress. Babu and Rao (1983) introduced a 35 days' water stress since 20 days after planting (DAP), then RWC and leaf water potential were determined under both water adequate and deficit conditions. After the 35-day dry period, the plant was wilted and a lower RWC (29.7%) and leaf water potential (-5.0 MPa) were found in stressed plants than non-stressed plants, in which RWC ranged between 87% to 100% and leaf water potential ranged from -1.15 to -0.15, respectively. This was then confirmed by Bennett et al (1984) and Boote and Ketring (1990), who reported the leaf water potential is less than -1.2 to -1.3 MPa for well-watered groundnut while -3.0 to -5.0 MPa for stressed plants. The same pattern was observed for turgor potential (Black et al., 1985), transpiration rate (Mohandas et al., 1989; Subramanian and Maheswari, 1990), stomatal conductance (Mohandas et al., 1989; Black et al., 1985). As moisture stress increases, stomata will start closing as a mechanism to reduce transpiration, which also cause reduction in photosynthesis by reducing the entry of carbon dioxide (Bhagsari et al., 1976). In addition, desiccation reduced leaf longevity and leaf area duration by slowing leaf expansion, and then further reduced the canopy photosynthesis (Black et al., 1985).

During peanut development, plants respond differently to drought based on the water requirement at different growth stages. At the vegetative stages (V_0 to V_n), water requirement is moderate, thus, the start of vegetative growth and flowing would not be delayed by drought stress (Boote and Ketring, 1990). Moreover, several studies indicated an association between high leaf area index and high biomass production with the peanut plant that subjected to the early season drought followed with an irrigation recovery (Puangbut et al., 2009ab). Even though the

rate of flower production would be reduced by drought stress during flowering, the total number of flowers per plant would not be affected during to a long duration of flowering stage (Gowda and Hegde., 1986; Janamatti et al., 1986). In addition, a significant burst in flowering would show immediately when the stress is alleviated, particularly when drought is imposed just prior to re-productive development (Janamatti et al., 1986). However, when the drought stress is introduced around 30-45 days after sowing, the first flush of flowers would have no peg formation; but the flowers produced after an immediate recovery irrigation would compensate the production loss (Gowda and Hegde., 1986). If the water stress is imposed during re-productive stages, such as pegging and pod development, it then would result in a drastic reduction in pod yield and fruit quality. The magnitude of reduction would depend on peanut cultivar and agronomic practices (Jogloy et al., 1996; Rucker et al., 1995). At the pegging stage, peg elongation would be delayed since this process is turgor dependent. Additionally, pegs would fail to penetrate the soil effectively, after 4 days of water withhold, where the soil surface becomes too dry for peg penetration (Boote and Ketring., 1990). Once pegs are in the soil, adequate pod zone moisture and darkness are essential for keeping pegs alive and pod development. Soil water deficit during this stage in pegging and root zone would reduce approximately 30% pod and seed growth rate. It is also frequently reported that pegging and seed formation response under drought stress varies substantially among different peanut cultivars (Nageswara Rao et al., 1998).

1.3.2 Effects of drought stress on nitrogen fixation

Almost all-metabolic processes involved in shoot and root growth could be influenced by water deficit, and severe water deficits will cause decreasing in enzymatic activity (Pandey et al., 1984). In particular, BNF in the symbiosis process of peanut and rhizobacteria is sensitively and

severely inhibited under water deficit, so the amount of nitrogen required for vegetative and reproductive stages of peanut would be compromised under drought conditions (Lenka and Mishra, 1973; Pimratch *et al.,* 2008). Lenka and Mishra (1973) reported treatment that irrigated at 75% depletion of available soil moisture has less nodulation and lower efficient nitrogen fixation when compared to the treatment with 25% depletion of available soil moisture. Pimratch *et al*. (2008) revealed a positive correlation between nitrogen fixation and biomass production under a long-term drought stress from 21 DAP until to harvest. In addition, the more severe the drought stress, the stronger the correlation was discovered. Lately more studies were conducted to investigate the effect of drought stress on the particular growth stages of peanut (Wunna *et al.,* 2009; Puangbut *et al.,* 2011; Dinh *et al.,* 2013). Peanut subjected to early season drought or pre-flowering drought stress followed by watering recovery could actually increase the transpiration efficiency and the nitrogen fixation efficiency due to the long-time recovery after drought stress (Puangbut *et al.,* 2011). Additionally, increased transpiration efficiency and improved nitrogen fixation could further contribute the biomass production and pod yield (Puangbut *et al.,* 2011). Wunna *et al.* (2009) reported the similar results that the correlation between nitrogen fixation traits and biomass production under pre-flowering drought was positive and significant, and the maintaining high nitrogen fixation under drought stress could be an important adaptive mechanism for peanut genotypes to achieve high yield under water limited conditions.

Under mid-season drought, the nitrogen fixation activity of nodule system would be seriously affected due to the essence that mid-growing season, from flowering to pod filling, has the highest water requirement (Peña-Cabriales and Castellanos, 1993), and peanut under mid-growing season is the most susceptible to drought stress (Dinh *et al*., 2013). Dinh *et al,* 2013 concluded the nitrogen fixation related traits and yield was highly reduced under mid-season

drought, in the meanwhile, a positive correlation between fixed nitrogen with biomass production and pod yield under mid-season drought was discovered. Due to the association reported between nitrogen fixation and pod yield under drought conditions, nitrogen fixation related traits could be considered as a drought tolerance character and genotypes selection criterion for peanut breeding (Dinh *et al.,* 2013). In contrast, there was also a literature reported that no effects of irrigation frequency (7, 14 and 21 days) on nodule number and nodule weight at the end of the season for groundnut grown on deep soil with high organic matter (Shimshi et al. 1967). Therefore, mechanism behind drought stress on nitrogen fixation is remaining unclear.

1.4 Methodologies on quantifying BNF

Investigation about response of peanut for nitrogen fixation traits under drought conditions, particularly under different growing stages, is extremely necessary.

The demand of regulating BNF to maintain the availability of consumable N for human and animal uptake and to develop more sustainable farming practice and natural ecosystem is strong and will continue to be fueled up. However, it will be extremely difficult to conduct research to identify, understand, and utilize the principal factors to regulate BNF for the optimal environment and agricultural productivity, unless BNF could be accurately quantified. There are several methodologies available, allows us to quantify BNF, which includes N balance method, N difference, ^{15}N natural abundance, ^{15}N isotope dilution, ureide methods, acetylene reduction and hydrogen evolution method (Unkovich et al., 2008; Herridge et al., 2008). In my thesis, we focus on ^{15}N natural abundance and acetylene (C_2H_2) reduction assay.

1.4.1 ^{15}N natural abundance

There are two main stable nitrogen isotopes; ^{14}N makes up vast majority of naturally occurring nitrogen, and ^{15}N occurs at 0.3663 atom% in atmospheric N_2 (Mariotti, 1983). Based

on the essence that the ^{15}N concentration in plant-available soil N differs significantly with ^{15}N concentration in atmospheric N_2, and it will be able to possible to quantify nitrogen fixation, if the ^{15}N concentrations from both resources are known. ^{15}N from atmosphere N_2 occurs at a constant value 0.3663 atom%, and ^{15}N uptake by plant from soil could be estimated by planting a non-N_2 fixing plant.

1.4.2 Acetylene reduction assay

Atmospheric nitrogen is converted to NH_3 by the enzyme called nitrogenase, which is also capable of reducing acetylene to ethylene. Both acetylene and ethylene could be rapidly detected and quantified using gas chromatography (Schollhorn and Burris, 1967; Hardy et al., 1968, 1973). Based on this fact, the nitrogenase activity, which representing nitrogen fixation activity, could be quantitatively measured at the certain time point for pot studies.

1.5 Plant physiological responses to drought

1.5.1 Root signaling under drought stress

Under drought stress, plant root system would induce signals to the shoots via xylem causing a serial of physiological changes which would eventually determine the level of adaptation to drought. Abscisic acid (ABA), cytokinins, ethylene, malate and other unidentified factors have been proved to involve in root-shoot signaling, where ABA is considered as an essential factor in controlling plant growth and transpiration (Long et al., 2019). ABA promotes the efflux of K^- ions from the guards cells, which leads to the loss of turgor pressure and further leads to stomata closure. In addition, dehydration of plants has been shown to increase ABA level up to 50-fold due to loss of cell turgor (Guerrero and Mullet, 1986). In addition to ABA, root produced cytokinins traveled from roots to the shoots and involved in response to nutrient deprivation which is important in drought adaption (Schachtman and Shin, 2007).

1.5.2 Photosynthesis and chlorophyll contents

Drought stress has a direct impact on the photosynthetic apparatus, essentially by disrupting all major components of photosynthesis including the thylakoid electron transport, the carbon reduction cycle and the stomatal control of CO_2 supply, together with an increased accumulation of carbohydrates, peroxidative destruction of lipids and disturbance of water balance (Allen and Ort, 2001). As one of the major chloroplast components for photosynthesis, the relative chlorophyll content has a positive relationship with photosynthetic rate. The decrease in chlorophyll content under drought stress has been considered a typical symptom of chlorophyll degradation.

1.5.3 Water relations

Relative water content (RWC), leaf water potential, stomatal resistance, rate of transpiration, leaf temperature and canopy temperature are important characteristics that influence plant water relations. RWC is usually considered as a measure of plant water status, reflecting the metabolic activity in plant tissues and considered as one of the most important indices for drought tolerance. It has been well noted that RWC relates to water uptake as well as water loss by transpiration. In response to drought stress, RWC, water potential and transpiration rate substantially decreased in leaves (Nayyar and Gupta, 2006; Siddique et al., 2001).

1.5.4 Osmolyte accumulation

Under drought stress, plants accumulate different types of organic and inorganic solutes in the cytosol to lower osmotic potential in order to maintain cell turgor (Anjum et al., 2011a). In order to maintain leaf turgor under drought, plants made osmotic adjustment by accumulation of proline, sucrose, soluble carbohydrates, glycinebetaine, and other solutes in cytoplasm improving water uptake from drying soil. Of these solutes, proline is the most widely studied due to the

essential role in stress tolerance. Proline accumulation is the first response of plants exposed to water-deficit stress in order to reduce injury to cells (Anjum et al., 2011b). In addition, proline also act as signaling molecule for modulating mitochondrial functions, influencing cell proliferation and triggering specific gene expression, which is essential for plant recovery (Szabados and Savoure, 2009). Besides the functions that have been discussed above, it also influences protein solvation, preserves the quaternary structure of complex proteins, maintains membrane integrity, reduces oxidation of lipid membrane, contributes free radicals scavenging (Ashraf and Foolad, 2007; Demiral and Turkan, 2004).

1.6 RNA sequencing (RNA-Seq) as a tool for breeding elite drought-tolerant varieties in peanut

1.6.1 Gene expressions in plants respond to drought

The multigenic, incompletely penetrant, quantitative nature of drought tolerance makes it difficult for breeding purposes. In response to water deficit, plants have developed several adaptive strategies, which display different degrees of tolerance determined by their genetic plasticity. One of the major molecular response to drought stress is alteration of gene expression that are related to different pathways with stress perception, signal transduction, regulators and synthesis of a number of compounds (Ramanjulu and Bartels 2002; Sreenivasulu et al. 2007). In the model plant, *Arabidopsis*, a large number of genes that are induced by drought stress at transcriptional level have been identified and characterized by microarray technology and other means (Seki et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007).

The resurrection plants have better capabilities to cope with severe drought conditions; hence, several studies have been conducted to discover what key genes are involved in enabling these plants to survive desiccation. The molecular aspects of desiccation tolerance in resurrection

plants such as *Craterostigma plantagineum* (Bartels et al. 1990; Bartels and Salamini 2001; Phillips et al. 2002; Bartels 2005), *Xerophyta viscosa* (Mundree et al. 2000; Mowla et al. 2002; Dahlia et al. 2003), *X. humilis* (Collett et al. 2003), *Sporobolus stapWanus* (Neale et al. 2000) and *Selaginella lepidophylla* (Iturriaga et al. 2000) reveal complex mechanisms of desiccation tolerance (Bernacchia and Furini, 2004). Although some mechanisms of stress response are common to all cells, there exist major differences in strategies adopted by plants to cope with desiccation stress (Ramanjulu and Bartels 2002; Smith-Espinoza et al. 2003). Realizing this, it would be more rewarding to explore crop species with higher levels of stress tolerance at molecular level. Evidences support the fact that stress responsive genes from tolerant species provide better protection to cellular structures due to existence of genes that code for structurally and/or functionally efficient stress proteins associated with stress adaptation (Waditee et al. 2002; Majee et al. 2004; Dastidar et al. 2006). To identify stress specific genes, it would be more rewarding to isolate the differentially expressed genes providing a clear picture of the transcriptome under stress from relatively drought tolerant crop.

1.6.2 Peanut as a model organism to study transcriptomic response to drought

Peanut improvement is still facing great challenge due to the low genetic diversity among different cultivated genotypes and limited genomic information for peanut. With the limited genomic information and genetic diversity for peanut, only two traits (nematode resistance and high oleic acid) have been improved in peanut by using molecular breeding. These two traits are relatively simple inherited in peanut with two dominant genes controlling the nematode resistance and two recessive genes controlling oleic fatty acid (Lopez et al., 2001). However, many considerable traits that interest breeders are complex and controlled quantitatively, e.g.

disease resistance, drought tolerance, and yield, and these traits usually have a substantial genotype × environment interaction, which produced numerous obstacles in peanut breeding.

With increased population growth and demand for peanut crops, breeders are under constant pressure to deliver high-performing varieties. Genetics and genomics tools have a great potential to enhance food production and food safety. The application of genetics tools for breeding has been shown to significantly increase the rate of genetic gain when compared with conventional breeding. More recently, the advent of next-generation sequencing (NGS) is revolutionizing molecular breeding through both genomic selection approaches (Collard and Mackill, 2008; Gupta et al., 2008) and single nucleotide polymorphisms (SNPs) identification, which can be further converted into marker-assisted selection (MAS) assays (Allen et al., 2013). NGS of steady state RNA (RNA-seq) gives unprecedented detail about the RNA landscape within a cell, which premise to unravel previously inaccessible complexities in the transcriptome. Not only expression levels of genes can be interrogated without specific prior knowledge, but comparisons of expression levels between genes within a sample can be made. It has also been demonstrated that splicing variants and SNPs can be detected through sequencing the transcriptome, which opens the opportunity to interrogate allele-specific expression and RNA editing. Thus, RNA-seq promises a great potential to breed elite peanut varieties and become more and more popular among plant breeders.

1.6.3 RNA-Seq technology and its advantages

Various technologies have been developed to deduce and quantify the transcriptome, including hybridization- or sequence-based approaches. Hybridization-based approaches typically involve incubating fluorescently labelled cDNA with custom-made microarrays or commercial high-density oligo microarrays. These hybridization-based approaches are high

throughput and relatively inexpensive. However, these methods have several limitations including reliance upon existing knowledge on genome sequence, high background levels owing to cross-hybridization, and a limited dynamic range of detection owing to both background and saturation of signals (Okoniewski and Miller, 2006; Royce et al., 2007). Comparing expression levels across different experiments is often difficult and can require complicated normalization methods. In contrast to microarray methods, sequence-based approaches directly determine the cDNA sequence. At beginning, Sanger sequencing of cDNA or EST libraries (Gerhard et al., 2004) was used, but this approach is relatively low throughput, expensive and generally not quantitative. Tag-based methods were developed to overcome these limitations, including serial analysis of gene expression (SAGE) (Velculescu et al., 1995; Harbers and Carninci, 2005), cap analysis of gene expression (CAGE) (Kodzius et al., 2006; Nakamura and Carninci, 2004; Shiraki et al., 2003) and massively parallel signature sequencing (MPSS) (Brenner et al., 2000; Peiffer et al., 2008 and Reinartz et al., 2002). These tag-based sequencing approaches are high throughput and can provide precise and 'digital' gene expression levels. However, most are based on expensive Sanger sequencing technology, and a significant portion of the short tags cannot be uniquely mapped to the reference genome. Additionally, only a portion of the transcript is analyzed, and isoforms are generally indistinguishable from each other.

As one of the sequence-based approaches, RNA-seq was recently developed to use deep-sequencing technologies for both mapping and quantifying transcriptome profiles. This method offers several key advantages over existing approaches. First, unlike hybridization-based approaches, RNA-Seq is not limited to detecting transcripts that correspond to existing genomic sequence. This makes RNA-Seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined. The second advantage of RNA-Seq relative to DNA

microarrays is that RNA-Seq has very low background signal since DNA sequences can be unambiguously mapped to unique regions of the genome. It provides a far more precise measurement of levels of transcripts and their isoforms than other methods. Nowadays, studies using RNA-Seq have already expanded our view of the extent and complexity of eukaryotic transcriptomes.

1.6.4 General RNA-Seq work flow

Step one: *cDNA library preparation*

The quality and integrity of starting RNA can influence the success of an RNA-Seq experiment. To assess RNA quality, methods such as ultraviolet (UV) absorbance, fluorescent dye-based quantification, agarose and acrylamide gel electrophoresis, the 2100 Bioanalyzer (Agilent Technologies) that calculates an RNA integrity number (RIN) with a value in the range 1-10 (fully degraded RNA-intact RNA) can be used (Schroeder et al., 2006). RNA-Seq protocols typically require 100 ng to 4 μg of the total RNA with a RIN value of at least 7. Subsequently, mRNA is selected, fragmented, and reverse transcribed to generate the cDNA library.

Step two: *next-generation sequencing*

The Illumina HiSeq platform is currently the most common applied next-generation sequencing technology for RNA-Seq. The Illumina HiSeq platform follows a standard sequencing protocol. Based on specific research questions and the organism under study, modifications can be made to the standard RNA-Seq protocol. For example, when profiling gene expression of relatively nonrepetitive genomes, the most suitable option is to sequence 50 bp from one end of the cDNA fragments (single end). When studying alternative splicing of mRNA, the sequence information per gene should be increased, which is achieved by sequencing 50 or 100 bp from both ends of the cDNA fragments (paired end). Moreover, expression and/or splice

variant analysis of large or highly repetitive genomes requires paired-end sequencing reads of 100 or 150 bp to accurately map the reads to the genome.

Step three: *alignment of RNA-Seq reads*

To perform a complete RNA-Seq analysis from reads to differential gene expression, a UNIX-like operating system such as Mac OS or Linux is recommended. After obtaining the RNA-Seq reads, their quality should be evaluated with a tool such as FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), which flags any potential abnormalities that may have occurred during library preparation or the sequencing reaction. Subsequently, reads are aligned to a reference genome or transcriptome. For alignment to transcriptome, software tools such as Bowtie (Langmead et al., 2009) and BWA (Li et al., 2009) are commonly used, whereas the splice aligner TopHat (Trapnell et al., 2009) is a popular choice for mapping reads to an annotated reference genome.

Step four: quantification of gene expression

Following alignment of RNA-Seq reads, the data need to be translated into a quantitative measure of gene expression, which can be achieved by counting the number of reads that map to each gene. Reads that are aligned to annotated transcripts comprising the transcriptome can be summarized relatively easily. Reads that are aligned to annotated coding regions of the genome can be summarized using tools such as those available in the HTSeq. Estimating the expression levels of individual splice variants of a gene is more complex, as they typically share a set of exons, and thus only a minor fraction of the reads will align uniquely to the distinct regions of a particular splice variant. Methods such as Cufflinks and MiSO use statistical models to estimate the proportion of reads which can be assigned to individual splice variants (Trapnell et al., 2010; Katz et al., 2010).

Step five: *normalization of RNA-Seq count data*

Following quantification of expression levels, a common objective is to identify genes that are differentially expressed between different conditions. To enable an accurate comparison of expression levels between different samples, the count data must first be normalized. Two important types of sequencing bias need to be considered when normalizing count data: within-sample bias, which is primarily caused by differences in transcript length, and between sample bias, which results mainly from differences in sequencing depth. One of the most widely used normalization methods is RPKM/FPKM (reads or paired-end fragments per kilobase of exon model per million mapped reads), which adjusts raw counts to the total gene length and the number of reads mapped within a sample, thereby simultaneously performing between- and within-sample normalization. When comparing the expression of the same gene between different samples, only between-sample bias should be considered. Consequently, normalization methods that are more robust to extreme values, such as the median count ratio (Anders and Huber, 2010) and trimmed mean of M-values (TMM) (Robinson et al., 2010), have been developed.

Step six: *determination of genes with differential expression*

Many statistical software has been developed to determine differential gene expression between samples. Bioconductor packages such as DESeq, edgeR, and baySeq that model count data using a negative binomial distribution are recommended (Anders and Huber, 2010; Hardcastle and Kelly, 2010; Robinson et al., 2010). Other packages or platforms such as MiSO, DEXSeq, and CuffDiff can handle testing for differences in alternative splicing between two samples (Andres et al., 2012; Katz et al., 2010; Trapnell et al., 2010).

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Chapter Two

Genotypic variability in symbiotic nitrogen fixation and carbon isotope discrimination among peanut (*Arachis hypogea* L.) genotypes under drought stress

2.1 Abstract

Drought stress is one of the major environmental factors limiting peanut productivity. The vulnerability of peanut to drought varies depending on genotypic characteristics, crop growth stages, and environmental conditions. The objective of this study was to evaluate the effects of drought stress on symbiotic nitrogen fixation and carbon isotope discrimination in various peanut genotypes. Two parental lines (Tifrunner and C76-16) and 14 recombinant inbred lines with varying drought tolerance characteristics were evaluated in rainout shelters under three irrigation regimes (irrigated control, middle-season drought with rehydration, and late-season drought with rehydration) using a split-plot design with a randomized complete block design within. Symbiotic nitrogen fixation capacity and carbon isotope discrimination in different genotypes were evaluated by measuring ^{15}N and ^{13}C natural abundance, respectively, for two years. Reduction in percentages of shoot N derived from the atmosphere (%Ndfa) was observed under two drought treatments in both years; a greater reduction was observed under middle-season drought than under late-season drought. Middle-season drought negatively affected carbon isotope discrimination in both years although no difference was observed under late-season drought. Variabilities in %Ndfa and carbon isotope discrimination were found among different genotypes. Under middle-season drought, both %Ndfa and carbon isotope discrimination were higher in drought tolerant lines than drought susceptible lines, and the most drought tolerant lines (AU-582, AU-586, and AU-587) had the highest N-fixing capacity under

both drought treatments in the study periods. Additionally, there was a positive correlation between %Ndfa and carbon isotope discrimination under both drought treatments. In most genotypes, %Ndfa remained unchanged after rehydration. Only a few genotypes showed a slight increase in %Ndfa after rehydration following mid-season or late-season drought. However, no consistent pattern was observed in either year. Our data suggested that unlike other traits, symbiotic nitrogen fixation in many peanut genotypes may not recover from the damage caused by mid- or late-season drought upon rehydration.

2.2 Introduction

Peanut (*Arachis hypogaea* L.) is one of the most important food legumes grown mainly in arid and semi-arid regions where drought is one of the major environmental factors limiting productivity (Akram et al., 2018; Balota et al., 2012; Songsri et al., 2008; Devi et al., 2017; Dinh et al., 2013; Pimratch et al., 2008a; Pimratch et al., 2009). The vulnerability of peanut to drought depends on crop growth stages and genotypic variability (Carter, 2015; de Lima Pereira et al., 2016; Devi et al., 2010, 2017; Dinh et al., 2013; Greenberg et al., 1992; Puangbut et al., 2009; Stansell et al., 1985). Previous studies showed that drought stress severely reduced pod yield in peanut during mid-growing season (i.e., flowering to pod development) when water requirement is the highest for growth, and moderately limited peanut yield at late-growing season (i.e., pod development to maturation) (de Lima Pereira et al., 2016; Patel and Golakya, 1988; Junjittakarn et al., 2016; Stansell et al., 1985; Stirling et al., 1989). Genotypic variations in several physiological characteristics, including water use efficiency (WUE), carbon isotope discrimination (Δ), specific leaf area (SLA) etc., associated with drought tolerance have been identified, offering great opportunities to breed high-yielding drought tolerant genotypes,

especially under middle-to-late season drought (Balota et al., 2012; de Lima Pereira et al., 2016; Pimratch et al., 2008a, 2008b).

As a leguminous plant, peanut forms symbiotic relationship with rhizobia resulting in fixation of atmospheric N_2, thus reducing or eliminating the necessity of N fertilization during growth. However, the symbiotic N_2 fixation (SNF) is sensitive to soil drying, thereby substantially compromising the amount of N acquired during reproductive stages of peanut (Devi et al., 2010; Sinclair et al., 1995; Pimratch *et al.,* 2008a, 2008b). Variations of SNF were reported among different peanut genotypes under drought stress (Devi et al., 2010, 2013; Furlan et al., 2012; Sinclair et al., 1995; Pimratch *et al.,* 2008a, 2008b), and a positive relationship between SNF and peanut pod yield was demonstrated (Pimratch *et al*., 2008a, 2009) indicating that peanut genotypes with high SNF capacity under drought stress are likely to be drought-tolerant (Dinh et al., 2013; Pimratch et al., 2004; Pimratch et al., 2008a, 2008b). Therefore, identification of high-yielding peanut genotypes with superior SNF ability under drought stress can be economically- and environmentally-beneficial; however, the information on responses of peanut genotypes to drought stress at different growth stages for N_2-fixation is lacking.

At the plant level, water use efficiency (WUE) is the ratio of biomass produced to the water transpired, which can be assessed through carbon isotope discrimination (Δ) (Farquhar and Richards, 1984). Under drought stress, Δ is usually negatively correlated with WUE, an important correlation to help to improve crop productivity in drought studies (Condon et al., 2002; Hall et al., 1994). However, breeding selection through WUE appeared to make slow progress or be unsuccessful in peanut due to the large genotype × environment (G × E) interactions (Arunyanark et al., 2008; Jongrungklang et al., 2011; Blum et al., 2005, 2009, and 2011). Under drought stress, the increased WUE mainly depends on reduced transpiration rate or

crop water use (Blum, 2009; Sinclair, 2012). However, the reduction is crucial for carbon assimilation, biomass production, and plant yield potential (Blum, 2005). In contrast, previous studies reported high yield associated with low WUE under limited water conditions in various crops including wheat (Monneveux et al., 2005), robusta coffee (Pinheiro et al., 2005), maize (Monneveux et al., 2007), and black bean (Polania et al., 2016a, 2016b). Due to the inconsistent results observed previously and the complexity of WUE and SNF under drought stress, a better understanding of the existing genetic variability in yield, SNF, and WUE, and the magnitude of the correlation among these characteristics are important for improving selection strategies to increase SNF and peanut yield under drought stress. Thus, the objective of the present study was to evaluate drought and rehydration effects on selected peanut genotypes in terms of SNF capacity and Δ under either middle-season or late-season drought to identify elite peanut genotypes possessing both high yield potential and SNF capacity.

2.3 Materials and Methods

2.3.1 Plant materials

Sixteen peanut genotypes with varying drought tolerant characteristics were selected based on previous yield trials under drought stress in 2013 and 2014 (data not shown). Tifrunner (drought susceptible), C76-16 (drought tolerant) and 14 recombinant inbred lines (RILs) derived from the cross 'Tifrunner x C76-16' were planted in three rainout shelters to create artificial drought conditions at the USDA-ARS National Peanut Research Laboratory in Dawson, Georgia. Of the 14 RILs, seven (AU-587, AU-586, AU-582, AU-580, AU-543, AU-539, AU-431) were identified as drought tolerant genotypes, and the other seven (AU-592, AU-517, AU-506, AU-499, AU-491, AU-483, AU-459,) as drought susceptible genotypes. Two non-N_2 fixing

reference plants were planted along with the 16 peanut genotypes; they were marigold and weeds in 2015, and marigold and mint in 2016.

2.3.2 Rainout shelter experiment

A split plot design with a randomized complete block design within was used to examine drought effects and genotypic variations. Three rainout shelters (5.5 m × 12.2 m each) were designated as three drought treatments: full irrigation, middle-season drought, and late-season drought. Each rainout shelter was equipped with sensors to close at the first drop of rain and a controlled irrigation system under each shelter (Blankenship et al., 1983).

Each shelter comprised three blocks; the 16 peanut genotypes and two reference plants were randomly distributed within each block (three blocks per shelter) as sub-plot treatments. Each genotype was planted in a single-row (15 x 120 cm) at a rate of 10 seeds m^{-1}. All three shelters were irrigated after sowing to provide uniform germination. The shelter for the full irrigation treatment was irrigated throughout the growing season based on evapotranspiration (ET) replacement for peanut as described by Stansell et al. (1976), and irrigations were triggered automatically when the average measurement of two sensors (5 cm and 20 cm) was below -60 kPa. The drought treatments consisted of a complete irrigation period followed by an irrigation withholding period. For middle-season drought in both 2015 and 2016, water withholding was initiated at 61 days after planting (DAP); for late-season drought, water withholding occurred 103 DAP in 2015 and 90 DAP in 2016. The irrigation was withheld consecutively for four weeks for each drought treatment. Irrigation was resumed at the end of each drought treatment and plant recovery was measured two weeks after irrigation resumed.

The experimental plots were 1.8 m deep and filled with Tifton sandy loam soil. Watermark moisture sensors (Irrometer, Riverside, CA) were placed at four depths (5 cm, 20 cm,

40 cm, and 60 cm) to monitor soil moisture and temperature in peanut geocarposphere. Soil water status and temperature were collected at 30 min intervals throughout the growing season. All treatments were subjected to agronomic management practices according to the University of Georgia best management practices for peanut.

2.3.3 Visual rating of drought stress

At the end of each drought period, the apparent drought tolerance of each genotype was assessed through visual ratings for both middle-season and late-season drought treatments. Ratings were based on a 10-point scale: 1 = not wilted, 2 = 20% wilted, 3 = 30% wilted, 4 = 40% wilted, 5 = 50% wilted, 6 = 60% wilted, 7 = 70% wilted, 8 = 80% wilted, 9 = 90% wilted, and 10 = 100% wilted.

2.3.4 Specific leaf area (SLA)

SLA was determined using the method describe by Dang et al. (2012). In order to measure SLA, leaf samples were collected once a week for four consecutive weeks during the drought period, and the samples were also obtained during the recovery stage after irrigation resumed. For each sampling time, fully expanded leaves (second nodal) were collected from two randomly selected plants in each genotype. Fresh collected leaves were immediately placed into plastic bags and put on ice until collection was completed. Immediately following complete collection of all samples, each leaf was placed into an individual Petri dish, fully submerged in deionized water, and placed under white light for 2 hours to ensure tissues were completed turgid. Afterwards, leaves were blotted dry and leaf area (LA) was immediately measured using a LI-3100 area meter (LI-COR Biosciences, Lincoln, NE, USA). Leaves were then placed in a 65 °C oven for 2 days to ensure complete dryness and subsequently weighed to obtain the leaf dry

mass (DW). Finally, SLA was calculated as the ratio of leaf area to leaf dry mass (LA/DW) for each leaf measured.

2.3.5 Shoot biomass and ^{15}N and ^{13}C natural abundance

One single plant was randomly harvested from each sub-plot. The selected plant was cut at the soil surface, washed with tap water, and dried at 60 °C for 72 hours. Shoot biomass of each plant was determined after oven drying. Entire dried shoot samples were first ground using a cyclone mill with 2 mm screen; representative subsamples were ground again using another cyclone mill with 1 mm sifter. Four mg of each resulted sample were weighed into a tin capsule and sent to the Staple Isotope Facility of the University of California, Davis, California to measure ^{15}N and ^{13}C natural abundance using an elemental analyzer interfaced to isotope ratio mass spectrometer.

The percentage of nitrogen derived from atmospheric nitrogen (%Ndfa) was determined using ^{15}N natural abundance method (Unkovich et al., 2008), and is expressed as:

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N non fixing reference plant} - \delta^{15}\text{N of } N_2 \text{ fixing legume}}{\delta^{15}\text{N non fixing reference plant} - B} \times \frac{100}{1},$$

where 'B' is the $\delta^{15}N$ of peanut shoots determined when plants were fully dependent upon N_2 fixation (Unkovich et al., 2008). In this study, the 'B' value used for peanut shoots was -1.536‰, which is an average value from several studies (Unkovich et al., 2008).

Carbon isotope discrimination was calculated by the following formula to assess WUE (Farquhar et al., 1989):

$$\Delta = \frac{\delta^{13}C_{atmosphere} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}}$$

where $\delta^{13}C_{atmosphere}$ = -8‰.

2.3.6 Statistical analysis

All statistical analyses were performed at a significant level of $\alpha = 0.05$, using SAS 9.4 (SAS Institute, Cary, NC). The homogeneity of variance assumption for all responses was first examined using Levene's test (PROC GLM). Then, the dataset was subjected to an analysis of variance (ANOVA; PROC MIXED) with a split plot design coupled with randomized complete block design. A comparison of different genotypes was also conducted using Tukey's honest significant difference (Tukey's HSD). In addition, Pearson's correlation (PROC CORR) was applied to the dataset for examining the correlations among different parameters.

2.4 Results

2.4.1 Shoot biomass, visual rating and specific leaf area

Effects of drought stress on shoot biomass did not show a consistent pattern (Data not shown). A large genotypic variability was found on shoot biomass in both growing seasons, and the effects of drought stress on biomass were either positive or negative. The visual rating did not appear to correlate with yield (Data not shown). Some of the drought tolerant lines had higher visual stress rating than drought susceptible lines. An overall reduction of SLA was observed in 2016 under middle-season drought ($P < 0.0001$), and the drought treatment significantly reduced SLA in drought susceptible lines of AU-483, AU-491, AU-499, and AU-592. There was no treatment difference in SLA measured between irrigated treatment and middle-season drought in 2015 (Fig. 2.1). Genotypic variability among different genotypes was observed; however, no significant differences between drought tolerant genotypes and drought susceptible genotypes were detected (Fig. 2.2).

2.4.2 Proportion of nitrogen derived from SNF

Overall, middle-season drought caused substantial reductions in %Ndfa ($P < 0.0001$), and the average reduction was 23.9% and 14.2% in 2015 and 2016, respectively, compared with the

irrigated controls. The responses of some peanut genotypes to middle-season drought did not change significantly (Fig. 2.3). For example, genotypes AU-543, AU-580, and AU-592 had similar %Ndfa levels when compared with the corresponding irrigated control in 2015 (Fig. 2.3a), and the %Ndfa levels of genotypes AU-491, AU-506, AU-517, AU-543, AU-580, AU-582, AU-586, and AU-587 did not show significant differences between treatments in 2016 (Fig. 2.3b). Of these genotypes, most were identified as drought tolerant lines. Similarity in %Ndfa response to late season drought was also found in both years with an overall significantly reduced %Ndfa levels ($P < 0.0001$; Fig. 2.3c and d). Interestingly, the number of genotypes with similar %Ndfa levels between irrigation and drought treatments under late-season drought was higher when compared with middle-season drought, and the overall level of %Ndfa was also higher when plants were subjected to late-season drought than middle-season drought.

Genotypic variability of %Ndfa was observed under middle-season drought in both years. As presented in Fig. 2.4a and 2.4b, drought-tolerant genotypes had higher %Ndfa than drought-susceptible ones. In 2015, the overall difference of the mean %Ndfa values between tolerant lines and susceptible lines was 5.59 ($P = 0.0218$), and the overall difference of mean %Ndfa values between tolerant and susceptible lines was 6.96 ($P = 0.006$) in 2016. Of the examined genotypes, some (i.e., AU-582, AU-586, and AU-587) had higher %Ndfa under mid-season drought across the years. There was also genotypic variation of %Ndfa in response to late-season drought (Fig. 2.4c and 2.4d). Of the examined genotypes, AU-587 displayed the highest level of %Ndfa under late-season drought in both years. Although there were differences among genotypes, no differences were found when grouping individual genotypes into drought tolerant lines and drought susceptible lines (Fig. 2.4c and 2.4d).

Effects of rehydration on SNF is displayed in Figure 2.5. In both 2015 and 2016, %Ndfa in most genotypes remained unchanged after rehydration. Only a few genotypes showed a slight increase in %Ndfa after rehydration. AU-506 recovered from middle season drought in 2015. AU-431, AU-459, C76-16 and Tifrunner recovered from middle season drought in 2016. In addition, %Ndfa for AU-517 significantly increased after late-season rehydration in 2015 (Fig. 2.5c). C76-16, Tifrunner and AU-539 recovered from late-season drought in 2016 (Fig. 2.5d).

2.4.3 Carbon isotope discrimination

Carbon isotope discrimination of six genotypes (AU-431, AU-459, AU-491, AU-499, AU-582 and AU-586) were reduced when middle-season drought occurred on 2015; however, nearly all genotypes were reduced on 2016 (Fig. 2.6a and 2.6b). The average differences of mean Δ between control and mid-season drought were 0.52 for 2015 (P_{2015} = 0.0002) and 0.93 for 2016 (P_{2016} = 0.0013). However, there were no impact of late-season drought on Δ when compared with the irrigated controls (Fig. 2.5c and 2.5d), although a few genotypes (e.g. Tifrunner, AU-491, AU-539, AU-543 and AU-586) had lower Δ in response to late-season drought. Notably, the drought tolerant line AU-582 was the only genotype being measured with a much higher value in 2016 for late-season drought treatment (Fig. 2.6d).

Among the examined genotypes, AU-587 in the 2015 trial and Tifrunner as well as AU-459 in the 2016 trial had the highest Δ, while AU-491 showed the lowest Δ in both years (Fig. 2.7). Although some drought susceptible genotypes were identified with high Δ after mid-season drought, the drought tolerant lines still displayed an overall higher Δ than drought susceptible lines under middle-season drought (P_{2015} = 0.0024; P_{2016} = 0.0449). Unlike middle-season drought, the responses of Δ between drought tolerant and susceptible lines to late-season drought were not consistent across years. Drought tolerant lines had significantly higher Δ values than

drought susceptible lines in 2015 ($P = 0.0002$), but not in 2016. The Δ was the highest for drought tolerant line AU-582 in both years, and the lowest numerically for drought susceptible line AU-491 in 2016 under late-season drought.

2.4.4 Correlation between %Ndfa, Δ and SLA

Correlations between %Ndfa and Δ were evaluated for both middle-season and late-season drought in 2015 and 2016 (Fig. 2.8, Table 2.1). Under middle-season drought, positive correlations ($r_{2015} = 0.42$, $P_{2015} = 0.0001$; $r_{2016} = 0.56$, $P_{2016} < 0.0001$) were found between %Ndfa and Δ in both years, but no correlation exhibited under late-season drought (data not shown). Weak positive correlations were also observed between SLA and %Ndfa ($r_{2016} = 0.20$, $P_{2016} = 0.0542$), and between SLA and Δ ($r_{2016} = 0.17$, $P_{2016} = 0.0887$) in 2016. There were no correlations between visual stress rating, shoot biomass, and %Ndfa or Δ.

2.5 Discussion

In this study, we determined effects of middle-season or late-season drought on selected peanut genotypes in the rainout shelters. The effect of drought stress on shoot biomass production did not show a consistent pattern among different genotypes in either year. In contrast, a study conducted in Thailand showed significant reductions in peanut shoot biomass under drought stress, and reductions were dependent on the severity of the stress (Pimratch et al., 2008a). This is likely caused by different peanut genotypes used and differences in environmental conditions in the two studies.

Reductions in SNF in selected peanut genotypes due to drought stress were found in the present study. Similarly, SNF reductions in different peanut genotypes and genetic variations in SNF determined by acetylene reduction were demonstrated under middle-season drought in a greenhouse study (Sinclair et al., 1995). Different levels of reductions were also reported in SNF

when middle-season drought occurred with different soil water availability. For example, a greater reduction was found at 1/3 field capacity, and less reduction was reported at 2/3 field capacity (Pimratch 2008a & 2009) indicating the level of the reduction corresponded to the severity of drought stress. In the parallel study by Pimratch (2008b), they found the reduction in nitrogenase activity was increased with increasing drought stress. In addition, drought stress also reduced nodule number and nodule dry weight and the reductions for these traits at severe drought stress were about two times the reductions under mild drought stress. This may explain the why drought stress reduced SNF and the level of the reduction corresponded to the severity of drought stress. Limited information on late-season drought stress affecting SNF and its related traits in peanut exist in the literature. Shimshi et al. (1967) demonstrated different irrigation frequencies (7, 14 and 21 days) at the end of the growing season had no impact on nodule number and weight in a soil with high organic matter content, indicating that SNF rate was likely to remain unchanged. However, a significant overall reduction for SNF under late-season drought was found in our study. In addition, a smaller reduction was observed under late-season drought than it was assessed under middle-season drought, indicating that there might be less stress imposed by the late-season drought because of the lower soil temperatures and higher soil moisture contents later in the season for both years (Fig. 2.9).

Genotypic variability in SNF reductions under drought stress was found in the present study, and it was supported by studies that nitrogenase activity of drought susceptible genotypes showed an earlier decrease than tolerant genotypes when the fraction of transpirable soil water was reduced under an early middle-season drought (Devi et al., 2010). Genotypic variability for SNF was also reported when peanuts were subjected to drought stress (Pimratch et al., 2008a and

2009). Therefore, those observations give strong support to the conclusion that drought stress significantly reduces SNF to different degrees depending on the genotypic characteristics.

Moreover, the drought tolerant lines used in this study were selected based on our previous yield study under drought stress, and they exhibited higher SNF capability under drought stress than the drought susceptible lines, especially under the mid-season drought. Therefore, it is possible that drought tolerant genotypes with high yield potential are more likely to maintain high SNF capabilities when middle-season drought occurred. However, no significant difference was found between drought tolerant lines and susceptible lines under late-season drought. It is likely that late-season drought imposed less stress than middle-season drought due to a higher level of soil water content later in the season (Fig. 2.9), or the drought stress was not enough to pose the difference between drought tolerant lines and drought susceptible lines. In addition, the ability of genotypes to have higher SNF under mild drought stress was largely due to their high SNF under well-watered conditions and partly dependent on their ability to maintain high SNF under mild water stress, whereas the ability of genotypes to have higher SNF under severe drought stress was dependent primarily on their ability to maintain high SNF. Although no overall difference was revealed between drought tolerant lines and susceptible lines, genotype AU-587, which is the best drought tolerant line identified from previous yield trials (Data not shown) exhibited highest SNF capacity in both drought treatments and in both years.

WUE is a parameter that has been widely considered in crop breeding programs for selection of drought tolerant traits, and it has been extensively studied under different conditions. The WUE could be estimated by Δ because there is a strong negative correlation between WUE and Δ in peanut (Hubick et al., 1986; Craufurd et al., 1999; Wright and Hubick, 1988; Wright et

al., 1994). In this study, we found that Δ significantly decreased under middle-season drought compared with the irrigation treatment, indicating that WUE increased. This result is consistent with previous studies (Wright et al., 1993; Craufurd et al., 1999), and the reduced transpiration rate and partial stomatal closure of peanut plants under severe water deficit conditions should be the physiological basis of the reduction in Δ (Wright et al., 1993; Brown and Byrd, 1997). In contrast, we did not observe significant reductions of WUE under late-season drought, and this might be due to the lower temperature lead to lower transpiration rate in the late-season in both 2015 and 2016 (Fig. 2.9). Additionally, a higher soil temperature and lower precipitation in 2016 accounted for the observation that more genotypes showed differences on carbon isotope discrimination between middle-season drought and irrigation treatment in 2016 than these in 2015. WUE is also considered an important determinant of plant yield under stress; however, the results on their relationship examined in various crops were not consistent when water deficit conditions varied (Hall et al., 1994; Matus et al., 1996; Monneveux et al., 2007; Morgan et al., 1993; Munoz et al., 1998; Ngugi et al., 1994 & 1996; Read et al., 1991; Saranga et al., 2004; Sayre et al., 1995; Specht et al., 2001 and Chen et al., 2013). For example, positive correlations between Δ and grain yield in wheat without irrigation or under postanthesis water stress indicated there was a negative relationship between WUE and grain yield (Morgan et al., 1993; Monneveux et al., 2005), while another study by Monneveux et al. (2007) reported an increased Δ in maize under drought stress but no correlation was found between Δ and grain yield. Monneveux et al. (2007) also reported a higher Δ in maize drought tolerant lines than in drought susceptible lines, which was in support of our findings that peanut drought tolerant lines also had a higher Δ (i.e., lower WUE). In pervious study in peanut by Chen et al. (2013), negative genetic correlations were found between $\delta^{13}C$ and two other traits (harvest index (HI) and yield) at both

irrigated and non-irrigated conditions. The correlation coefficients ranged from -0.22 (*P<0.01*) under irrigated conditions to -0.37 (*P<0.01*) under non-irrigated conditions. The correlation coefficients ranged from -0.44 in irrigated conditions to -0.98 in non-irrigated conditions. Due to the positive correlation between $\delta^{13}C$ and WUE, therefore, a negative correlation between WUE and HI and yield exist in peanut especially under drought conditions. Taken together, we hypothesize that plants with high WUE under drought conditions are not likely to be associated with high yield potential, and high WUE may even lead to yield reduction.

At the plant level, WUE is the ratio of biomass produced to the water transpired, and the change of WUE is largely dependent on the changing of the water transpired. Therefore, the greater WUE under drought stress should mainly depend on the traits that reducing transpiration and crop water-use. Nevertheless, the water usage is crucial for physiological metabolism, plant production and yield, thus it is not reliable to select drought tolerant genotypes only considering high WUE. Instead of WUE, another parameter, effective use of water which maximizing soil moisture capture towards stomatal transpiration was suggested to be a better indicator for enhancing plant yield production under drought stress (Blum, 2009). However, the genotypic variability of Δ are still valuable and it indicates the enhancement potential of WUE in drought study if we have a better understanding the relationship between WUE and yield.

After rehydration, %Ndfa in most genotypes remained unchanged in both 2015 and 2016, which indicated drought significantly reduced %Ndfa and the adverse effects are unrecoverable in most genotypes. In addition, peanuts under late-season drought recovered easier than under mid-season drought, since more genotypes were recovered from late-season drought than mid-season drought. This may due to the fact that late-season drought imposed less stress on peanut than mid-season drought. The results confirmed the findings by Furlan et al., (2012) that SNF

activity remained the same for drought and rehydration treatments. However, our results showed that some drought tolerant genotypes may be recovered after drought stress. However, more studies need to be done to identify the underlying physiological mechanism for the recovery.

Drought effect on the SLA did not present a consistent pattern in 2015 and 2016. In 2016, the middle-season drought significantly reduced SLA although most of genotypes remained unchanged. Notably, the reduction was mainly occurred in drought susceptible genotypes indicating the SLA of drought susceptible genotypes appeared to be more sensitive to middle-season drought than drought tolerant genotypes. Moreover, the positive correlations between SLA, %Ndfa and Δ indicate that plants with higher SLA could have a higher %Ndfa and Δ.

In conclusion, our study demonstrated that SNF and Δ in peanut were significantly reduced under drought stress. The more severe drought stress led to higher reduction in SNF and Δ, which was supported by the middle-season drought presented a higher impact on SNF and Δ than the late-season drought. Genotypic variability in SNF and Δ was observed among 16 different peanut genotypes under drought treatments, and genotype AU-587 was determined as the best performing genotype under both middle and late-season drought. The drought tolerant lines selected based on yield under stress showed higher SNF rate and Δ than the drought susceptible lines. Due to the complexity of plant-water relations under drought stress, future studies are needed to determine associations among SNF, Δ, and yield, and to investigate the enhancement of water usage efficiency of traits of interests.

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Table 2.1 Pearson correlation coefficients among %Ndfa, carbon isotope discrimination and SLA in peanut leaf tissue under middle-season drought in 2015 and 2016.[†]

	2015 %Ndfa	2015 Δ	2015 SLA	2016 %Ndfa	2016 Δ	2016 SLA
2015 %Ndfa	1.00	0.42***	NS			
2015 Δ		1.00	NS			
2015 SLA			1.00			
2016 %Ndfa				1.00	0.56***	0.20*
2016 Δ					1.00	0.17*
2016 SLA						1.00

[†]* $P \leq 0.1$; ** $P < 0.05$; *** $P < 0.01$; NS = non-significant, $\alpha = 0.05$.

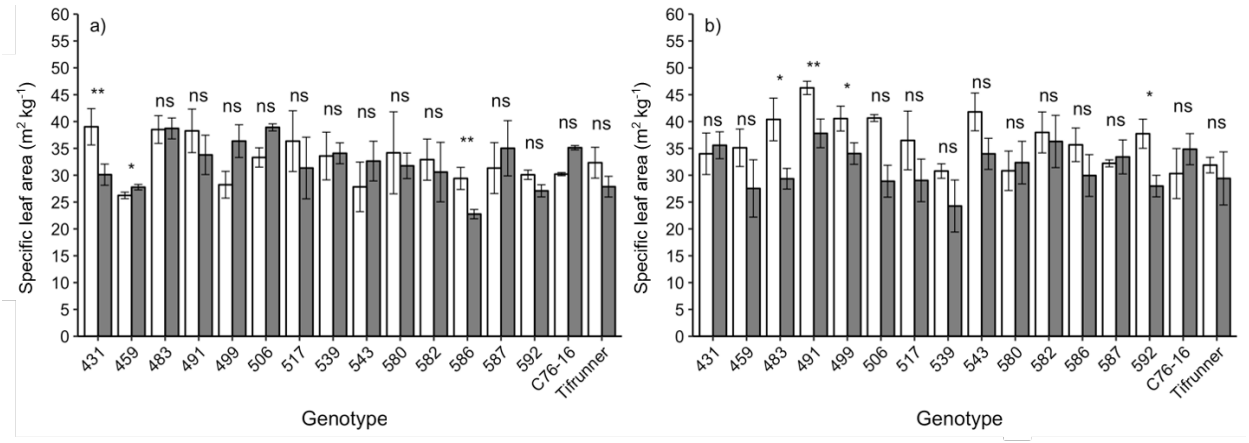

Figure 2.1 Effects of middle-season drought treatment on specific leaf area (SLA) among peanut genotypes in 2015 (a) and 2016 (b). Gray and white bars (means ± standard errors) represent mid-season drought and irrigation treatments, respectively. Asterisks denote significant differences between irrigation and middle-season drought treatments for each genotype based on Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

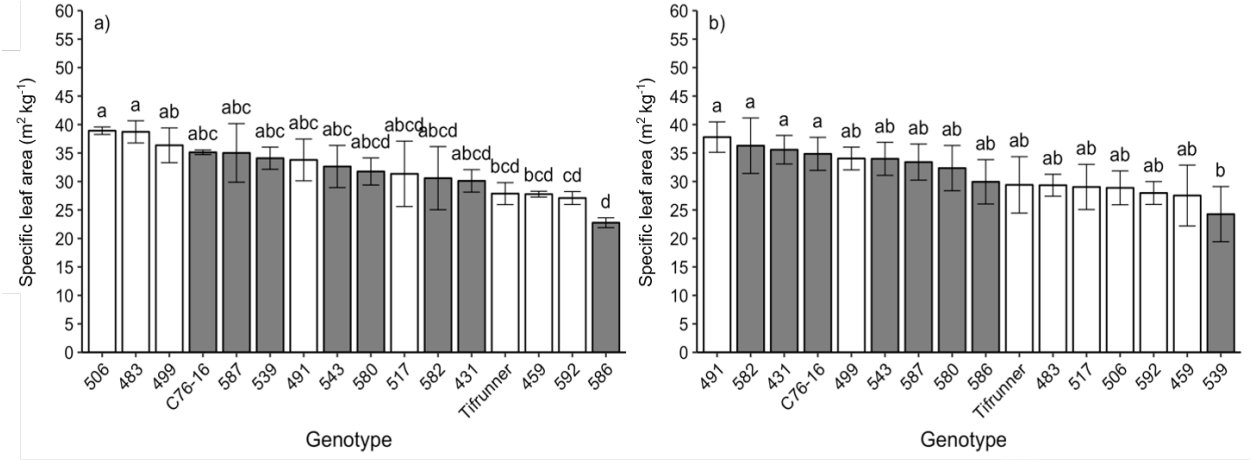

Figure 2.2 Variability in specific leaf area (SLA) among peanut genotypes under middle-season drought in 2015 (a) and in 2016 (b). Gray and white bars (means ± standard errors) represent drought tolerant and drought susceptible genotypes, respectively. Different letters denote significant difference among 16 different peanut genotypes based on Tukey's test ($\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

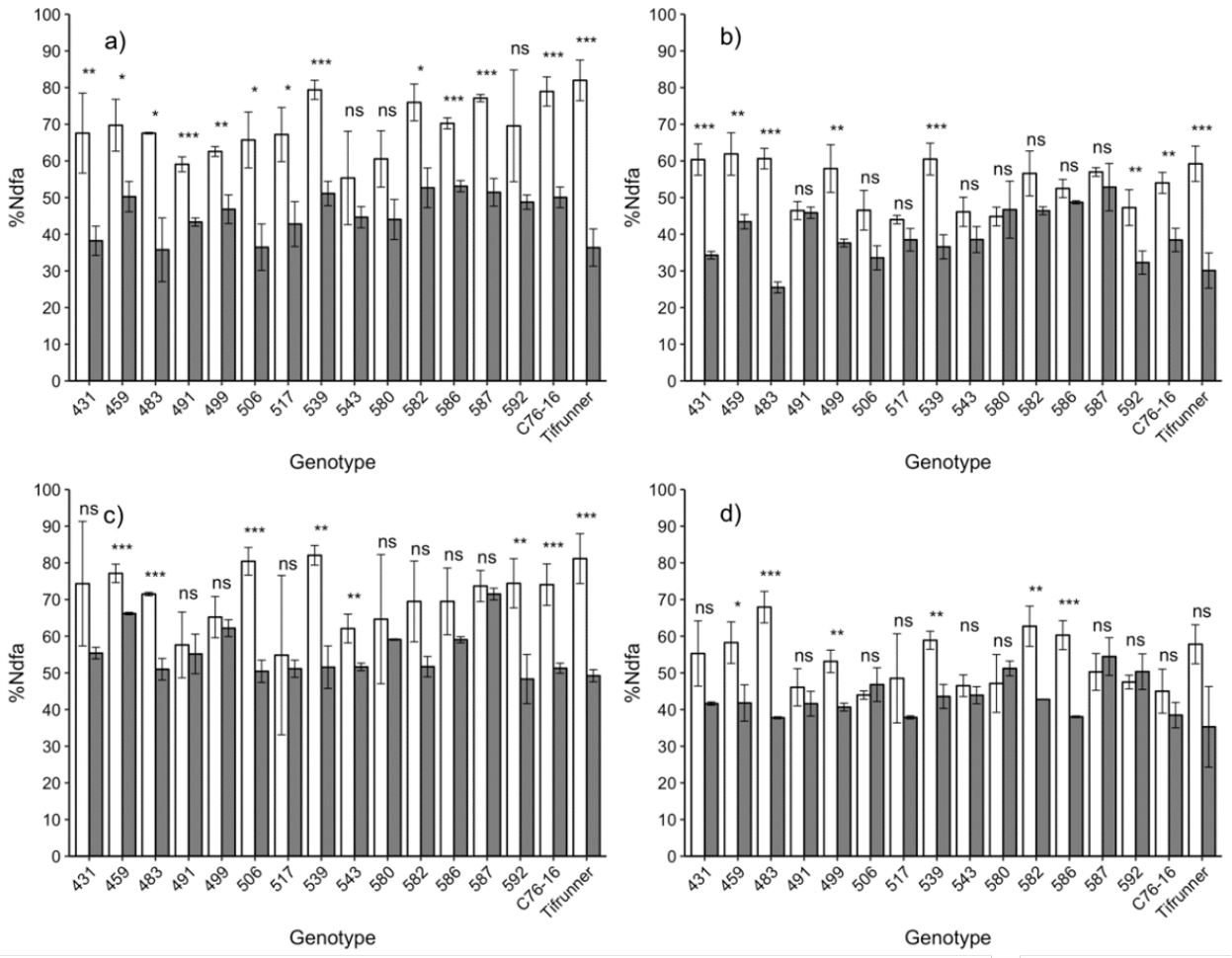

Figure 2.3 Effects of drought treatments on %Ndfa among peanut genotypes: a) irrigation and middle-season drought in 2015; b) irrigation and middle-season drought in 2016; c) irrigation and late-season drought in 2015; and d) irrigation and late-season drought in 2016. Gray and white bars (means ± standard errors) represent drought and irrigation treatments, respectively. Asterisks denote significant differences between irrigation and middle-season drought treatments for each genotype based on Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

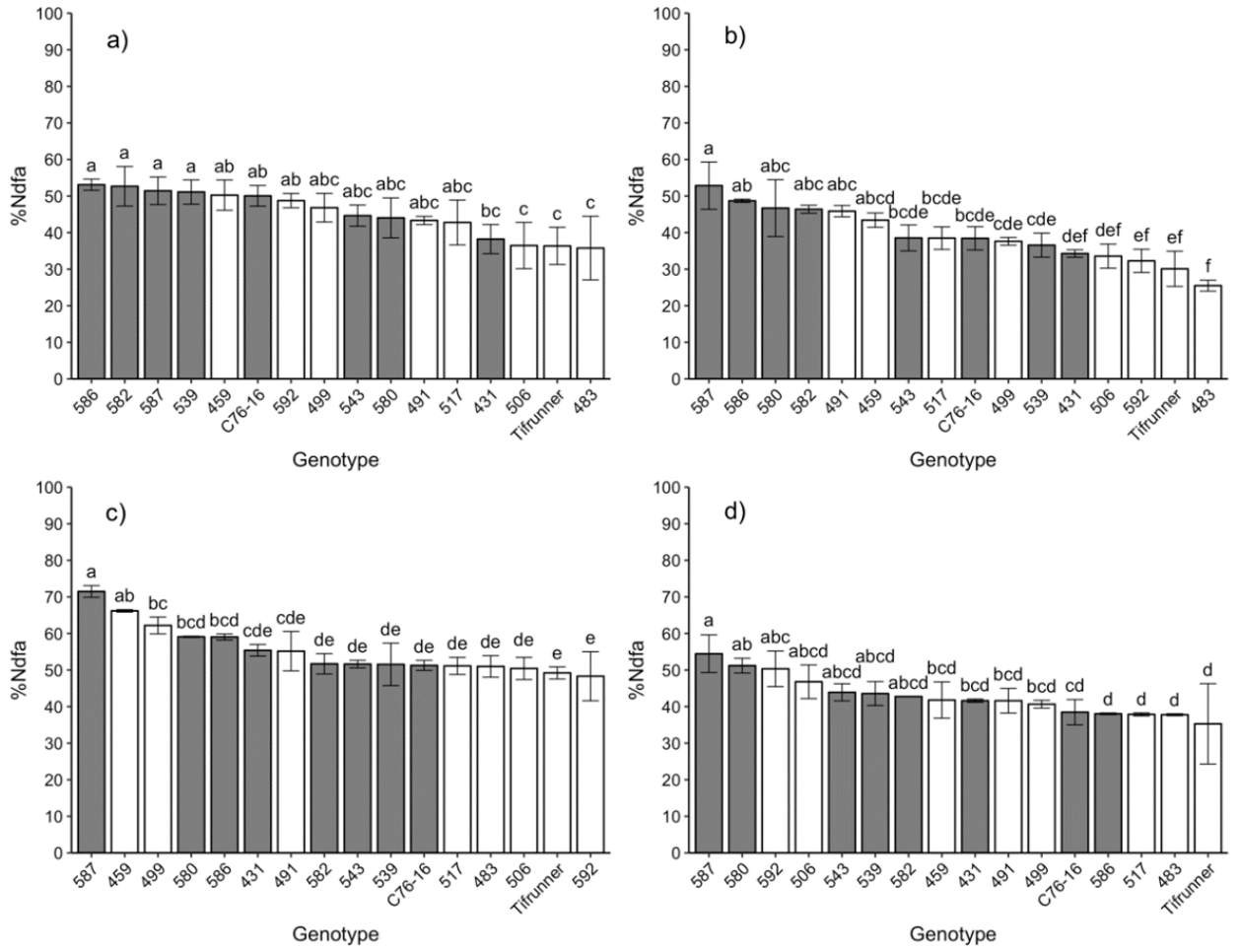

Figure 2.4 Variability in %Ndfa among peanut genotypes under middle-season drought in 2015 (a) and 2016 (b), and late-season drought in 2015 (c) and 2016 (d). Gray and white bars (means ± standard errors) represent drought tolerant and drought susceptible genotypes, respectively. Different letters denote significant difference among 16 different peanut genotypes based on Tukey's test ($\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

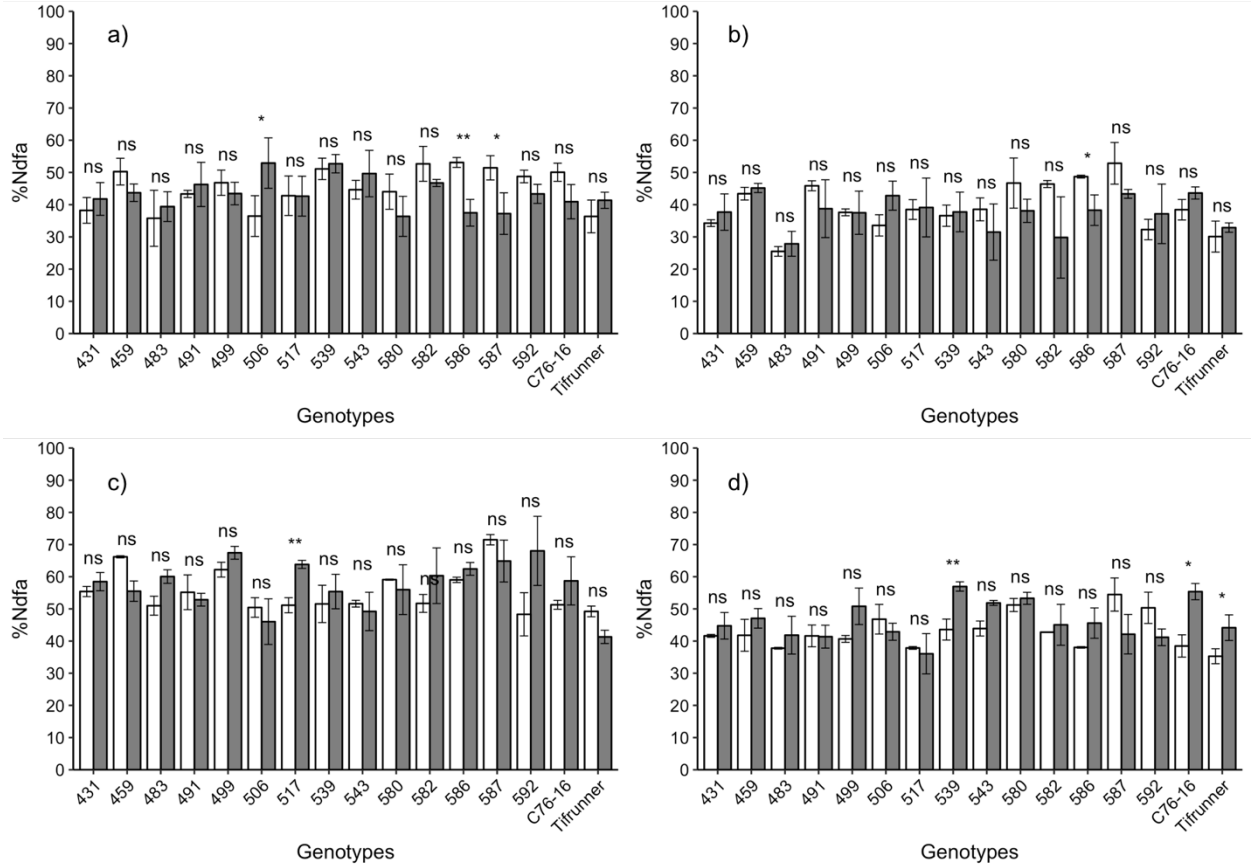

Figure 2.5 Effects of rehydration on %Ndfa among peanut genotypes: a) middle-season drought and rehydration in 2015; b) middle-season drought and rehydration in 2016; c) late-season drought and rehydration in 2015; and d) late-season drought and rehydration in 2016. Gray and white bars (means ± standard errors) represent drought and rehydration treatments, respectively. Asterisks denote significant differences between rehydration and middle-season drought treatments for each genotype based on Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

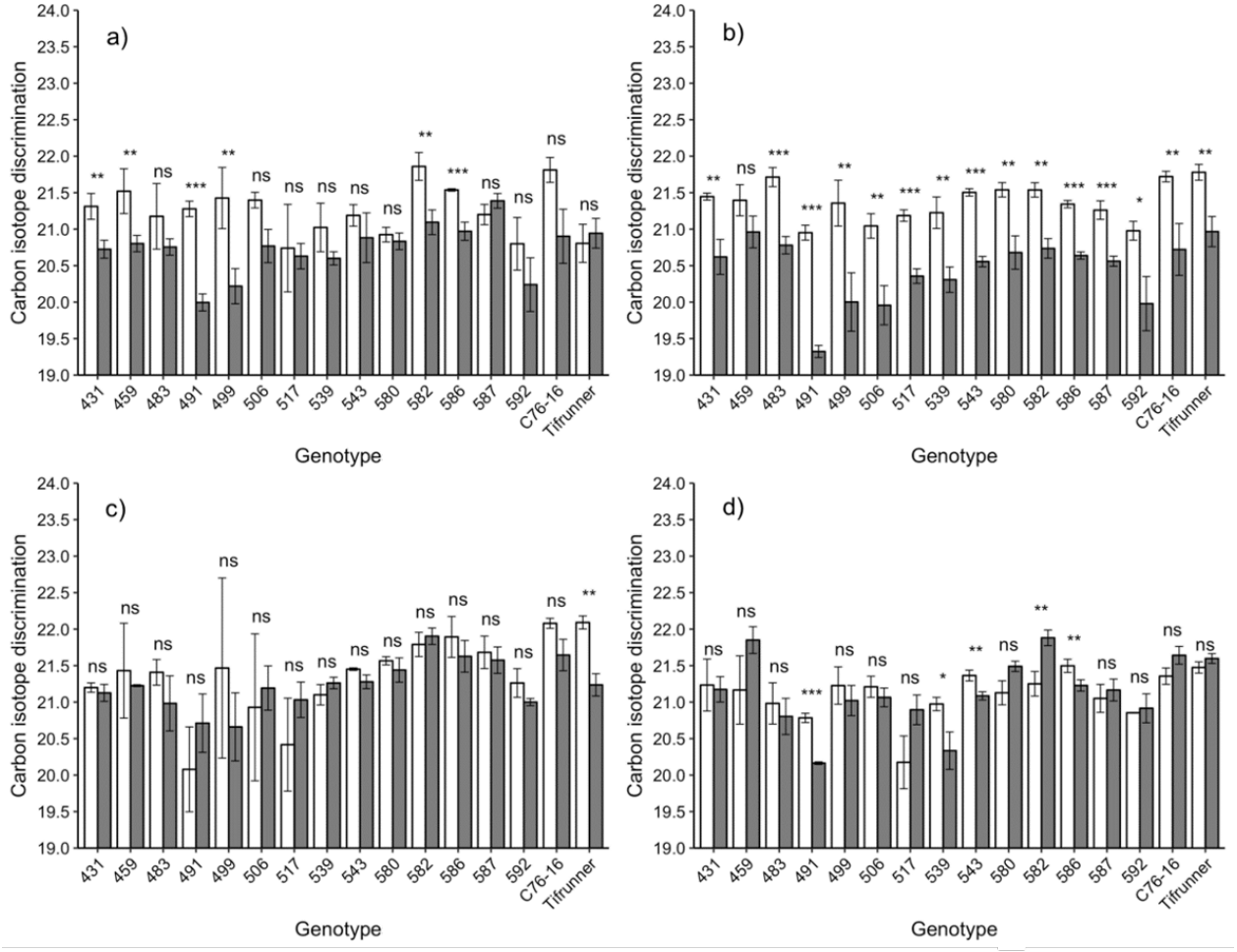

Figure 2.6 Carbon isotope discrimination among peanut genotypes: a) irrigation and middle-season drought in 2015; b) irrigation and middle-season drought in 2016; c) irrigation and late-season drought in 2015; and d) irrigation and late-season drought in 2016. Gray and white bars (means ± standard errors) represent drought and irrigation treatments, respectively. Asterisks denote significant differences between irrigation and middle-season drought treatments for each genotype based on Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

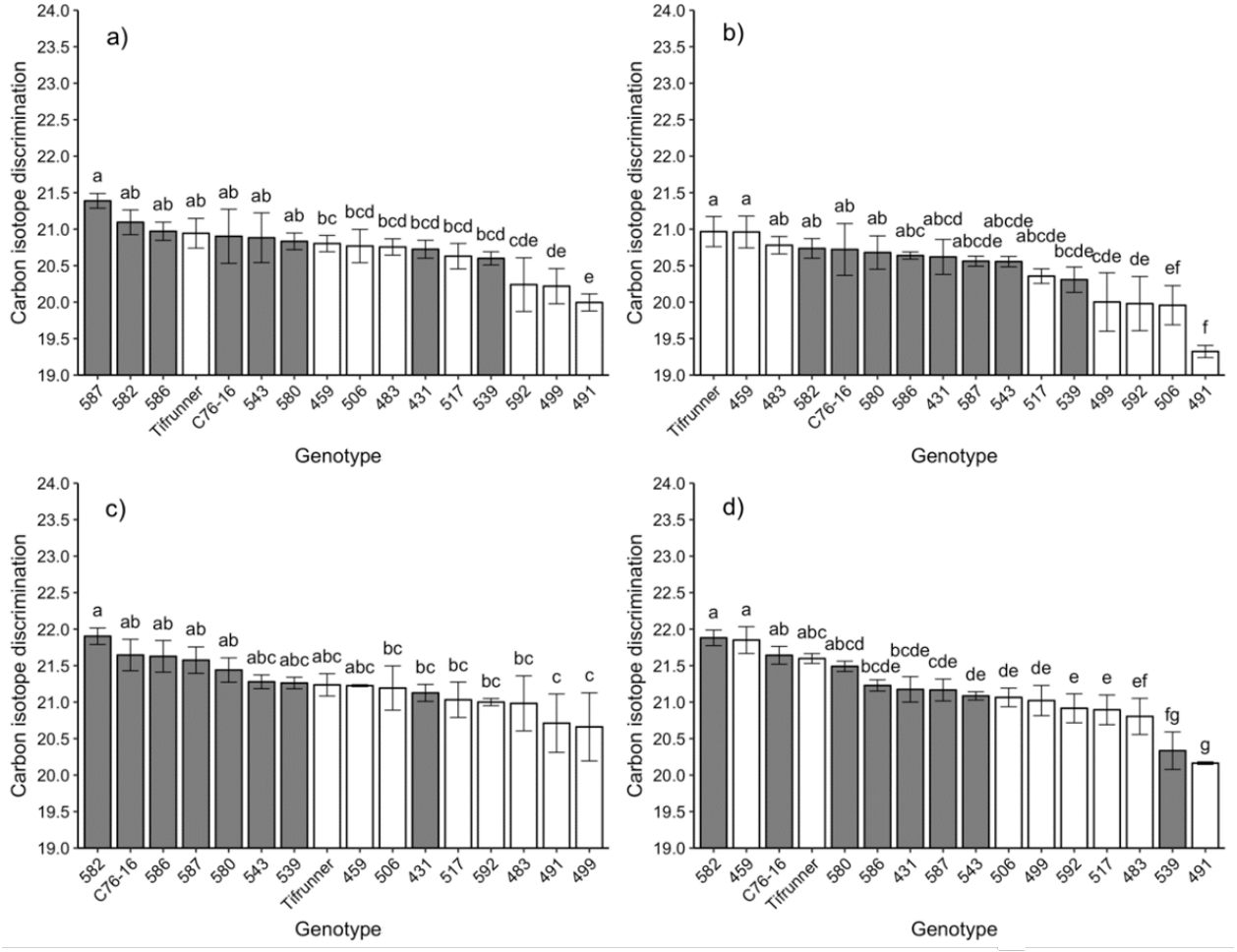

Figure 2.7 Variability in carbon isotope discrimination among peanut genotypes under middle-season drought in 2015 (a) and 2016 (b); and late-season drought in 2015 (c) and 2016 (d). Gray and white bars (means ± standard errors) represent drought tolerant and drought susceptible genotypes, respectively. Different letters denote significant difference among 16 different peanut genotypes based on Tukey's test ($\alpha = 0.05$). Drought tolerant genotypes: C76-16, 587, 586, 582, 580, 543, 539, and 431; drought susceptible genotypes: Tifrunner, 592, 517, 506, 499, 491, 483, and 459.

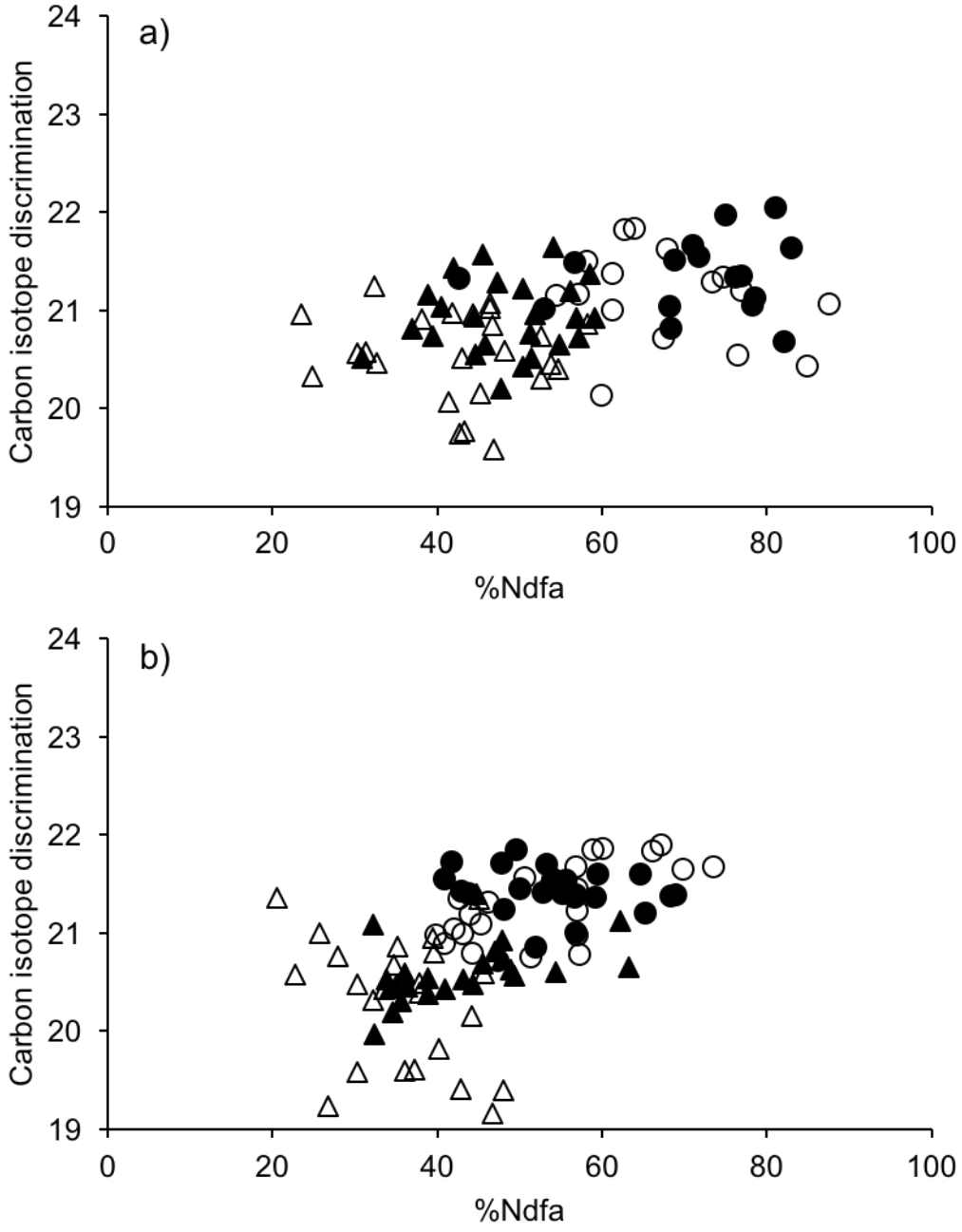

Figure 2.8 The correlations between carbon isotope discrimination and %Ndfa under middle-season drought in 2015 (a) and 2016 (b). Black markers represent drought tolerant genotypes, and white markers represent drought susceptible genotypes. Circles represent irrigation treatments and triangles indicate the middle-season drought treatment.

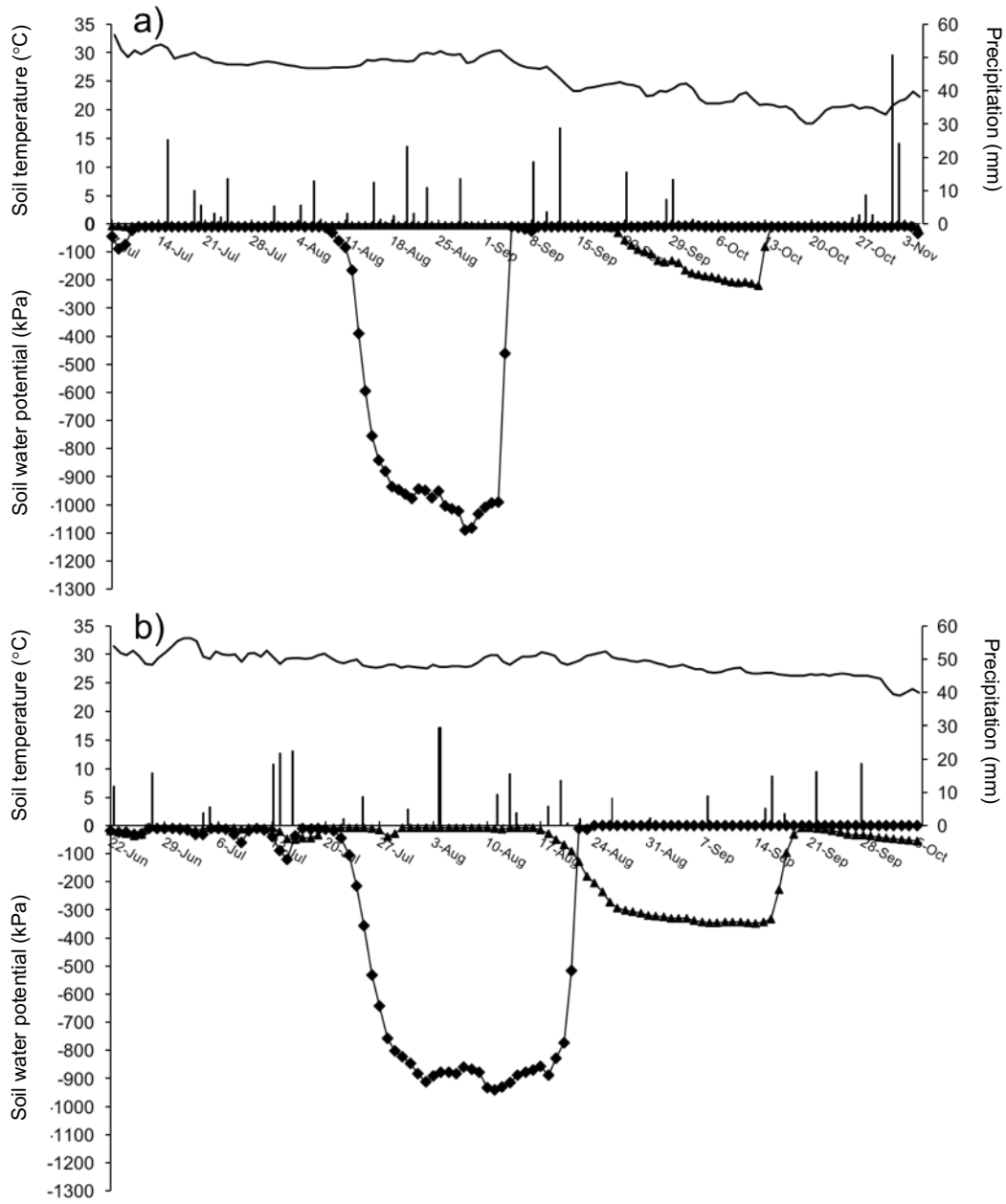

Figure 2.9 Daily precipitation, soil water potential and soil temperature at 20 cm depth in 2015 (a) and 2016 (b). Square represents middle season drought and triangle indicates late season drought treatment.

Chapter Three

Transcriptome profile reveals drought induced genes preferentially expressed in response to water deficit in peanut (*Arachis hypogaea* L.) genotypes

3.1 Abstract

Cultivated peanut (*Arachis hypogaea*) is one of the most widely grown food legumes in the world being valued for its high protein and unsaturated oil contents. Drought stress is one of the major constraints that limits peanut production. The objective of this study was to identify the drought responsive genes preferentially expressed under drought stress in different peanut genotypes. To accomplish this, four genotypes (drought tolerant: C76-16 and AU-587; drought susceptible: Tifrunner and AU-506) used in a rainout shelter experiment were examined. Whole-transcriptome sequencing analysis identified 7,780 genes differentially expressed in Tifrunner and 9,767 in AU-506. Of the 7,780 genes in Tifrunner, 5,310 genes were up-regulated and 2,470 were down-regulated. For the drought tolerant genotypes, 12,348 DEGs were identified in AU-587, including 7,172 up-regulated genes and 5,176 down-regulated genes. In C76-16, a total of 13,005 DEGs were identified with 7,718 up-regulated genes and 5,287 down-regulated genes. A total of 2,457 DEGs were shared by all four genotypes. Functional analysis of the shared DEGs identified a total of 139 enriched gene ontology (GO) terms consisting of 86 biological processes, and 53 molecular function, and defense response, reproductive process and signaling pathways were significantly enriched. Total of 43 significantly enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways were also identified, and the most enriched pathways are those process involved in metabolic pathways, biosynthesis of secondary metabolites, plant circadian rhythm, phenylpropanoid biosynthesis, starch and sucrose metabolism, etc. This

research expands our current understanding of the mechanisms that facilitate peanut drought tolerance and shed light on breeding advanced peanut lines to combat drought stress.

3.2 Introduction

Cultivated peanut (*Arachis hypogaea* L.) is an important legume that grown mainly on arid and semi-arid regions where peanut productivity is usually limited by water deficit (Balota et al., 2012; Brasileiro et al., 2015; Songsri et al., 2008; Dinh et al., 2013; Pimratch et al., 2008, 2009). Drought stress between the growth stages of flowering to pod development (middle-growing season) leads to severe reduction in peanut pod yield due to the highest water requirement during this period (Stansell et al., 1985; Sterling et al., 1989). How to sustain and even increase peanut production to meet growing population needs of while environmental conditions are deteriorating is a major challenge that peanut industry faces. Developing drought-tolerant varieties that can be cultivated in the different drought-affected locations is a priority for many peanut breeding programs (Zhao et al., 2018). Unfortunately, the traditional breeding approaches achieves little progress because drought-stress tolerance is a polygenic trait, and very little is known about the molecular signaling and regulatory mechanisms of peanut under drought stress because its complex genetic background.

The vulnerability of peanut to drought stress depends on genotypic variability (Devi et al., 2010; Dinh et al., 2013; Greenberg et al., 1992; Puangbut et al., 2009; Stansell et al., 1985). Genotypic variations in several physiological characteristics associated with drought tolerance, including transpiration, photosynthesis rate, have been identified, offering great opportunities to breed high-yielding drought tolerant genotypes, especially under middle season drought (Balota et al., 2012; Pimratch et al., 2008). The advent of RNA-sequencing (RNA-Seq), a technique for genome-wide gene expression analysis, provides powerful alternatives to facilitate the

production of drought tolerant genotypes in a more efficient manner (Li et al., 2014; Zhao et al., 2018). Recently, gene structures and expression profiles in many crops, including wheat, corn, soybean, peanut, in response to stress conditions were determined with RNA-Seq technology (Brasileiro et al., 2015; Chen et al., 2013; Li et al., 2014; Long et al., 2019; Mathioni et al., 2011; Petre et al., 2012; Ruan et al., 2018; Zhao et al., 2018). Large-scale screening of peanut has identified some drought-related genes. For example, increased transcript levels of basic leucine zipper (*bZIP*) transcription factors genes were observed from the wild relative of cultivated peanut, *A. duranensis,* when the plant were subjected to drought with 18% soil water content (Guimarães et al., 2012). Likewise, Li et al. (2014) reported 621 genes that were rapidly induced under water deficit conditions and the main drought response mechanisms in peanut function through the abscisic acid (ABA) dependent pathway. In addition, more than 4,000 genes were identified to be associated with drought stress, including 224 transcription factors and genes involved in photosynthesis-antenna proteins, carbon metabolism and the citrate cycle (Zhao et al., 2018).

Since the genome sequence of the cultivated peanut Tifrunner was released recently (Bertioli et al., 2019), a more accurate transcriptome assembly can be obtained by mapping to the reference genome. Thus, the objective of the present study was to discover drought-induced genes by comparing drought tolerant and susceptible lines under drought stress by mapping RNA-Seq data to the cultivated peanut reference genome. The data developed in this work could provide comprehensive insight in molecular mechanisms that underlie drought tolerance and resource to further molecular research in peanut.

3.3 Materials and Methods

3.3.1 Plant materials and experimental design

The experiments were performed using four cultivated peanut genotypes, Tifrunner (susceptible), C76-16 (tolerant), AU-587 (tolerant) and AU-506 (susceptible), which were selected based on the drought study conducted in 2015 and 2016. AU-587 and AU-506 are two recombinant inbred lines (RILs) derived from the cross 'Tifrunner x C76-16' which represent the highest and lowest drought tolerant level. A split-plot design with randomized complete block design (RCBD) within was adopted in this study. All seeds were planted in a single-row (15 x 120 cm) at a rate of 10 seeds m^{-1} under rainout shelters at the USDA-ARS National Peanut Research Laboratory in Dawson, Georgia to create artificial drought stress. Two rainout shelters were designated with two treatments including: full irrigation and middle-season drought, and each shelter consists of three blocks. Irrigation was provided for both treatments right after seed sowing to provide uniform germination. The irrigated treatment (designated as 'irrigated control') received complete irrigation throughout growing season based on evapotranspiration (ET) replacement described by Stansell et al. (1976). The drought treatment (designated as 'treatment') received fully irrigation at the beginning of the growing season until 61 days after planting (DAP). Water-deficit stress was applied at 61 DAP by withheld water consecutively for four weeks. Besides the water treatment, all other agronomic management practices were applied according to the University of Georgia best management practices for peanut.

3.3.2 RNA extraction and library construction

Fully expanded leaves (second nodal) were randomly collected from each genotype at the end of drought period in 2016. Leaf samples of each genotype were flash frozen using liquid nitrogen and stored at -80 °C until RNA extraction. Three leaflets randomly collected from each biological replication were pooled and approximately 0.2 g pooled leaf samples were ground in liquid nitrogen for RNA extraction. Total RNA was extracted using a modified CTAB method

(Yin et al., 2011) and purified using a Direct-Zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). The purity and integrity of RNA were analyzed using NanoDrop ND-1000 UV spectrophotometer (Thermo Scientific, Wilmingtong, USA) and Agilent 2100 Bioanalyzer (Agilent, USA), respectively. A total of 24 libraries (4 genotypes × 2 treatments × 3 replicates) were constructed and subsequently sequenced using an Illumina HiSeq 4000 instrument at the Beijing Genomics Institute (BGI).

3.3.3 Bioinformatics analysis

3.3.3.1 Quality control, alignment and genome-guided assembly

The raw reads from RNA-seq were trimmed with Trimmomatic (Bolger et al., 2014). Clean reads were obtained by removing the adaptor sequences, ambiguous 'N' nucleotides, and low-quality reads from the raw data. The read quality was assessed using FastQC (Andrews, 2010) before and after trimming. High quality clean data was subjected to the downstream analyses. The RNA-seq data analysis pipeline followed the protocol described by Trapnell et al., (2012). Each sample was mapped to the reference genome by Tophat2 (Kim, 2013) with all the parameters setting to default. The cultivated peanut genome and the annotation file (Bertoli et al., 2019) were used as reference for alignment. The alignment files of the 24 samples from Tophat2 were input into Cufflinks (Trapnell et al., 2012) for transcripts reconstruction.

To identify the novel transcript sequences, all the assemblies were compared with the reference annotation using Cuffcompare. Novel transcript sequences were then compared to the 'nr' database at NCBI by BLASTX to achieve gene functional annotation.

3.3.3.2 Identification of differentially expressed genes (DEGs)

The gene expression levels were represented by the expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM), which was

calculated on the basis of the length of the gene and reads count mapped to this gene. The DEGs analysis were performed using Cuffdiff (FDR < 0.05) (Trapnell et al., 2012). The DEGs were identified through the comparison of gene expression between 'irrigated control' and 'drought treatment' samples for each genotype. The calculated *P*-value was then adjusted through false discovery rate (FDR) correction. Genes with adjusted *P*-values < 0.05 were considered to be significantly differentially expressed.

3.3.3.3 Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

GO terms for gene models available in genome annotation were directly retrieved from the 'GFF' file downloaded at PeanutBase website (http://peanutbase.org). GO terms for the novel transcripts were assigned using Blast2Go (Conesa et al., 2005). To combine the GO terms of the annotated genes and novel genes, the GO enrichment analysis for DEGs was performed using AgriGO (http://systemsbiology.cau.edu.cn/agriGOv2/) (Tian et al., 2017). GO terms with FDR-adjusted *P*-value < 0.05 were considered as significantly enriched by differently expressed genes. The enriched GO terms were subsequently visualized using REVIGO (Supek et al., 2011). To identify important pathways involved by the DEGs, the transcripts were assigned to the KEGG pathways using the web server (http://www.genome.jp/kaas-bin/kaas_main) against the *Arabidopsis thaliana, Glycine max, A. duranensis* and *A. ipaensis* gene datasets using the bi-directional best hit (BBH) method. KEGG enrichment analysis was conducted on KOBAS 3.0 webserver (http://kobas.cbi.pku.edu.cn/index.php) (Wu et al., 2006).

3.4 Results

3.4.1 Genome-guided assembly and annotation of novel transcripts

To assess the global transcriptome profile of peanut leaf samples in response to drought stress, RNA-Seq analysis was performed using peanut leaves under drought treatments. RNA-seq of 24 samples of the four genotypes with three replicates under 'irrigated control' or 'treatment' generated a total of 1,059,869,097 pairs of 100-bp cleaned reads (197.41Gb) with an average of 44.16 million read pairs per library. By comparing the accumulated size of sequences generated in this study to the total size of overall transcript sequences of *A. hypogaea* (109.0 M), the sequence generated in this study was more than 1854.6 times coverage of the known peanut transcript sequences with an average coverage of 77.27 times per library. After trimming, 87.81% of the raw reads, including 930,991,527 paired reads and 77,462,493 un-paired reads, survived (Table 3.1). The cleaned reads were mapped to the cultivated peanut genome and overall mapping rate per library ranged from 62.20% to 79.30%, with an average mapping rate of 73.42% (Table 3.1).

Through the genome-guided assembly, a total of 73,575 genes were assembled for Tifrunner, 73610 genes were assembled for C76-16, 73898 genes were assembled for AU-587 and 73900 genes were assembled for AU-506, respectively. There were 66437 (90.30%), 66445 (90.27%), 66373 (89.82%) and 66378 (89.82%) assembled genes matched to genes annotated from the cultivated peanut reference genome in Tifrunner, C76-16, AU-587 and AU-506, respectively (Table 3.2), resulting in 7138, 7165, 7525 and 7522 novel genes were identified in Tifrunner, C76-16, AU-587 and AU-506, respectively.

To explore the potential functions of these novel transcript sequences, they were blasted against the 'nr' database at the NCBI. In total, 2063 GO terms were assigned to the novel transcript sequences. In addition, there were 248 KEGG Orthology (KO) terms were assigned the novel transcripts.

3.4.2 Differentially expressed genes.

The DEGs were determined between 'irrigated control' and 'treatment' samples of each genotype. For the drought susceptible genotypes, there are totally 7780 genes were differentially expressed in Tifrunner and 9,767 were differentially expressed in AU-506 (Table 3.2). Of the 7,780 genes in Tifrunner, the expression of 5,310 genes were increased and the level of the remaining 2,470 genes were decreased. For genotype AU-506, 6,052 genes were upregulated, and 3715 genes were downregulated in the drought treatment (Table 3.2). For the drought tolerant genotypes, 12348 DEGs were identified in genotypes AU-587, including 7,172 up-regulated genes and 5176 down-regulated genes. In addition, a total of 13,005 DEGs were identified in C76-16 showed either up-regulation (7,718 genes) or down-regulation (5,287 genes). Among the DEGs identified, 6,410, 10,210, 10,605 and 8,065 DEGs were annotated with the reference genome in Tifrunner, C76-16, AU-587 and AU-506, respectively (Table 3.2).

Pairwise comparison of the DEGs from the four genotypes was performed to investigate which genes failed to respond to drought stress in drought susceptible genotypes as well as those normally involved in drought tolerant genotypes (Fig. 3.1). A total of 5,704 DEGs, including 3,668 up-regulated genes and 2,035 down-regulated genes, were shared by AU-506 and two drought tolerant genotypes (Fig. 3.1A&C). Tifrunner shared 4,611 DEGs (3,246 up-regulated and 1,365 down-regulated) with the drought tolerant genotypes (Fig. 3.1B&D). Among the identified DEGs in the drought tolerant lines, 3,860 genes were shared between AU-587 and C76-16, with 10,315 DEGs exclusively detected in the two drought tolerant genotypes. Moreover, there were 2,457 DEGs identified in all four genotypes, and these genes were used in the subsequent GO and KEGG enrichment analysis.

Among those 2,457 DEGs shared by all four genotypes, we set log2-fold change of > 2 and < -2 as threshold to select the most significant DEGs. After filtering, only 250 genes were determined as significant DEGs. Then, 78 unique genes with different expression profiles were showed in Figure 3. Among these 78 genes, 76 genes were up-regulated under drought stress, while 2 genes were down-regulated in response to drought stress.

3.4.3 Gene Ontology enrichment and functional classification of DEGs

Go assignments were used to classify the functions of DEGs. GO enrichment analysis was performed on the 2,457 genes to identify processes and functions over-represented in the DEGs. The 2,457 drought responsive DEGs were assigned into 139 enriched GO terms consisting of 86 biological process and 53 molecular functions (Table 3.3). The most significantly enriched GO term in biological processes was cellular protein modification process, followed by protein modification process. Several other protein modification related process, including protein dephosphorylation, protein ubiquitination, protein modification by small protein conjugation, protein metabolic process, and protein serine etc. It indicated the role of protein in drought response. In addition, a cluster of GO terms related to defense response, such as response to stimulus, response to heat, response to abiotic stimulus, and response to temperature stimulus was also observed. Furthermore, many reproductions related GO terms including pollination, pollen-pistil interaction, reproduction and reproductive process were highly enriched indicating the effects of drought stress on reproduction process. There are also many GO terms that are related to signaling (signaling and signal transduction) and regulation (regulation of transcription, regulation of RNA biosynthetic, regulation of RNA metabolic etc.) were enriched. Additionally, kinase related GO terms were observed, including protein serine/threonine kinase activity, kinase activity, protein kinase activity in molecular function (Fig

3.2B). There are also many GO terms are related to oxidation-reduction process, such as oxygen, oxidoreductase activity and dioxygenase activity were observed in molecular function.

3.4.4 KEGG pathway enrichment analysis of DEGs

A total of 741 KEGG Ontology (KO) terms were assigned to those 2,457 DEGs that were common in both drought tolerant genotypes and drought susceptible genotypes. The KEGG enrichment analysis was conducted against *Arabidopsis thaliana* gene dataset using KOBAS 3.0 (Table 3.4). There are 43 pathways were significantly enriched (FDR-adjusted P-value < 0.05) and the most enriched pathways including metabolic pathways, biosynthesis of secondary metabolites, circadian rhythm-plant, phenylpropanoid biosynthesis, starch and sucrose metabolism etc. (Figure 3.5). Photosynthesis related KEGG pathways including photosynthesis-antenna proteins and carbon fixation in photosynthetic organisms were observed. More interestingly, proline related pathway, arginine and proline metabolism was enriched which has been widely reported to be associated with drought tolerance.

3.5 Discussion

Transcriptome data are valuable resources for discovering gene expression levels, characterizing new alleles and developing molecular markers associated with drought responses in plant by investigating plants under abiotic or biotic stress. However, studies focusing on transcriptome of peanut under drought stress are limited. Li et al. (2014) demonstrated 47,842 unigenes with 621 induced DEGs (≥ 1.5 fold change compared with control) in the seedlings of peanut *(Arachis hypogaea* L.) cultivar Yueyou7 in South China under water deficit condition, and 22 putative transcription factor (TF) genes were reported as drought responsive. They concluded that the main drought response mechanism in peanut function is through the ABA-dependent pathway. RNA-Seq analyses on two wild relatives of peanut under drought

conditions, *A. stenosperma* (7,722 contigs) and *A. duranensis* (12,792 contigs), classified TF transcripts into 25 and 20 families, respectively (Guimarães et al., 2012). A more recent study assembled 51,554 genes in cultivated peanut root samples under drought conditions, where 4,648 DEGs were identified by comparing the irrigation and drought treatment (Zhao et al., 2018). In contrast, the present study analyzed the transcriptome of four peanut lines by mapping the sequenced library to the cultivated peanut reference genome and thus provided a more thorough dataset showing gene regulations under drought stress. We reported 73,575, 73,898, 73,900 and 73,610 genes for Tifrunner, AU-587, AU-506 and C76-16 with 7,780, 13,005, 9,767 and 12,348 DEGs in each genotype, respectively.

Majority of the DEGs were involved in secondary metabolite biosynthesis, photosynthesis, and response to heat and abiotic stimulus. This is supported by several studies reporting plant stress resistance systems (Farooq et al., 2009, Zhao et al. 2018). Some of the DEGs in response to drought stress were involved in 11 enriched KEGG pathways (carbon fixation in photosynthetic organisms, starch and sucrose metabolism, photosynthesis-antenna proteins, porphyrin and chlorophyll metabolism, cysteine and methionine metabolism, circadian rhythm-plant, pyruvate metabolism, amino sugar and nucleotide sugar metabolism, glycine, serine and threonine metabolism, phenylpropanoid biosynthesis, and phenylalanine metabolism), which is consistent with the results demonstrated in a recent study on peanut (Zhao et al. 2018). DEGs enriched in carbon metabolism pathway, starch and sucrose metabolism pathway, and photosynthesis-antenna proteins suggesting plant photosynthesis was affected due to decreasing CO_2 assimilation rate under mid-season drought stress (Farooq et al., 2009). Similarly, several genes involved in ascorbate and aldarate metabolism, carbon fixation, and photosynthesis were also reported to drought stimuli in other plants including *Boehmeria nivea* and *Chrysanthemum*

morifolium (Liu et al., 2013; Xu et al., 2013). However, our data indicated that ribosome as well as plant hormone signal transduction were not enriched in peanut in response to mid-season drought, suggesting plant host, growth stages, sampling dates and treatments might play roles in the demonstrated variability.

Drought stress significantly affected the transcripts of some key genes related to secondary metabolism. For example, protein ubiquitination are demonstrated to regulate plant drought stress response (Liu et al., 2013a; Zhang et al., 2013). Our GO enrichment analysis indicated peanut ubiquitin-related genes were highly enriched in both drought-tolerant and drought-susceptible genotypes. Fifty DEGs were identified to correlate with protein ubiquitination. For example, *Arahy.4AP7UE* played a role in protein ubiquitination which may negatively regulates abscisic acid (ABA) and drought response. In support of our data, ubiquitin-related gene *AhUBC2* was shown to enhance drought tolerance through regulating the expression of stress responsive gene (Wan et al., 2011).

Among the enriched KEGG and GO, many signaling related GO terms were enriched indicating the potential importance of related pathway in peanut plants under drought stress. Interestingly, many ABA pathways related DEGs were significantly induced by drought stress, including *Arahy.UN6GTT, Arahy.RRZ6LI, Arahy.KS1HEQ, Arahy.H5H05M,* and *Arahy.KLG2UC*. Plant hormones play important roles throughout the lives of plants, including during growth, under environmental stress, and senescence (Davis. 2013). ABA, as an important plant hormone being produced in the roots in response to drought, was widely studied for its role in regulating guard cell movement to close stomata (Li et al., 2014). ABA functions in plants under drought by regulating the development of reproductive tissues via massive transcriptional reprogramming events under long-term drought stress, which further reduce the plant growth and

crop yield (Degenkolbe et al., 2009; Sreenivasulu et al., 2012). The previous study in peanut identified 279 DEGs that are significant overlaps in expression between the water deficit only and water deficit + ABA treatment groups indicating the significant role of ABA in signaling under drought (Li et al., 2014). Furthermore, combining the reproduction related pathway that enriched in this study, we speculated that the induced ABA related DEGs may further affect the reproduction process of peanut under drought stress due to the four-week drought period. This confirms the previous finding that ABA regulates the development of reproductive tissues under long-term drought (Degenkolbe et al., 2009; Sreenivasulu et al., 2012). Besides the ABA pathway related genes, we also found a range of ethylene-related and auxin signaling pathway-related genes were differentially expressed in the peanut under drought-stress conditions. In summary, these results show that the mechanism underlying drought tolerance in peanut involves a complex network of multiple hormones. However, the underlying regulatory mechanisms still need to be further studied. Therefore, studying plant hormone signaling pathways will be crucial for understanding the regulatory mechanism in peanut drought tolerance.

Analysis of genes involved in the drought-stress response

Late embryogenesis abundant (LEA) proteins are mainly low molecular weight (10-30 kDa) proteins, which are involved in protecting higher plants from damage caused by environmental stresses, especially drought (dehydration). The present study identified two LEA genes (*arachy. RD0T5B* and *arachy. XWYH2Z*) are shared by all four genotypes, five LEA genes (*arachy.3IB3IU, arachy.Q07BGG, arachy. R9W6MW, arachy.B0SKQG* and *arachy.FY9BZZ*) are shared uniquely in drought tolerant genotypes and only one LEA gene (*arachy.*P4KHGY) shared in drought susceptible genotypes. In our study, more LEA genes were up-regulated in drought tolerant genotypes than it in drought susceptible genotypes. This indicated the essential role of

LEA proteins in peanut under drought stress, especially in drought tolerant genotypes. Accumulation of LEA proteins has also been found to occur in peanut roots when peanut plants under drought stress (Zhao et al., 2018). With regard to the many peanut LEA gene subfamilies, the precise functions are still enigmatic, and further research should be performed to elucidate the possible roles of these genes in peanut stress tolerance.

TFs

TFs (sequence-specific DNA-binding factors) are proteins that bind to specific DNA sequences, thereby controlling the RNA transcription rate for genes (Latchman et al., 1997). TFs may perform their functions alone or with other proteins in a complex by promoting (as an activator) or blocking (as a repressor) the recruitment of RNA polymerase to specific genes. In legumes, different TF subfamilies might show different regulation under stress (Udvardi et al., 2007). In our study, many TFs families have been identified and many of them have been reported to be involved in the plant drought-tolerance system. In the present study, most of the TFs were enriched in MYB, WRKY and ERF. In the present study, five genes from MYB family were highly induced under drought stress in all four genotypes. In addition, nine MYB TFs were highly induced particularly in the two drought tolerant genotypes and only one MYB TF was induced only in the two drought susceptible genotypes. This indicated the significance of the MYB family in drought stress, especially in drought tolerant genotypes. The MYB family has been described to act through the abscisic acid (ABA) signaling cascade to regulate stomatal movement and therefore water loss regulation in *Arabidopsis* and rice (Yanhui et al., 2006 and Dai et al., 2007).

The present study demonstrated that mid-season drought alters the transcriptome profile in four peanut genotypes with varying drought tolerant level. Thousands of novel genes of

cultivated peanuts were identified and annotated. The DEGs involved in circadian rhythm-plant, phenylpropanoid biosynthesis, starch and sucrose metabolism, and photosynthesis-antenna proteins," etc. were enriched. In addition, ABA-related pathway was considered as one of the most important mechanism underlying drought tolerance in peanut. This study provided insights into putative peanut response against drought stress.

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Table 3.1 Summary of library, trimming and alignment of reads to A. hypogea genome in each library

Genotype	Sample type	Input read pairs	Both surviving	Forward only surviving	Reverse only surviving	Dropped	Overall alignment rate (%)
Tifrunner	Control	43170429	37947881 (87.90%)	3481624 (8.06%)	608537 (1.41%)	1132387 (2.62%)	77.60%
Tifrunner	Control	48861831	43973662 (90.00%)	3082838 (6.31%)	754300 (1.54%)	1051031 (2.15%)	73.40%
Tifrunner	Control	43839412	39489936 (90.08%)	2774014 (6.33%)	685366 (1.56%)	890096 (2.03%)	76.90%
587	Control	40070955	36200463 (90.34%)	2424462 (6.05%)	624743 (1.56%)	821287 (2.05%)	79.30%
587	Control	44781655	40323747 (90.05%)	2875811 (6.42%)	656639 (1.47%)	925458 (2.07%)	72.90%
587	Control	44683142	40559180 (90.77%)	2486187 (5.56%)	704413 (1.58%)	933362 (2.09%)	73.20%
506	Control	46447905	41940433 (90.30%)	2887090 (6.22%)	689080 (1.48%)	931302 (2.01%)	64.80%
506	Control	47081614	42489538 (90.25%)	2967321 (6.30%)	710662 (1.51%)	914093 (1.94%)	65.90%
506	Control	48467014	43354569 (89.45%)	3122297 (6.44%)	839644 (1.73%)	1150504 (2.37%)	75.50%
C76-16	Control	44107057	30307111	1502648	9723368	2573930	67.90%

			(68.71%)	(3.41%)	(22.04%)	(5.84%)	
C76-16	Control	44738941	39605524 (88.53%)	3111979 (6.96%)	757947 (1.69%)	1263491 (2.82%)	62.20%
C76-16	Control	44941724	40406587 (89.91%)	2744593 (6.11%)	770588 (1.71%)	1019956 (2.27%)	67.70%
Tifrunner	Treatment	44640385	39841817 (89.25%)	2962824 (6.64%)	805756 (1.80%)	1029988 (2.31%)	78.70%
Tifrunner	Treatment	34479724	30039097 (87.12%)	2622507 (7.61%)	691319 (2.01%)	1126801 (3.27%)	78.50%
Tifrunner	Treatment	44888435	39964474 (89.03%)	3022199 (6.73%)	830020 (1.85%)	1071742 (2.39%)	77.40%
506	Treatment	43660502	39093910 (89.54%)	2863022 (6.56%)	722662 (1.66%)	980908 (2.25%)	70.80%
506	Treatment	37280523	32601953 (87.45%)	3329725 (8.93%)	591735 (1.59%)	757110 (2.03%)	78.60%
506	Treatment	50216886	43881482 (87.38%)	4466693 (8.89%)	803551 (1.60%)	1065160 (2.12%)	71.50%
587	Treatment	49500427	43151072 (87.17%)	4570321 (9.23%)	773842 (1.56%)	1005192 (2.03%)	74.40%
587	Treatment	41764992	36311859 (86.94%)	3889085 (9.31%)	667580 (1.60%)	896468 (2.15%)	75%
587	Treatment	44072172	38394486 (87.12%)	4033529 (9.15%)	702205 (1.59%)	941952 (2.14%)	75.10%

C76-16	Treatment	47419319	41051649 (86.57%)	4630751 (9.77%)	720477 (1.52%)	1016442 (2.14%)	74.90%
C76-16	Treatment	40858205	35399986 (86.64%)	3911482 (9.57%)	635605 (1.56%)	911132 (2.23%)	72.40%
C76-16	Treatment	39895848	34661111 (86.88%)	3699491 (9.27%)	652797 (1.64%)	882449 (2.21%)	77.40%

Table 3.2 Summary of library and alignment of reads to *A. hypogea* genome in each library

Genotype	Total Genes	Annotated	DEGs	Up-regulated	Down-regulated	Annotated DEGs
Tifrunner	73575	66437	7780	5310	2470	6410
587	73898	66373	13005	7718	5287	10605
506	73900	66378	9767	6052	3715	8065
C76-16	73610	66445	12348	7172	5176	10210

Table 3.3 The enriched GO terms of the DEGs that common in drought tolerant genotypes and drought susceptible genotypes.

GO term	Description	Number in input list	Number in BG/Ref	FDR
GO:0006464	cellular protein modification process	1137	3209	4.10E-22
GO:0036211	protein modification process	1137	3209	4.10E-22
GO:0043412	macromolecule modification	1178	3355	1.50E-21
GO:0006468	protein phosphorylation	940	2637	2.00E-19
GO:0006793	phosphorus metabolic process	1229	3587	1.20E-18
GO:0006796	phosphate-containing compound metabolic process	1225	3579	1.60E-18
GO:0016310	phosphorylation	998	2905	1.10E-15
GO:0008037	cell recognition	98	181	3.40E-12
GO:0009856	pollination	98	181	3.40E-12
GO:0044706	multi-multicellular organism process	98	181	3.40E-12
GO:0048544	recognition of pollen	98	181	3.40E-12
GO:0009875	pollen-pistil interaction	98	181	3.40E-12

GO:0044702	single organism reproductive process	105	202	1.30E-11
GO:0032501	multicellular organismal process	113	225	2.80E-11
GO:0044703	multi-organism reproductive process	105	206	5.80E-11
GO:0007154	cell communication	435	1193	8.90E-11
GO:0022414	reproductive process	109	220	1.90E-10
GO:0000003	reproduction	109	220	1.90E-10
GO:0051704	multi-organism process	108	218	2.20E-10
GO:0008152	metabolic process	5124	17453	1.20E-08
GO:0044699	single-organism process	2592	8647	1.30E-07
GO:0050789	regulation of biological process	992	3152	1.60E-06
GO:0065007	biological regulation	1051	3355	1.70E-06
GO:0050794	regulation of cellular process	974	3097	2.30E-06
GO:0009765	photosynthesis, light harvesting	23	30	4.20E-06
GO:0006470	protein dephosphorylation	63	128	1.20E-05
GO:0010468	regulation of gene expression	569	1758	3.40E-05
GO:0044710	single-organism metabolic process	1757	5879	4.20E-05

GO:1901565	organonitrogen compound catabolic process	59	121	4.20E-05
GO:0051252	regulation of RNA metabolic process	554	1712	4.50E-05
GO:0019219	regulation of nucleobase-containing compound metabolic process	561	1738	5.20E-05
GO:2001141	regulation of RNA biosynthetic process	552	1709	5.30E-05
GO:0006355	regulation of transcription, DNA-templated	552	1709	5.30E-05
GO:1903506	regulation of nucleic acid-templated transcription	552	1709	5.30E-05
GO:0016567	protein ubiquitination	55	112	6.50E-05
GO:0044237	cellular metabolic process	3180	10932	7.10E-05
GO:0051171	regulation of nitrogen compound metabolic process	563	1752	7.60E-05
GO:0010556	regulation of macromolecule biosynthetic process	557	1734	8.20E-05
GO:2000112	regulation of cellular macromolecule biosynthetic process	557	1734	8.20E-05
GO:0009889	regulation of biosynthetic process	557	1734	8.20E-05
GO:0071704	organic substance metabolic process	3747	12961	8.20E-05
GO:0031326	regulation of cellular biosynthetic process	557	1734	8.20E-05
GO:0044763	single-organism cellular process	1501	5011	0.0001
GO:0032446	protein modification by small protein conjugation	55	114	0.00011

GO:0044711	single-organism biosynthetic process	446	1366	0.00013
GO:0009987	cellular process	3940	13694	0.00021
GO:0007165	signal transduction	334	999	0.00025
GO:0023052	signaling	334	999	0.00025
GO:0044700	single organism signaling	334	999	0.00025
GO:0044267	cellular protein metabolic process	1392	4656	0.00027
GO:0070647	protein modification by small protein conjugation or removal	57	123	0.00029
GO:0016311	dephosphorylation	77	180	0.00029
GO:0050896	response to stimulus	844	2748	0.00038
GO:0060255	regulation of macromolecule metabolic process	579	1834	0.00038
GO:0044238	primary metabolic process	3472	12070	0.00052
GO:0006950	response to stress	575	1827	0.00058
GO:0019222	regulation of metabolic process	579	1841	0.00058
GO:0043648	dicarboxylic acid metabolic process	48	102	0.00093
GO:0009628	response to abiotic stimulus	49	105	0.00098
GO:0031323	regulation of cellular metabolic process	564	1800	0.0011

GO:0080090	regulation of primary metabolic process	564	1802	0.0012
GO:0006952	defense response	342	1056	0.003
GO:0044260	cellular macromolecule metabolic process	2328	8056	0.003
GO:0055114	oxidation-reduction process	984	3302	0.0068
GO:0009308	amine metabolic process	49	113	0.0094
GO:0044281	small molecule metabolic process	595	1948	0.011
GO:0019538	protein metabolic process	1629	5612	0.013
GO:0043170	macromolecule metabolic process	2597	9085	0.013
GO:0015914	phospholipid transport	19	33	0.016
GO:0015748	organophosphate ester transport	19	33	0.016
GO:0006022	aminoglycan metabolic process	24	46	0.019
GO:0009266	response to temperature stimulus	35	77	0.027
GO:0009408	response to heat	35	77	0.027
GO:0006040	amino sugar metabolic process	24	47	0.027
GO:0044723	single-organism carbohydrate metabolic process	237	731	0.032
GO:0044283	small molecule biosynthetic process	190	573	0.032

GO:0034654	nucleobase-containing compound biosynthetic process	709	2372	0.032
GO:1901362	organic cyclic compound biosynthetic process	794	2676	0.037
GO:0016053	organic acid biosynthetic process	161	479	0.041
GO:0009066	aspartate family amino acid metabolic process	29	62	0.043
GO:0009081	branched-chain amino acid metabolic process	14	23	0.045
GO:0006629	lipid metabolic process	319	1017	0.045
GO:0009082	branched-chain amino acid biosynthetic process	10	14	0.045
GO:0051716	cellular response to stimulus	398	1292	0.049
GO:0043650	dicarboxylic acid biosynthetic process	8	10	0.049
GO:0006537	glutamate biosynthetic process	8	10	0.049
GO:0004674	protein serine/threonine kinase activity	722	1849	5.30E-26
GO:0016301	kinase activity	1072	2982	2.10E-23
GO:0004672	protein kinase activity	957	2649	7.90E-22
GO:0016773	phosphotransferase activity, alcohol group as acceptor	1055	2984	1.00E-20
GO:0016740	transferase activity	2096	6693	4.20E-12
GO:0016772	transferase activity, transferring phosphorus-containing groups	1218	3740	1.20E-11

GO:0043565	sequence-specific DNA binding	285	714	1.20E-11
GO:0003700	transcription factor activity, sequence-specific DNA binding	364	1021	1.50E-07
GO:0001071	nucleic acid binding transcription factor activity	364	1021	1.50E-07
GO:0003824	catalytic activity	5256	18125	2.20E-06
GO:0030554	adenyl nucleotide binding	1753	5820	1.10E-05
GO:0032559	adenyl ribonucleotide binding	1727	5732	1.10E-05
GO:0005506	iron ion binding	322	927	1.80E-05
GO:0016791	phosphatase activity	121	299	5.00E-05
GO:0042578	phosphoric ester hydrolase activity	159	418	8.10E-05
GO:0005524	ATP binding	1447	4811	0.0001
GO:0016597	amino acid binding	41	76	0.0001
GO:0004721	phosphoprotein phosphatase activity	71	157	0.00011
GO:0030246	carbohydrate binding	143	375	0.00019
GO:0004722	protein serine/threonine phosphatase activity	38	70	0.00019
GO:0001883	purine nucleoside binding	1858	6291	0.00027
GO:0017076	purine nucleotide binding	1887	6394	0.00027

GO:0032555	purine ribonucleotide binding	1858	6291	0.00027
GO:0032550	purine ribonucleoside binding	1858	6291	0.00027
GO:0032553	ribonucleotide binding	1884	6388	0.0003
GO:0001882	nucleoside binding	1863	6325	0.00042
GO:0032549	ribonucleoside binding	1862	6322	0.00042
GO:0097367	carbohydrate derivative binding	1898	6451	0.00042
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	332	1009	0.0013
GO:0043167	ion binding	1600	5433	0.0014
GO:0016491	oxidoreductase activity	1098	3668	0.0018
GO:0010333	terpene synthase activity	47	101	0.0018
GO:0035639	purine ribonucleoside triphosphate binding	1578	5370	0.0022
GO:0004806	triglyceride lipase activity	36	72	0.0024
GO:0000166	nucleotide binding	2207	7659	0.0073
GO:0008889	glycerophosphodiester phosphodiesterase activity	11	14	0.0073
GO:0030247	polysaccharide binding	56	132	0.0073

GO:0016838	carbon-oxygen lyase activity, acting on phosphates	48	109	0.0073
GO:1901265	nucleoside phosphate binding	2207	7659	0.0073
GO:0001871	pattern binding	56	132	0.0073
GO:0051213	dioxygenase activity	136	385	0.012
GO:0043169	cation binding	1478	5075	0.013
GO:0004012	phospholipid-translocating ATPase activity	19	33	0.015
GO:0005548	phospholipid transporter activity	19	33	0.015
GO:0046872	metal ion binding	1471	5059	0.016
GO:0020037	heme binding	284	883	0.017
GO:0036094	small molecule binding	2221	7751	0.018
GO:0031406	carboxylic acid binding	44	103	0.026
GO:0016725	oxidoreductase activity, acting on CH or CH2 groups	6	6	0.028
GO:0048037	cofactor binding	362	1158	0.028
GO:0016165	linoleate 13S-lipoxygenase activity	22	43	0.041
GO:0070402	NADPH binding	7	8	0.043
GO:0016639	oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor	8	10	0.048

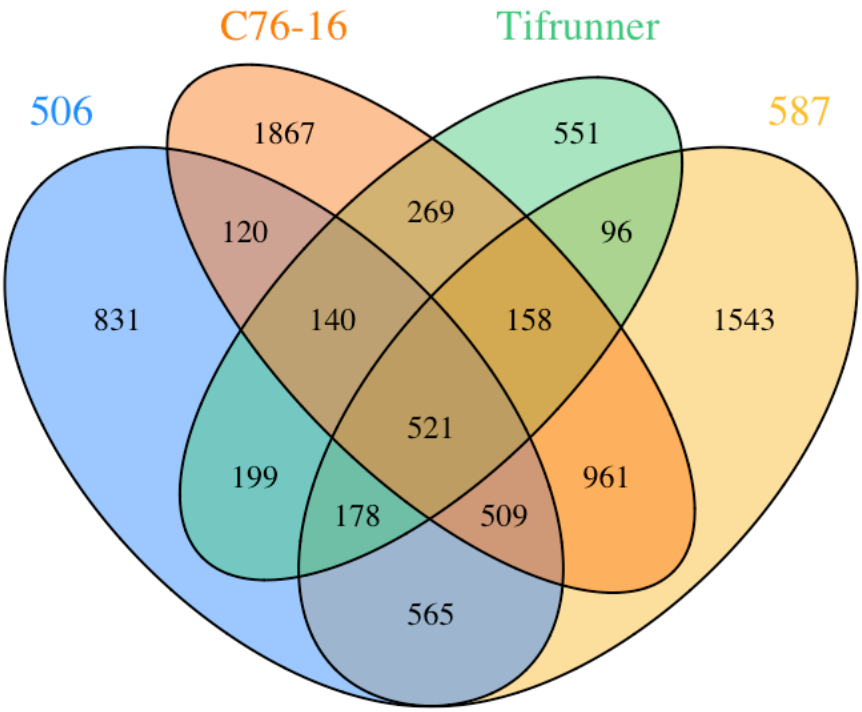
Table 3.4 The enriched KEGG ontology terms of the DEGs that common in drought tolerant genotypes and drought susceptible genotypes.

Term	Database	ID	Input number	Background number	P-Value	Corrected P-Value
Metabolic pathways	KEGG PATHWAY	KO01100	388	1910	7.38E-52	4.89E-50
Biosynthesis of secondary metabolites	KEGG PATHWAY	KO01110	255	1076	4.02E-43	2.21E-41
Circadian rhythm - plant	KEGG PATHWAY	KO04712	34	36	9.43E-20	2.70E-18
Phenylpropanoid biosynthesis	KEGG PATHWAY	KO00940	48	157	3.10E-12	5.48E-11
Starch and sucrose metabolism	KEGG PATHWAY	KO00500	53	202	3.16E-11	4.95E-10
Photosynthesis - antenna proteins	KEGG PATHWAY	KO00196	17	22	1.22E-09	1.52E-08
Alanine, aspartate and glutamate metabolism	KEGG PATHWAY	KO00250	23	48	1.77E-09	2.14E-08
Stilbenoid, diarylheptanoid and gingerol biosynthesis	KEGG PATHWAY	KO00945	21	46	1.73E-08	1.93E-07
Phenylalanine metabolism	KEGG PATHWAY	KO00360	20	42	2.17E-08	2.40E-07
Amino sugar and nucleotide sugar metabolism	KEGG PATHWAY	KO00520	36	135	3.06E-08	3.35E-07
alpha-Linolenic acid metabolism	KEGG PATHWAY	KO00592	18	36	6.17E-08	6.41E-07
Isoquinoline alkaloid biosynthesis	KEGG PATHWAY	KO00950	14	23	3.01E-07	2.87E-06
Phenylalanine, tyrosine and tryptophan biosynthesis	KEGG PATHWAY	KO00400	21	57	3.17E-07	3.02E-06

Plant-pathogen interaction	KEGG PATHWAY	KO04626	38	167	4.22E-07	3.94E-06
Tyrosine metabolism	KEGG PATHWAY	KO00350	17	40	8.64E-07	7.55E-06
Limonene and pinene degradation	KEGG PATHWAY	KO00903	17	44	2.46E-06	1.99E-05
Valine, leucine and isoleucine degradation	KEGG PATHWAY	KO00280	17	48	6.36E-06	4.66E-05
beta-Alanine metabolism	KEGG PATHWAY	KO00410	15	40	1.27E-05	8.98E-05
Ascorbate and aldarate metabolism	KEGG PATHWAY	KO00053	15	41	1.61E-05	0.00011201
Tropane, piperidine and pyridine alkaloid biosynthesis	KEGG PATHWAY	KO00960	14	36	1.77E-05	0.00012194
Carbon fixation in photosynthetic organisms	KEGG PATHWAY	KO00710	18	69	0.000104	0.00060361
Glucosinolate biosynthesis	KEGG PATHWAY	KO00966	9	19	0.000177	0.00096339
Glutathione metabolism	KEGG PATHWAY	KO00480	20	93	0.000408	0.00201203
Terpenoid backbone biosynthesis	KEGG PATHWAY	KO00900	15	58	0.000412	0.00203127
Diterpenoid biosynthesis	KEGG PATHWAY	KO00904	9	22	0.000417	0.00204405
Arginine and proline metabolism	KEGG PATHWAY	KO00330	14	53	0.000531	0.00252985
Base excision repair	KEGG PATHWAY	KO03410	12	43	0.000869	0.00387834
AGE-RAGE signaling pathway in diabetic complications	KEGG PATHWAY	KO04933	8	20	0.000979	0.00428699

Biosynthesis of amino acids	KEGG PATHWAY	KO01230	38	255	0.001263	0.00538193
Pentose and glucuronate interconversions	KEGG PATHWAY	KO00040	17	81	0.001351	0.00572059
Cysteine and methionine metabolism	KEGG PATHWAY	KO00270	21	112	0.001387	0.00584628
Nucleotide excision repair	KEGG PATHWAY	KO03420	15	69	0.001878	0.0077515
Ubiquinone and other terpenoid-quinone biosynthesis	KEGG PATHWAY	KO00130	10	35	0.001983	0.00814361
Inositol phosphate metabolism	KEGG PATHWAY	KO00562	14	68	0.004012	0.01497196
Thiamine metabolism	KEGG PATHWAY	KO00730	5	11	0.005897	0.02069711
Monoterpenoid biosynthesis	KEGG PATHWAY	KO00902	4	7	0.00763	0.02585832
Pyruvate metabolism	KEGG PATHWAY	KO00620	15	85	0.010056	0.03283136
Porphyrin and chlorophyll metabolism	KEGG PATHWAY	KO00860	10	48	0.012979	0.04046835
Glycine, serine and threonine metabolism	KEGG PATHWAY	KO00260	13	72	0.013615	0.04225667
Pyrimidine metabolism	KEGG PATHWAY	KO00240	18	116	0.015576	0.04708738
Fatty acid elongation	KEGG PATHWAY	KO00062	8	35	0.01621	0.04871628
DNA replication	KEGG PATHWAY	KO03030	10	50	0.016307	0.04893789
2-Oxocarboxylic acid metabolism	KEGG PATHWAY	KO01210	13	74	0.01635	0.04899404

A


C76-16
Tifrunner
506
587
1867
551
120
269
96
831
140
158
1543
521
199
961
178
509
565

B

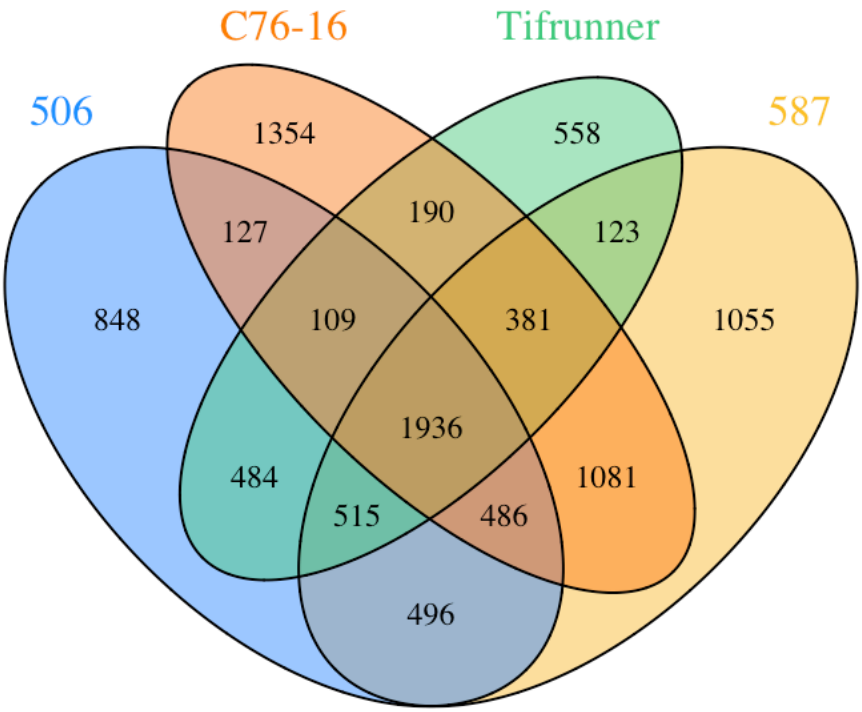
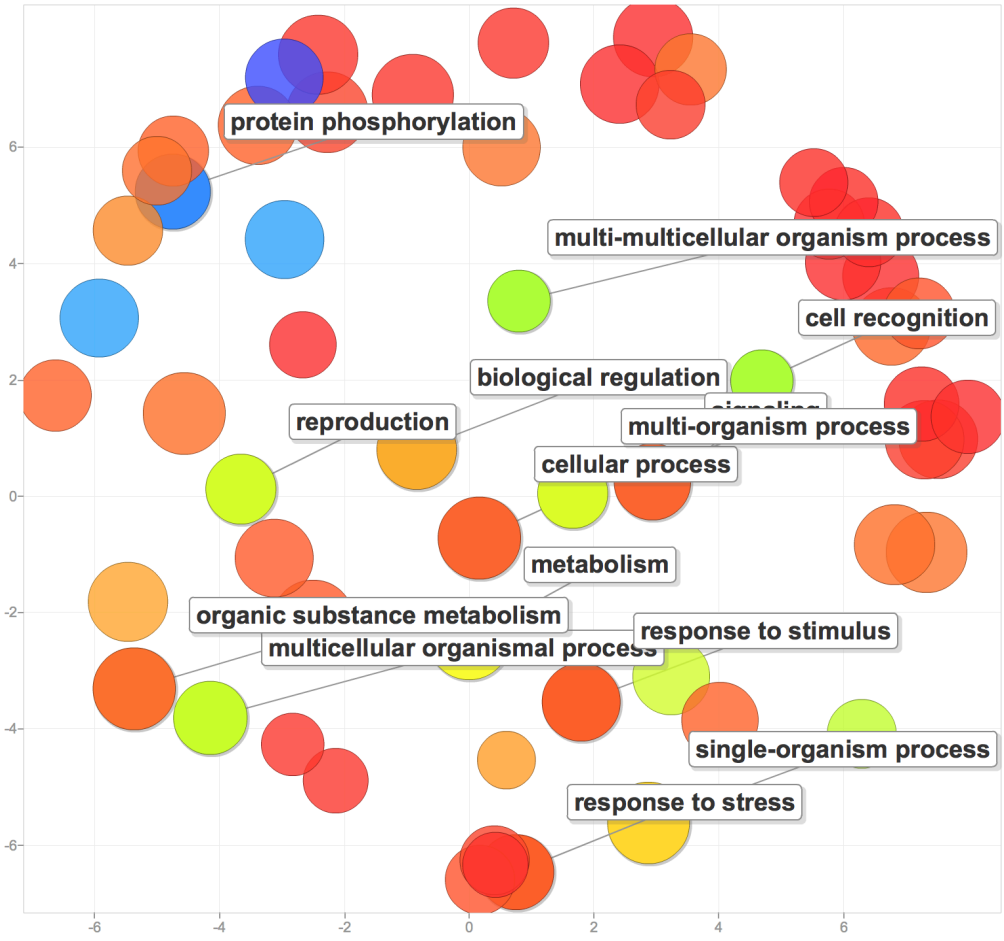


Figure 3.1 Comparison of the annotated DEGs between the drought susceptible genotypes, Tifrunner and AU-506, and drought tolerant genotypes, C76-16 and AU-587 showing (A) up regulated genes, (B) down-regulated genes.

A


protein phosphorylation
multi-multicellular organism process
cell recognition
biological regulation
reproduction
multi-organism process
cellular process
metabolism
organic substance metabolism
response to stimulus
multicellular organismal process
single-organism process
response to stress
6
4
2
0
-2
-4
-6
-6
-4
-2
0
2
4
6

B

Figure 3.2 Significantly enriched GO categories in differentially expressed genes identified in all four genotypes (A) Enriched GO categories in biological process. (B) Enriched GO categories in molecular function.

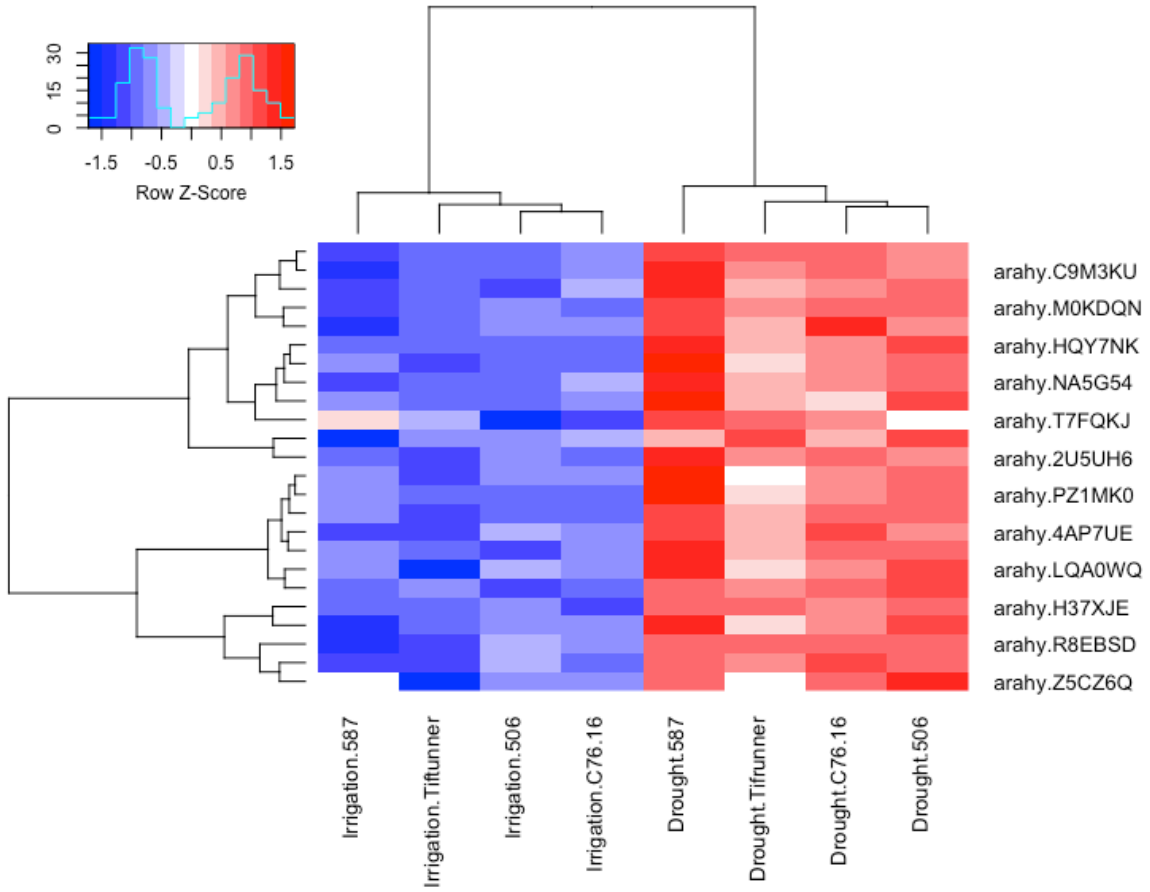


Figure 3.3 Expression profiles of the ABA related differentially expressed genes shared by all four genotypes under irrigated and drought treatments. Log10 transformed FPKM values were used. "Blue" color indicates no expression or low expression level; and "red" color indicates high expression level

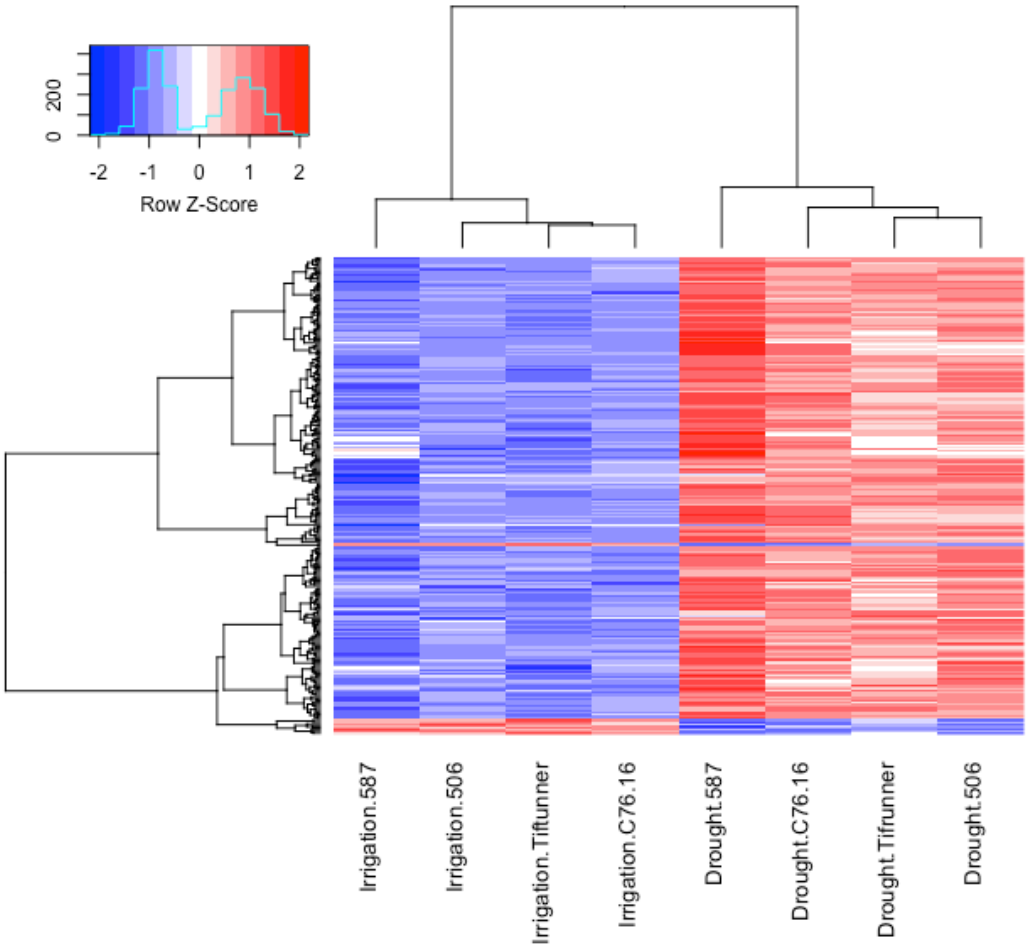


Figure 3.4 Expression profiles of the differentially expressed genes shared by all four genotypes under irrigated and drought treatments. Log10 transformed FPKM values were used. "Blue" color indicates no expression or low expression level; and "red" color indicates high expression level

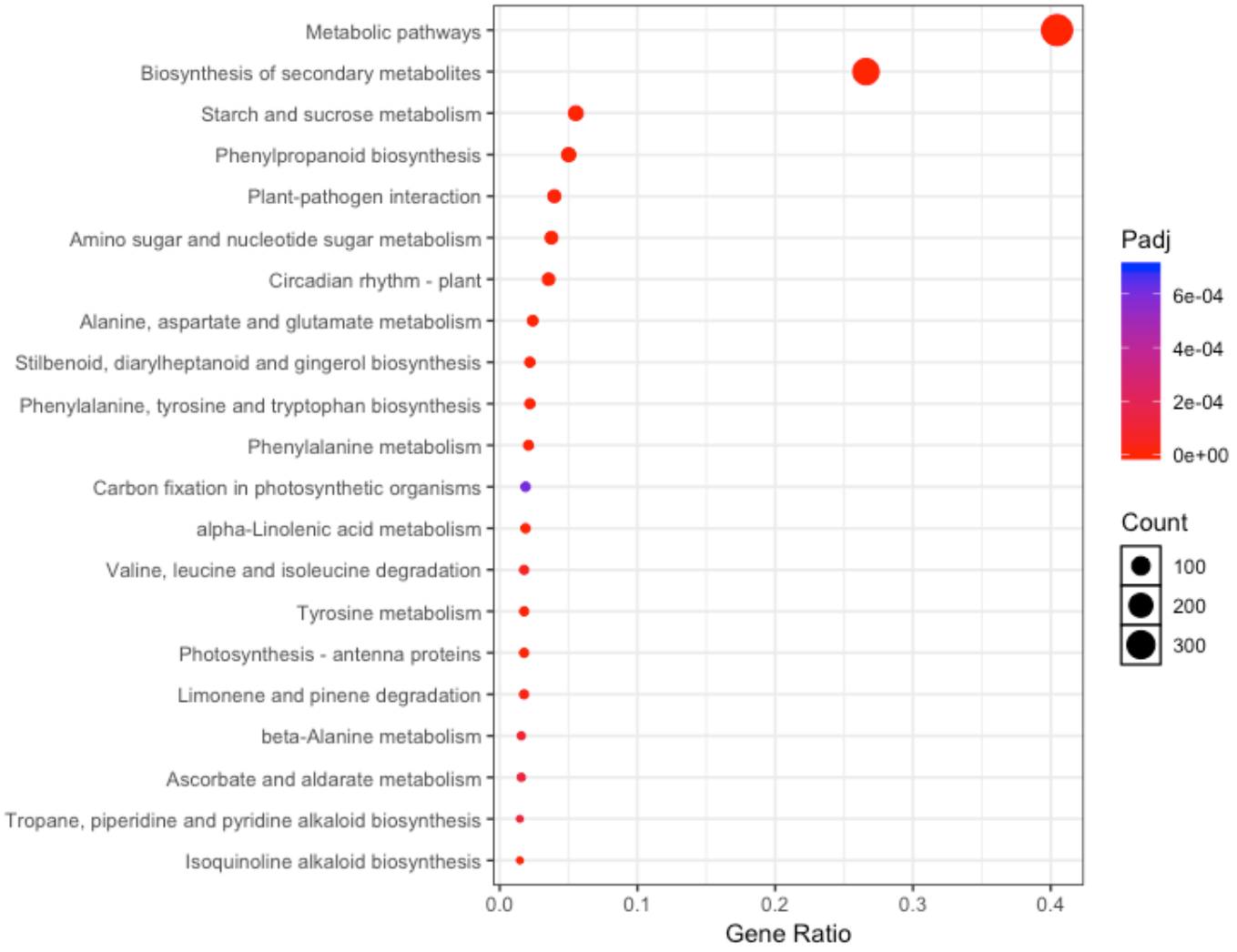


Figure 3.5 KEGG pathway enrichment analysis based on the differentially expressed genes shared by all four genotypes under irrigated and drought treatments.

Chapter Four

Research Summary and Future Perspectives

4.1 Research Summary

The research projects mainly focused on investigation of the effects of drought stress on symbiotic nitrogen fixation in various peanut genotypes and identification drought responsive genes preferentially expressed to drought stress in different peanut genotypes. In the first study, SNF and carbon isotope discrimination were evaluated and compared between different genotypes under three irrigation regimes. We demonstrated the mid-season drought had a greater impact on nitrogen fixation and carbon isotope discrimination than late-season drought. In the meantime, drought resistant lines have higher nitrogen fixation rate and carbon isotope discrimination than drought susceptible lines. Significant genotypic variability was also found in nitrogen fixation and carbon isotope discrimination among peanut genotypes after drought treatments. Additionally, the genotype AU-587 performed the best under both mid-season and late season drought. After identified the genotypes with highest and lowest drought tolerance, transcriptome analysis was performed to identify the drought responsive genes in the second study. Both unique and well-known drought responsive genes were identified in this study. Then, GO term enrichment and biochemical pathway analysis showed that drought stress caused changes in the following pathways, including protein modification related pathways, stress responses, reproduction, signaling, oxidation-reduction process and photosynthesis. These findings suggest that drought tolerance of peanut may be related to the regulation of hormone

biosynthesis and signaling, reduction of oxidative damage, stabilization of cell proteins and structures, and maintenance of energy and carbon supply.

4.2 Future Perspectives

The results achieved from these two studies provide great resources for understanding the drought stress regulatory mechanism of peanut and guides future efforts attempting to breed drought tolerant peanut genotypes. However, further efforts are still needed on investigating the molecular signaling and regulatory mechanisms of peanut under drought stress because of the complex genetic background. Additional detailed analyses of the drought responsive genes and pathways identified in this study should be performed to characterize their possible functional roles in drought tolerance of peanut. After identifying the possible functions of the drought responsive genes, targeted gene modification and marker assisted selection could be carried out for selection the drought tolerant genotypes. Overall, this study provides novel insights into peanut responses to drought stress.

Appendix

Characterization of ACC deaminase producing *Bradyrhizobium sp.* Isolated from peanut nodules

Introduction

The gaseous hormone ethylene (C_2H_4) synthesized in plant tissues from precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is involved in multiple physiological and developmental processes in plants, such as tissue differentiation, lateral bud development, seedling emergence, leaf and flower senescence, root hair development and elongation, anthocyanin synthesis, fruit ripening and degreening and the production of volatile compounds responsible for aroma in fruits (Abeles et al. 1992; Spaink 1997; Bleecker and Kende 2000). Ethylene also regulates plant responses to biotic and abiotic stresses (Abeles et al. 1992; Roman et al. 1995; O'Donnell et al. 1996; Penninckx et al. 1998). Under ambient conditions, plants produce required levels of ethylene, conferring beneficial effects on plant growth and development; however, the plant often significantly increases endogenous ethylene production in response to biotic and abiotic stresses, which has adverse effects on plant growth and is thought to be responsible for plant senescence (Abeles et al. 1992; Woltering and Van Doorn 1988; Nayani et al. 1998; Ali et al. 2012)

Plant growth-promoting rhizobacteria (PGPR) are a group of free-living saprophytic bacteria that can be found in the rhizosphere in association with the root system and directly or indirectly enhance the growth and development of the plant (Kloepper and Beauchamp 1992; Liu et al. 1995). These bacteria are beneficial to plant growth as plants are often subjected to biotic or abiotic stresses that induce the production of ethylene such as salt stress (Cheng et al. 2007; Mayak et al. 2004; Zahir et al. 2009), flooding stress (Grichko and Glick 2001), drought stress

(Mayak et al. 2004), heavy metal stress (Belimov et al. 2005; Stearns et al. 2005), and pathogen attack (Wang et al. 2000). PGPR produced enzyme ACC deaminase can cleave ethylene precursor ACC to ammonia and α-ketobutyrate (Jacobson et al. 1994; Glick et al., 1998), thereby lowering the level of synthesized ethylene in plant under various stresses (Glick et al. 1998). In turn, decreased ethylene levels allow the plant to be more resistant to a wide variety of environmental stresses (Saraf et al., 2010).

Drought stress is one of the major agricultural problems limiting the growth of plants and the production of crops in most of the arid and semiarid regions of the world. Drought stress affects the plant-water relations at both the cellular and whole-plant level, causing both specific and non-specific reactions and damage. However, several studies have reported that inhibitory effects of ethylene induced by drought stress might have been reduced through the ACC deaminase activity of the PGPR. Inoculation of plants with PGPR containing ACC deaminase partially or completely eliminated the "drought stress-imposed effects" on root and shoot growth, fresh and dry weights, and number of leaves per plant of peas (Zahir et al., 2008). This might be due to suppression of the stress-induced accelerated synthesis of ethylene by the ACC deaminase activity of these PGPR in the inoculated roots. Sharp increases in ACC levels and, consequently, ethylene synthesis in plants under drought stress conditions has been frequently reported (Apelbaum and Yang 1981). The rhizobacteria having ACC deaminase activity are effective in promoting plant growth and water use efficiency under drought conditions, by lowering the ethylene or ACC accumulation whose higher levels have inhibitory effects on root and shoot growth, It is highly likely that rhizobacteria containing ACC deaminase might have decreased the drought stress induced ethylene in inoculated plants, which resulted in better growth of plants even at low moisture levels. Therefore, inoculation with rhizobacteria containing ACC

deaminase could be helpful in eliminating the inhibitory effects of drought stress on the growth of plants. Dodd et al. (2005) investigated the physiological responses of pea (*pisum sativum* L.) to inoculation with ACC deaminase bacteria *V. paradoxus* 5C-2 under moisture stress and watering condition. The bacterial effects were more pronounced and more consistent under controlled soil drying (moisture stress conditions). In addition, several studies have reported the presence of ACC deaminase in *Rhizobium* and *Bradyrhizobium.* Therefore, we attempted to isolate and characterize ACC deaminase producing *Bradyrhizobium* from peanut nodules to further determine the benefits of ACC deaminase producing *Bradyrhizobium* to drought-stressed plants.

Materials and methods

Plant Materials

Three cultivated peanut genotypes with varying drought tolerance characteristics were selected based on previous yield trial under drought stress in 2015. Tifrunner (susceptible), C76-16 (resistant) and AU-587, a recombinant inbred line (RIL) derived from the cross 'Tifrunner x C76-16' were used in this study. The seeds were planted in a mixture of peat and fine sandy loam soil (3:4) in a 6 gallons container (top diameter: 35.5 cm; bottom diameter: 30 cm; height: 30 cm). Representative soil sample were collected and sent to Soil testing lab on 06/09/2016 for soil test.

A total of 72 seeds of each genotype line was planted in six of 36-cell trays on 06/03/2016. Seeds were inoculated with *Rhizobium* (Histick N/T) (7oz/100 lbs of seed) prior to planting. After planting, all cells were fully irrigated to saturation. Three weeks later, 24 plants of each genotype were selected based on the size of plant for transplanting into individual pot. A

randomized complete block design with four irrigation treatments and three replications were applied. Irrigation treatments include mid-season drought, mid-season irrigation, late-season drought and late-season irrigation. Each treatment was consisting of 18 plants (6 plant of each genotype), respectively.

Irrigation treatments

Based on the soil weight and water holding capacity (WHC), plants were equally irrigated with tap water to maintain water content at 100% WHC. Water withholding were initiated right after irrigation at 60 DAP for mid-season drought treatment, and 90 DAP for late-season drought treatment. A seven-day drought treatment were applied to both mid-season and late-season drought. At the end of drought treatment, a seven-days recovery was applied to stressed plants. Besides the irrigation practice, all culture practices were followed the peanut production practices published by the University of Georgia. All pots were rotated every three days to minimize differences in ambient of the greenhouse.

Root tissue sampling

For the irrigated treatments, the plants were watered three days before sampling; however, the plants from drought treatments were harvested immediately after the seven-day drought treatment. In the meantime, the root tissues were carefully recovered and washed. The roots were placed on a paper towel to absorb the water and air dry for about 15 minutes. All roots were placed on ice and transferred to the lab. The *Bradyrhizobium* sp. were isolated from peanut nodules and stored at -80 °C.

Isolation of *Bradyrhizobium* sp. from peanut nodules

Effective nodules (i.e. nodules with a pink center) were collected from the central tap root system. Two nodules were carefully removed from each root sample. The root nodules were

washed with distilled water and soaked in 95% ethanol for 60 sec. Then, the nodules were rinsed with sterile distilled water 3 times and soaked in 3% H_2O_2 for two minutes. After surface sterilization, a drop of water was added, and the nodules were crushed to obtain a cloudy suspension. The nodule suspension was then be transferred to Mannitol-Yeast extract-Congo red agar plate (YMA: Mannitol, 10 g; yeast extract, 0.2 g; NaCl, 0.1 g; $CaCO_3$, 3 g; Congo red, 2.5 ml 1 % solution; Agar, 15 g; Distilled water, 1000 ml) (Vincent, 1970). The plates were incubated at 28 °C until appearance of single colonies. Single colonies were transferred to YMA several times until the pure culture was achieved.

Screening for ACC deaminase activity

The ACC deaminase activity of *Bradyrhizobium* isolates was screened based on the ability of the isolate to use ACC as a sole nitrogen source. ACC deaminase activity was induced followed by Ma et al., 2003. *Bradyrhizobium* isolates were streaked out on YMA plates (Vincent, 1970) and incubated at 28 °C for 4-7 days to obtain single colony. *Bradyrhizobium isolates* were then transferred into 5 ml of Tryptone-Yeast extract broth (TY: 5 g of Tryptone, 3 g of Yeast Extract and 0.44 g of $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water) (Atlas, 1993) and incubated at 28 °C for 2-4 days with shaking (100 rpm) until they reached stationary phase. The bacterial solutions were adjusted to approximately same CFU/mL, and 1 mL of the solutions were centrifuged for 10 mins at 10,000 x g to obtain bacterial pellet. The pellet was washed twice with 1 mL 0.1 M Tris-HCl (pH = 7.5) and then resuspended in 2 mL M9 minimal medium supplemented with 5 mM ACC. The resulting bacterial solution was incubated at 25 °C with shaking (100 rpm) for 36-40 hours, and cells were harvested and washed twice as described above for ACC deaminase activity assay (stored at -20 °C).

ACC deaminase activity

ACC deaminase activity was determined according to the method described in Penrose and Glick (2003) which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The number of the µmol of α-ketobutyrate produced was determined by comparing the absorbance at 540 nm of the sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µmol. All of the rhizobial cells harvested above were suspended in 200 µL of 0.1 M Tris-HCl (pH=8.5). Then, ten microliters of toluene (5% final concentration, v/v) were added to the cell suspension and vortexed at the highest speed for 30s. The 200 µL toluenized cells were then mixed with 20 µL of 0.5 M ACC and incubated at 30 □C for 15 min. Following incubation, 1 mL of 0.56 M HCl were added into the mixture and then vortex again. After vortex, the mixture was centrifuged at 10,000 rpm for 1 min at room temperature. One milliliter of the supernatant was removed and mixed with 800 µL of 0.56 M HCl and 300 µL of the 2,4-dinitrophenylhydrazine reagent for incubation at 30 °C for 30 min. Following the addition and mixing of 2 mL of 2M NaOH, the absorbance of the mixture was measured at 540 nm.

Protein concentration determination

The protein concentration of toluenized cells was determined by the method of Bradford (1976). A 26.5-µl aliquot of the toluene-labilized bacterial cell sample was used for the ACC deaminase enzyme assay was first diluted with 173.5 µl of 0.1 M Tris-HCl (pH = 8.0) and then boiled with 200 µL of 0.1 N NaOH for 10 min. After the cell sample was cooled to room temperature, the protein concentration was determined by measuring the absorbance at 595 nm immediately after mixing the solution with 200 µL of Bradford's reagent. Bovine serum albumin was used to establish a standard curve.

Rhizobium fingerprinting

Single colonies of rhizobium isolates were grown in 5 ml of TY broth at 28 °C for 2-4 days with shaking (100 rpm) until they reached stationary phase. Genomic DNA was isolated using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The DNA was quantified with a NanoDrop ND-1000 UV spectrophotometer (Thermo Scientific, Wilmingtong, USA), and were stored at -20 °C. The reaction mixture (25 ul) contained 1 x GoTaq Master Mix (Promega), 0.5 µM of each primer, and 2.5 µl of the template DNA. All reactions began with a hold at 95 °C for 5 min, followed by 35 cycles of 94 °C 1 min, 60 °C 1 min and 72 °C for 1 min. A final elongation step was performed at 72 °C for 5 mins.

Characterization of 16S rRNA gene

Bacterial genomic DNA was isolated as described above and the 16S rRNA gene was amplified by PCR using a universal primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Amplification reactions were performed in a total volume of 25 µL using the following program: 95 °C for 3 min, followed by 30 cycles consisting of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1.5 min and a final extension period of 72 °C for 10 mins. The PCR product (approx. 1,465 bp) was purified and sequenced (GeneScript Bioteck Corp.).

Characterization of ACC deaminase gene

The known ACC deaminase gene (blr0241) for *Bradyrhizobium* sp. was obtained on NCBI and the primers 5’-ATGCTGGAAAAATTCGCGCGC-3’ and 5’-CTAGCCGTTTCTGAACGCGTAGC-3’ was designed to amplify the ACC deaminase gene. The DNA was amplified using the following program: 90 seconds at 95 °C, 35 cycles of 1 min denaturation at 94 °C and 50 s at 58 °C, and 1 min of elongation at 72 °C. Then, a final

elongation of 5 min at 72 °C was included. Following PCR, bands were extracted, cloned, and sequenced. The sequences (*acdS* and 16S rRNA genes) obtained were compared with the existing database of *acdS* and 16S rRNA gene.

Leonard jar experiment

Isolated strains with positive ACC deaminase activity were selected to test for nodulation efficacy. Freshly inoculated Yeast extract-Mannitol broth was incubated at 30 °C for 2-4 days. After incubation, 1 mL of rhizobia solution was transferred into sterilized 1.5 ml Eppendorf tube and centrifuged at 8000 rpm for 5 mins. The pellet was collected and washed twice with 1 mL sterilized saline (0.85% NaCl solution) and resuspended in 1 mL saline as inoculum used in Leonard jar experiment. Leonard jar experiment (Somasegaran and Hoben et al., 2012) was conducted to examine the nodulation efficacy for the strains with positive ACC deaminase activity. Seeds were surface sterilized with 3% NaClO solution for 5 min and washed 10 times with sterile water. Clean seeds were soaked in sterile water overnight to absorb water. The next morning, seeds were transferred to 0.75% water agar and covered with moisturized sterilized filter paper. The plate with seeds were incubated at 25 °C in the growth chamber for two days before transplanting to Leonard jar containing Broughton and Dilworth N-free plant nutrient solution (Broughton and Dilworth, 1971) to provide nitrogen free environment. The germinated seeds were transplanted at 2.5 cm depth of the sands in the Leonard Jar. After transplanting, the seeds were inoculated with 1 mL of inoculum. The Leonard jars were placed under artificial grow light (6:00 am to 8:00 pm) at room temperature. The plants were harvested at 35 days after planting (DAP), followed by roots examination for nodulation.

Determination of nitrogen fixation potential

This experiment was conducted in 30 oz Styrofoam water cup using sand as growth medium. Three strains were selected from Leonard jar experiment. Four replications for each strain and 2 controls (1 negative control and 1 fertilized nitrogen control) with a total of 20 cups were used in the experiment. Sand was washed with tap water three times and soaked in the water overnight, followed by overnight oven-dried at 70 ˚C. After autoclaving, 1 kg of sand were filled into the Styrofoam cup. Three germinated seeds were transplanted into one cup at 2.5 cm depth in the cup. Each seed was inoculated with 1 ml of inoculum. The plants were grown in the growth chamber with 100% humility, 14 hours artificial grow light (6:00 am to 8:00 pm), 30 ˚C daytime temperature and 25 ˚C nighttime temperature. After 10 DAP, one germinated seed was pulled out to make sure all the seeds have uniform growth initially. In addition, the cups were weighed every day and water content was adjusted to field capacity. All the plants were harvested at 60 DAP. Shoot biomass, root biomass, nodule count was taken at harvest.

Results

ACC deaminase activity

By isolating the strains from the peanut nodules, a total of 87 stains were isolated using YMA with Congo red from all treatments. Then, PCR-based DNA fingerprinting was used to determine the similarity of the different strains. In addition, ACC deaminase activity was screened for all of the 87 strains and 14 strains were identified as ACC deaminase producing bacteria, which means16% of isolates displayed the ACC deaminase activity. Different strains displayed different ACC deaminase activity ranging from 0.173 to 1.162 (Table 1). We found that strain 2, 7, 9, 27, 31, 32, 66 and 69 has the similar highest ACC deaminase activity and Strain 23, 56 and 60 showed the lowest ACC deaminase activity. However, the strains displayed considerably high ACC deaminase activity did not derive from same genotype and same

treatment. There are seven strains are isolated from irrigated treatment and seven strains were isolated from the drought treatment. In addition, no differences were displayed between the drought treatment irrigated treatment.

Isolation and characterization of rhizobia 16S rRNA

The 16S rDNA gene sequences from the above mentioned 14 ACC deaminases producing bacteria were PCR amplified and the approximately 1,400-bp products were then sequenced. The sequences were blast to SILVA and GreenGenes database for identification. Analysis of these 14 sequences indicated that 5 of the strains had greater than 99% identity with the *Bradyrhizobium* sp., while two of *Mesorhizobium* sp., one of *Rhizobium* sp., three of *E.* coli, one of *Labrys* sp*.,* one of *Caulobator* sp., and one of *Burkhoderia* sp were also identified (Table 1).

Isolation and characterization of rhizobia ACC deaminase genes

After these 14 ACC deaminases producing bacteria were identified, the ACC deaminase gene (*acdS*) were successfully amplified from three different strains (9, 31 and 69) using the designed primers. The resulting PCR product is approximately 1.01 kb for these three strains. Among these three sequences, it was observed that the *acdS* genes of these strains are identical to each other. When these sequences were compared with the ACC deaminase gene sequences in the GenBank database, they showed > ()% identities at the nucleotide level compared with the *acdS* genes of *Bradyrhizobium diazoefficiens USDA 110.*

IAA Concentration

IAA concentration of all of the 14 strains were determined and displayed in Table 2. The IAA concentrations varied from 0.1424 µg/ml to 41.14 µg/ml. Strain 56 displayed the highest IAA concentration (41.14 µg/ml) among all 14 strains and strain 7 also displayed a considerable

high IAA concentration (16.38 µg/ml). In addition, strain 32 showed the lowest IAA concentration (0.1424 µg/ml) among all 14 strains.

Leonard Jar Experiment

Since not all of strains were identified as *Bradyrhizobium* sp., more chemical tests were done for characterization. In addition, Leonard jar experiment was carried out to determine the nodulation efficacy. Among all 14 isolates, only three strains (9, 31 and 69.) were determined to be able to nodulate peanut root. Then, nitrogen fixation potential was determined. Nodule count data and shoot biomass data was displayed in Figure 1 and Figure 2. Among those three strains, we found strain 9 showed significantly higher nodule count than other two strains (33). In addition, strain 69 also displayed a higher nodule numbers than strain 31. Moreover, a significant difference between all the treatments was also found in Figure 2. We found that strain 9, 31 and 69 displayed a similar level of shoot biomass with the nitrogen control. However, the treatment, without N control, showed the lowest shoot biomass among all five treatments,

Appendix Table 1. ACC deaminase activity of strains isolated from peanut nodules.

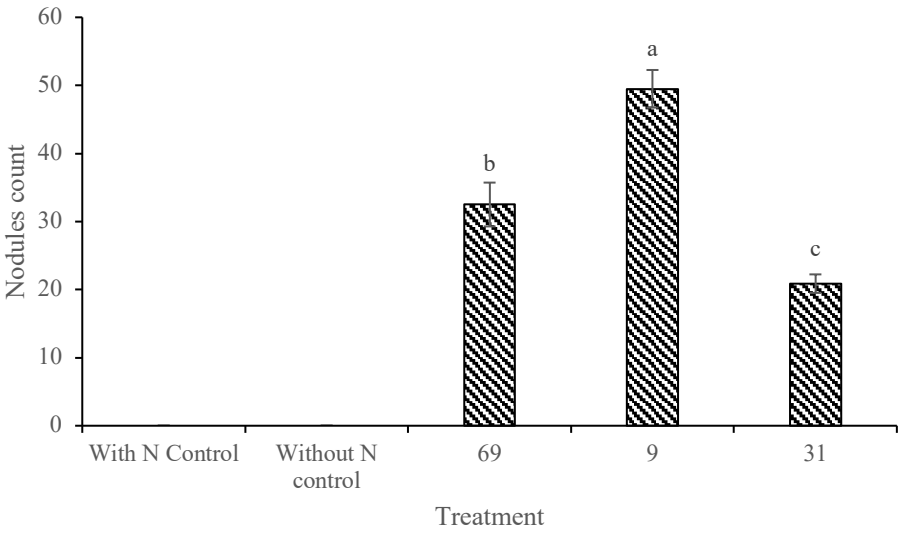
Pot #	Genotype	Treatment	ACC deaminase activity (umols/mg/h)	Taxonomy
2	587	Irrigated	1.162 ± 0.13	*Bradyrhizobium sp.*
6	587	Drought	0.522 ± 0.30	*Bradyrhizobium sp.*
7	Tifrunner	Drought	1.031 ± 0.11	*Labrys sp.*
9	587	Drought	0.910 ± 0.25	*Bradyrhizobium sp.*
10	Tifrunner	Irrigated	0.780 ± 0.30	*Mesorhizobium sp.*
23	Tifrunner	Drought	0.201 ± 0.09	*E. coli*
27	Tifrunner	Irrigated	0.897 ± 0.17	*E. coli*
28	C76-16	Irrigated	0.417 ± 0.09	*Burkhoderia sp.*
31	Tifrunner	Drought	0.831 ± 0.12	*Bradyrhizobium sp.*
32	C76-16	Drought	1.010 ± 0.12	*Mesorhizobium sp.*
56	C76-16	Drought	0.173 ± 0.10	*Caulobator sp.*
60	587	Irrigated	0.315 ± 0.11	*Bradyrhizobium sp.*
66	C76-16	Irrigated	0.877 ± 0.03	*Rhizobium sp.*
69	Tifrunner	Irrigated	1.301 ± 0.20	*E. coli*

Appendix Table 2. IAA concentration and PCR results of strains isolated from peanut nodules.

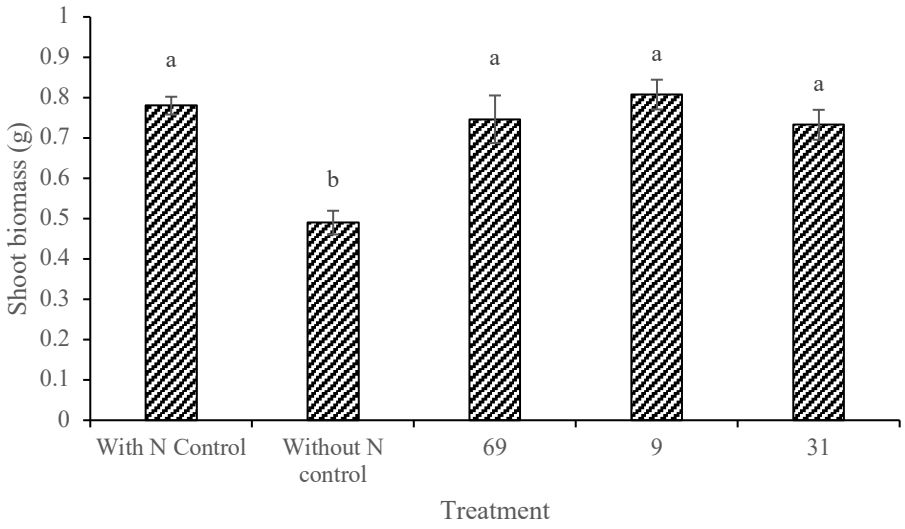
Pot #	Genotype	Treatment	Taxonomy	IAA concentration	pH change	PCR results (Duan)
2	587	Irrigated	*Bradyrhizobium sp.*	7.9028	7-8	-
6	587	Drought	*Bradyrhizobium sp.*	0.6979	7-8	-
7	Tifrunner	Drought	*Labrys sp.*	16.3750	7	+
9	587	Drought	N/A	4.5521	7-8	-
10	Tifrunner	Irrigated	*Mesorhizobium sp.*	2.7292	7	-
23	Tifrunner	Drought	*E. coli*	1.6701	6-7	-
27	Tifrunner	Irrigated	*E. coli*	1.3056	7-8	-
28	C76-16	Irrigated	*Burkhoderia sp.*	0.8889	7-8	-
31	Tifrunner	Drought	*Bradyrhizobium sp.*	1.4965	7-8	-
32	C76-16	Drought	*Mesorhizobium sp.*	0.1424	6	-
56	C76-16	Drought	*Caulobator sp.*	41.1319	7-8	-
60	587	Irrigated	*Bradyrhizobium sp.*	1.1146	7-8	-
66	C76-16	Irrigated	*Rhizobium sp.*	5.4896	6-7	+
69	Tifrunner	Irrigated	*E. coli*	2.5556	7-8	-

Appendix Table 3. Characterization of the strains isolated from peanut nodules.

Pot #	Genotype	Treatment	Taxonomy	Peptone glucose		Streptomycin		Non-Streptomycin	
				R1	R2	R1	R2	R1	R2
2	587	Irrigated	*Bradyrhizobium sp.*	+	-	+	+	+	+
6	587	Drought	*Bradyrhizobium sp.*	-	-	+	+	+	+
7	Tifrunner	Drought	*Labrys sp.*	+	+	+	+	+	+
9	587	Drought	*Bradyrhizobium sp.*	+	+	+	+	+	+
10	Tifrunner	Irrigated	*Mesorhizobium sp.*	+	+	+	+	+	+
23	Tifrunner	Drought	*E. coli*	+	+	-	-	+	+
27	Tifrunner	Irrigated	*E. coli*	+	+	+	+	+	+
28	C76-16	Irrigated	*Burkhoderia sp.*	+	+	+	+	+	+
31	Tifrunner	Drought	*Bradyrhizobium sp.*	+	+	+	+	+	+
32	C76-16	Drought	*Mesorhizobium sp.*	+	+	-	-	+	+
56	C76-16	Drought	*Caulobator sp.*	+	+	+	+	+	+
60	587	Irrigated	*Bradyrhizobium sp.*	+	+	+	+	+	+
66	C76-16	Irrigated	*Rhizobium sp.*	-	-	+	+	+	+
69	Tifrunner	Irrigated	*E. coli*	+	+	-	-	+	+



Appendix Figure 1. Nodules count of peanut inoculated with different ACC deaminase producing strains. Different letters denote significant difference among five different treatments based on Tukey's test.



Appendix Figure 2. Shoot biomass of peanut inoculated with different ACC deaminase producing strains. Different letters denote significant difference among five different treatments based on Tukey's test.

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