Physiological, phytohormonal and molecular responses of soybean to soil drying

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Declaration

Except where references are made to other sources, I declare that the contents in this thesis are my own work and have not been previously submitted, in part or in full, for the award of a higher degree elsewhere.

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Abstract

Soybean is an important global crop for human and animal nutrition, but its production is affected by environmental stresses such as drought. Crops adapt to these stresses by producing and transporting multiple internal signals (such as phytohormones) between roots and shoots. Understanding the relationships between physiological (water potential and stomatal conductance), biochemical (phytohormones) and gene expression changes can inform cultivar selection and management approaches, offering opportunities to enhance water-limited yields.

Soybean (*Glycine max* (L.) Merr., genotypes Williams 82 (W82), Jindou 21 (C12), Union (C08), Long Huang 1 (LH1) and Long Huang 2 (LH2)) were grown under soil drying conditions to investigate relationships between leaf xylem sap and leaf tissue ABA concentrations, stomatal conductance and leaf water potential among different genotypes. Stomatal conductance was better explained by variation in leaf xylem sap ABA concentration than leaf tissue ABA concentration or leaf water potential in most of the genotypes studied, which is physiologically important as stomatal closure limits soybean yields. Thus, limited ABA accumulation may be useful as a marker for breeding plants under drought conditions, assuming plants can access sufficient soil moisture at depth.

The role of the ABA in root-shoot communication was investigated by exposing plants to a combination of soil drying and stem girdling (which disrupts basipetal phloem transport) to determine the dependence of ABA accumulation on tissue water relations. Shoot-sourced ABA was necessary to allow maximal root ABA accumulation, and maintain root-to-shoot ABA signalling, in response to soil drying. Shoot to root ABA translocation also maintained high stomatal conductance by preventing foliar ABA accumulation under well-watered conditions. However, decreased stomatal conductance (by 20%) of well-watered plants one day after girdling may involve other hormones, induced by wounding effects prior to any ABA (xylem or tissue) accumulation.

Within 26 hours of girdling, root ACC concentrations increased 15-fold and leaf ABA, JA and SA concentrations increased 1.5-, 6- and 1.5-fold respectively. In contrast,

root GA3, GA4 and ABA concentrations decreased to 0.6-, 0.4- and 0.2-fold respectively. During this time (when there was limited soil drying), only leaf ABA and JA accumulation was highly negatively correlated with stomatal closure. Furthermore, leaf iP and SA concentrations were negatively and positively correlated respectively, and root ACC and ABA concentrations were negatively and positively correlated respectively, with soil moisture. Over the entire experiment in all plants, soil drying induced stomatal closure was negatively, positively and negatively correlated with foliar tZ, GA4 and ABA accumulations respectively, while root GA3, ABA and JA accumulations were negatively correlated with soil water content. Rapid leaf JA accumulation in response to girdling and soil-drying induced ABA accumulation in leaves and roots independently of girdling suggest that both hormones interact to stimulate stomatal closure. Girdling failed to disrupt the positive correlation between root and leaf ABA concentrations, but induced negative correlations between root and leaf JA and tZ concentrations, possibly due to carbohydrate depletion in the roots.

Understanding the role of local hormone synthesis *versus* root-to-shoot signalling in regulating ABA and JA accumulation of each tissue can be facilitated by gene expression analysis via RNA-seq and qRT-PCR. The majority of the ABA biosynthesis, catabolism and signalling genes were upregulated in the roots of girdled plants prior any change in leaf and root water relations, and were sustained as the soil dries. Girdling upregulated the expression level of JA biosynthesis and signalling genes in both roots and leaves. Soil drying up-regulated the ABA biosynthesis and catabolism in roots and leaves, while up-regulating JA biosynthesis and signalling genes in roots and leaves of intact plants. Thus girdling more rapidly increases the number of up-regulated genes in the selected (JA and ABA pathway) genes in roots than in leaves.

Taken together, this thesis furthers our understanding of relationships between leaf and root phytohormonal communication in co-ordinating physiological responses to soil drying. Further studies of cross-talk between different hormones, including their intermediate metabolites, seems necessary to help understand how plants respond under drought conditions.

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Chapter 1 – General Introduction

1.1 Soybean (*Glycine max*)

1.1.1 The importance of soybean

The soybean genus *Glycine* comprises two subgenera, *Soja* and *Glycine*. The subgenus *Soja* contains cultivated soybean (*Glycine max* (L.) Merr.) and the wild soybean (*Glycine soja*) which is a wild ancestor of the cultivated soybean. Both *Soja* species are annual, but the subgenus *Glycine* consists of 25 perennial species, such as *Glycine canescens* from Australia (Newell and Hymowitz, 1983; Singh, 2006; Qiu and Chang, 2010). Soybean (*Glycine max*) originates from, and was domesticated in, China 5,000 years ago with about 23,000 different cultivars in all Asia, from where was introduced to the USA and South America. It is one of the major cultivated crops worldwide (along with maize, rice and wheat) as a source of protein for humans and as a high-quality animal feed (FAO, 2003; López-López *et al.*, 2010). Soybean is grown for vegetable oil and meal to feed animals, and the seed comprises 40-42% protein content, 35% carbohydrate, 20% oils, and 5% ash (Robert, 1986; Zhang *et al.*, 2010). Soybean is also the most cultivated oilseed crop worldwide, comprising around 6% of the total land under cultivation, with its acreage increasing year on year (Fig. 1.1; Goldsmith, 2008; Sulieman *et al.*, 2015).

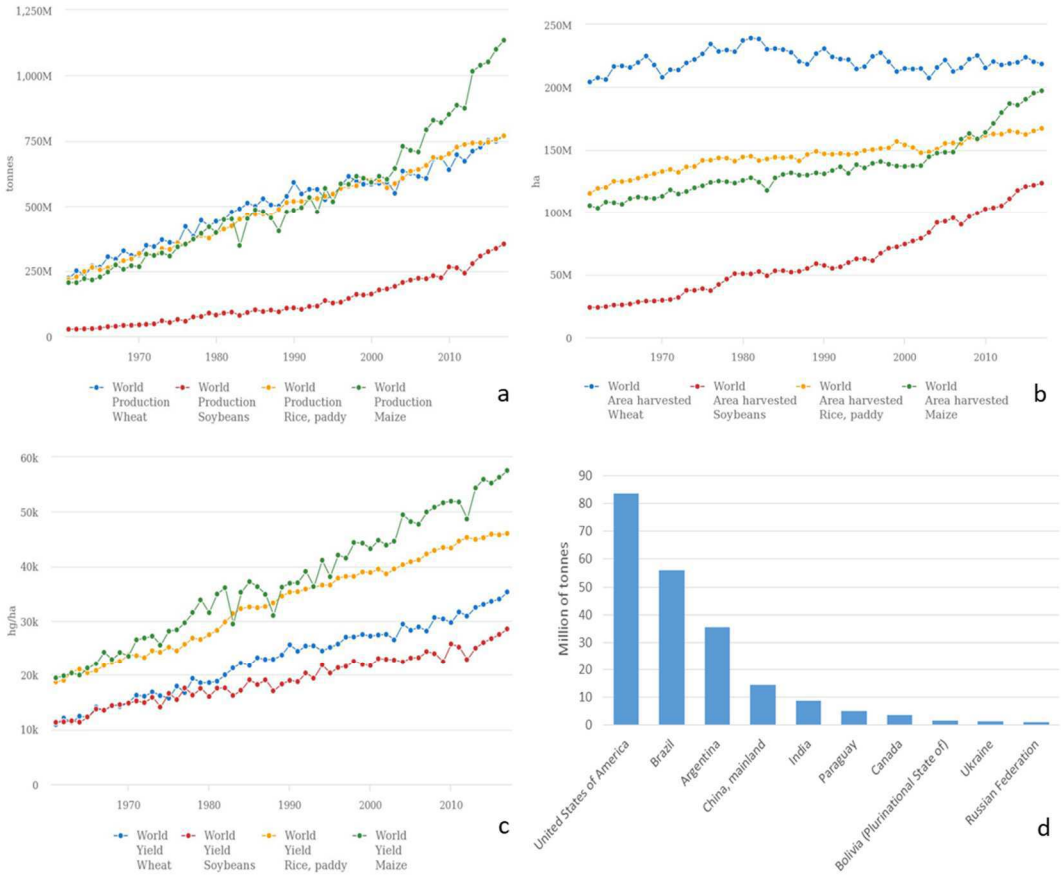


Figure 1.1. World yield data per area cultivated (a), total world production (b), total world area cultivated (c) of wheat (blue), soybean (red), rice (yellow) and maize (green), and production of the top 10 producers Data: (FAO, 2017).

Soybean production is dominated by five countries (USA, Brazil, Argentina, China and India) which contribute more than 92% of the world soybean production (Fig. 1.1d; Leff *et al.*, 2004; Rodríguez-Navarro *et al.*, 2011; FAO, 2017). The three highest producers (USA, Brazil and Argentina) have used different approaches for increasing soybean yield. The USA has used large amounts of nitrogen fertilisers for increasing the production and yield. Many years ago, Brazil and Argentina ceased using nitrogen fertilisers, and instead have used repeated rhizobial inoculations as seed treatments (Alves *et al.,* 2003; Salvagiotti *et al.,* 2008). Furthermore, world

soybean production has increased yearly by 4.6% in the last 30 years, reaching 250M tonnes in 2017 (Fig. 1.1a; Masuda and Golsmith, 2009), outstripped only by maize (475M tonnes). The production per area cultivated (Fig. 1.1b) followed the same pattern as world production, while soybean yields have increased by 1000 kg ha^{-1} in the last 30 years. According to the area harvested, soybean production has increased more than other crops over the same period, by 125M hectares (Fig. 1.1c).

In South American countries, the expansion of the total cropped area (by about 63% over the last 50 years), has replaced native crops and pasture, and greater land use has increased soybean production at an annual rate of 6% (Wingeyer *et al.*, 2015). In addition, Argentina, the world´s largest soybean exporter (46% in 2017/2018) has suffered fluctuating rainfall in the last 15 years, therefore soybean yields were affected by periods of drought (USDA Statistics, 2018). Potentially, soybean yields could be enhanced by improving tolerance to certain environmental stresses such as drought and salinity, which currently decrease yield by about 40% (Szilagyi, 2003; Le *et al.*, 2012).

1.1.2 Soybean production and drought stress

Since global climate change has changed rainfall frequency dramatically over the last hundred years, certain climatic areas now experience longer drought seasons (Boyer, 1982; Hirt and Shinozaki, 2004; Solomon *et al.*, 2007). Thus drought stress is the most devastating of the environmental stresses that could decrease soybean productivity (Szilagyi, 2003; Le *et al.*, 2012). Plants respond to drought at physiological, biochemical, and molecular levels (Mochida *et al.*, 2010; Tran *et al.*,

2009). Understanding these complex responses may offer opportunities to improve crop management and genetics to enhance drought tolerance of soybean (Pathan *et al.*, 2007).

The damage that plants suffer during drought stress depends on many factors including genotype, development status, soil water depletion and the duration of the water deficit (Mahajan and Tuteja, 2005; Reddy *et al.*, 2004). All these factors interact with each other to cause significant changes at the physiological, biochemical and molecular levels, which translates into a drop in crop performance. However, progress has been made in understanding mechanisms of plant drought tolerance through studying the aforementioned processes, especially physiological (water relations), metabolism (hormones) and genetic factors (gene expression) (Atkinson and Urwin, 2012; Cattivelli *et al.*, 2008). Since phytohormones could change physiological behaviour, such as stomatal movement, an increased capacity to detect and quantify different plant hormones makes it possible to evaluate the physiological function of hormones throughout the plant in response to water stress (Peleg and Blumwald, 2011; Robert-Seilaniantz *et al.*, 2011; Vanstraelen and Benková, 2012).

Plants adapt to drought conditions by escaping, avoiding or tolerating drought (Levitt, 1980; Athar and Ashraf, 2009). Drought escape means that plants complete their life cycle, or time their phenological development, during periods of sufficient water supply, before water deficit becomes severe enough to cause damage (Blum, 1988). Drought avoiding plants either maximise their water uptake from the root system and/or minimize their water loss by closing their stomata or restricting

growth, thereby maintaining their water status (Price *et al.*, 2002). Drought tolerant plants maintain a certain level of physiological activity even under severe drought stress conditions, completing their life cycle while having an acceptable yield even though their water status is decreased (Tardieu and Tuberosa, 2010).

The time, severity and duration of stress changes many aspects of plant physiology. Decreased soil water potential can restrict stomatal conductance, thereby limiting both photosynthesis and biomass accumulation (Ma *et al.*, 2006; Vikram *et al.*, 2011). Whole plant water loss is related to the foliar area and stomatal conductance, with fine stomatal regulation over minutes or hours in response to re-watering. In some species, stomatal responses to soil drying are more sensitive than other physiological variables, and sensitive stomatal closure could (Chaves *et al.,* 2003; Lei *et al.*, 2006a) allow soybean to avoid leaf water deficit.

1.2 Plant signalling models and molecular responses to soil drying

1.2.1 Hydraulic signalling

Hydraulic signalling, which is transmitted by changes in water potential gradients throughout different tissues, could regulate turgor and water status of guard cells to control water uptake and initiate stomatal closure, since a lower relative water content can lead to stomatal closure (Brodribb *et al.,* 2003; Franks, 2013). Short-term stomatal behaviour responds to changes in leaf water balance, explained by metabolic mediated response of the guard cells to a local hydraulic status (hydro-active local feedback). On the other hand, equivalence of hydraulic supply and

hydraulic demand from the action of stomatal effectors, of the water potential gradient between epidermal cells, makes its explanation difficult (Buckley, 2005).

Plant water potential and turgor often decrease as the soil dries, therefore turgor loss could suppress cell expansion and growth. Tissues can actively accumulate osmolytes to maintain a positive turgor and prevent damage to cellular integrity, since accumulating osmotically actives solutes can maintain a positive turgor over a wider water potential range preventing degradation in cell integrity (Melkonian *et al.*, 1982; Setter, 2012). Thus physiological processes such as stomatal movement could be sustained by optimal cell turgor pressure, since the osmolytes accumulated into guard cells can be retained therefore maintaining guard cell turgor (Morgan, 1984; Blum, 1996). Thus stomatal conductance and leaf water potential are important factors in plant water relationships (Franks and Farquhar, 1999; Sperry, 2000; Jongdee *et al.*, 2002; Tang *et al.*, 2015).

Stomatal closure is induced by hydropassive or hydroactive mechanisms (Murata and Mori, 2014). Hydropassive stomatal closure occurs in conditions of low humidity and high air movement, where the cells surrounding the guard cells rapidly decrease turgidity due to the evaporation (Wang *et al.*, 2001). The hydro-active negative feedback that affect stomatal closure is produced by the desiccation of the entire plant (root and shoot), where solutes are actively expelled from the subsidiary cells in response to external/internal factors, decreasing their osmotic potential causing these cells to become more mouldable (Buckley, 2005; Kaiser and Legner, 2007). Factors that promote stomatal changes through the hydro-active route (opening or closing) are mediated biochemically, requiring an active feedback

response of guard cell´s water potential changes in water status in or near the epidermis under different stress conditions. Decreased soil water content, and so the availability of water for the plant, affects physiological processes such as stomatal behaviour (opening and closing) and plant water potential under drought stress conditions.

1.2.2 Chemical signalling

Chemical signalling comprises many phytohormones, of which ABA's role as signal of soil drying has been highlighted (Fig. 1.2). Phytohormones play an essential role in plant development, conferring tolerance to biotic and abiotic stress (Catinot *et al.*, 2008; Peleg and Blumwald, 2011). Many studies have focused on changes in their concentrations and their impact on subsequent signalling in response to the stress perceived.

ABA is synthesised throughout the plant in response to decreased cellular turgor, but there has been considerable debate as to whether ABA is synthesised first in the roots or shoots, as with an earlier debate on which tissue first perceived (in terms of decreased water status) drying soil (cf. Kramer 1988; Passioura 1988). One school of thought is that ABA is primarily synthesized in the root in response to decreased root turgor, then transported in the xylem sap to the shoot where it accumulates in the leaf apoplast to initiate stomatal closure (Davies and Zhang, 1991; Dodd, 2005; Wilkinson *et al.*, 2012; Puertolas *et al.*, 2013). An opposing paradigm is that this hormone is synthesized in the aerial parts of the plant (e.g. the leaves and the stem) in response to decreased turgor, and then is transported

towards the roots (McAdam *et al.*, 2016a; Manzi *et al.*, 2015), where it enhances root hydraulic conductance.

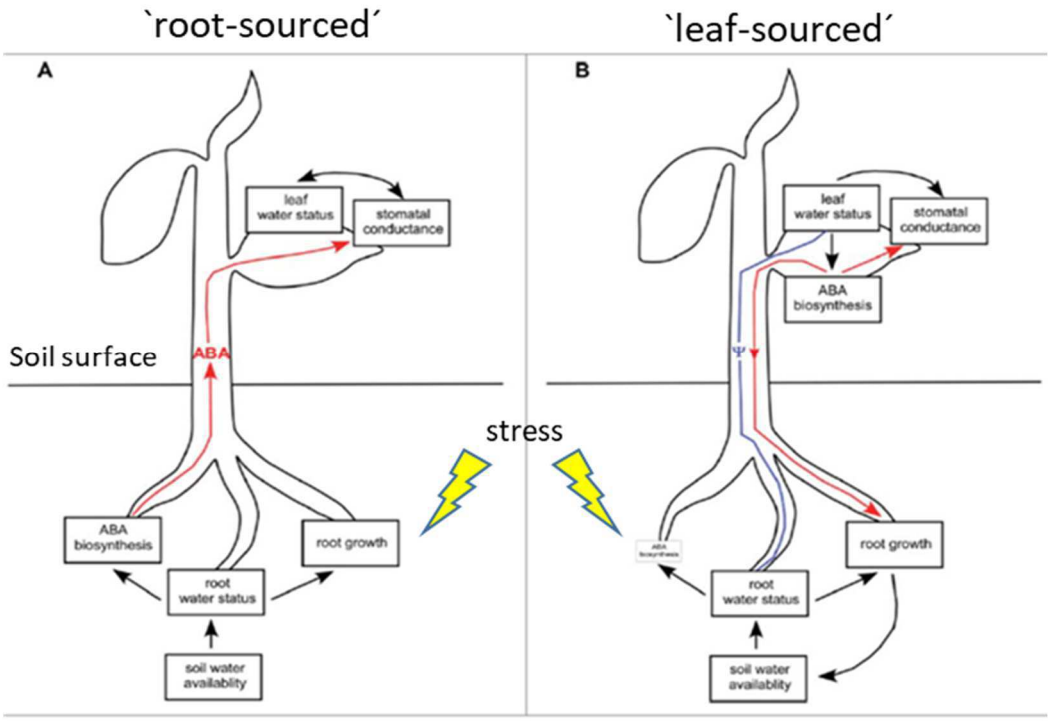


Figure 1.2. Diagram of `root-sourced´ model for ABA biosynthesis and transport by Davies and Zhang, 1991 (A) and `leaf-sourced´ model for water status and ABA biosynthesis and transport by McAdam *et al.*, 2016a (B) (redrawn from McAdam *et al.*, 2016a).

Phytohormones

Abscisic Acid

Among all the endogenous phytohormones that exist in plants, ABA is regarded as a primary chemical signal that initiates stomatal closure in response to soil drying (Wilkinson and Davies, 2002). ABA plays a key role in regulating crop vegetative and reproductive development, with higher concentrations accumulating within the plant during drought (Liu *et al.*, 2005a). It is well-known that ABA concentrations

increase in response to drought conditions and its biosynthesis proceeds via a series of chemical reactions transforming carotenoid precursors into ABA (Schwartz *et al.,* 2003; Schroeder and Nambara 2006; Ikegami *et al.,* 2009). ABA could affect plant biomass accumulation with stomatal movement regulating photosynthetic rates, and could inhibit shoot expansion during the early stages of drought stress (Trewavas and Jones 1991; Tardieu *et al.,* 2010). However, growth analyses of ABA-deficient mutants show that ABA is required to constrain ethylene production (Sharp *et al.* 2000), which may be synthesised in response to drying soil (Sobeih *et al.* 2004).

Under drought stress, ABA accumulation causes rapid and gradual responses in different tissues. Rapid stomatal closure prevents plant desiccation, since the guard cells decrease in volume, closing the pore. With further soil drying, ABA is gradually accumulated in the leaf and is transported basipetally to the roots, to promote root growth to allow water uptake from deeper in the soil profile. Root ABA accumulation is necessary to maintain root growth of plants in drying soil (Sharp *et al.,* 1994). Thus this long-distance transport between different organs within the plant has made this phytohormone a critical messenger regulating many physiological processes (Kuromori *et al.*, 2010; Kuromori *et al.*, 2014a; Zhang and Davies, 1990). In addition, a balance of ABA biosynthesis, catabolism and transport affects tissue ABA concentrations, which ultimately affect the ABA response (Finkelstein, 2013). The sensitivity of different tissues to ABA will determine the level of response to this phytohormone, where ABA signalling can be amplified by pH increases in xylem / apoplast, where anionic ABA is retained (Liu *et al.*, 2004,

2005b; Merilo *et al.,* 2015). So it is important to understand how ABA concentrations are governed in different tissues as the soil dries.

It is crucial to understand how ABA promotes tolerance to abiotic stress and regulates certain key processes, from germination to senescence. For the presence of ABA to induce an action, biologically active ABA must accumulate at the site of perception (Seiler *et al.*, 2014). In recent years, technological advances in sequencing have produced an immense collection of transcriptome data from plants under different abiotic stresses, (Huang *et al.*, 2008; Cramer *et al.*, 2011). Different transcriptional changes due to physiological and morphological adaptations to environmental stress are associated with an extensive complex of molecular mechanisms. Therefore, a greater understanding of each metabolic step in the synthesis and signalling of ABA that is affected under drought stress could lead to the development of drought-tolerant crops (Wang *et al.,* 2006).

Jasmonates

Jasmonic acid (JA), one of the jasmonates, is formed from lipid derivatives produced from fatty acid oxidation, and regulates various responses, such as stomatal movement in plants under different environmental stresses, including water scarcity (Balbi and Devoto, 2008; Murata and Mori, 2014; Taiz and Zeiger, 2010; Wasternack, 2007).

Jasmonate concentrations increase in a similar way to ABA as the soil dries, and promote stomatal closure (Evans, 2003; Munemasa *et al.*, 2007; Acharya and Assmann, 2009), and may interact to effect stomatal closure (Muñoz-Espinosa *et*

al., 2015). For example, the *aba2-2* mutant in *Arabidopsis* (which is constitutively ABA-deficient) showed impaired MeJA-induced stomatal closure (Hossain *et al.*, 2011), but is still unclear how that process occurs. Also, *Arabidopsis* mutants with impaired JA (*aos*), ABA (*aba2*) and ascorbate (*vtc1*) biosynthesis were all more sensitive than wild type to drought, with ABA a key player in regulating stomatal behaviour while JA controlled ascorbate levels (Brossa *et al.,* 2011). Furthermore, the JA precursor 12-oxo-phytodienoic acid (OPDA) was involved in stomatal movement in conjunction with ABA (Savchenko *et al.,* 2014; de Ollas and Dodd, 2016), since OPDA uncouples the conversion to JA with 12-OPDAs genes having a drought-responsive regulator. With ABA, the hormone JA is involved in the promotion of stomatal closure, but at the same time, other studies have proposed that drought stress prevents the conversion of the precursor of 12-oxo-phytodienoic acid (OPDA) to JA (Savchencko *et al.*, 2014; Kazan, 2015; de Ollas and Dodd, 2016). Since OPDA could act individually or in conjunction with ABA to promote stomatal closure, this is another step that could lead to increased drought tolerance. Thus, whether both hormones (JA and ABA) regulate stomatal movement via a similar mechanism is of interest.

Furthermore, using chemical inhibitors of JA (salicylhydroxamic acid, SHAM) and ABA (norflurazon, NFZ) biosynthesis, it was shown that a rapid and transient increase in JA was required for root ABA accumulation in citrus (*Citrus paradisi* × *Poncirus trifoliate*) experiencing severe drought (de Ollas *et al.,* 2012). However, JA did not accumulate in shoots of drought stressed tomato and *Brassica* plants, which may cause by differences in the level of drought stress between the studies

(Savchenko *et al.*, 2014). Alternatively, exogenous JA applications (spraying 0.5 mM JA) increased leaf fresh weight and relative water content of different *Brassica* species in combination with drought stress (Alam *et al.*, 2014). Thus it might be that JA, and its intermediate metabolites (such as OPDA), could interact in conjunction with ABA to modulate stomatal behaviour, thereby avoiding water losses under drought conditions. Furthermore, how JA accumulation under short- or long-term drought stress treatments affects different plant physiological processes, such as growth inhibition or root morphogenesis under non-stress conditions, or whether JA-induced changes in these parameters confers drought acclimation, remains unknown (Rossato *et al.,* 2002; Harb *et al.*, 2010; Santino *et al.,* 2013).

Cytokinins

Cytokinins (CKs) are adenine-derivative molecules with diverse active forms, such as zeatin, zeatin riboside, and isopentyladenine which are found in higher plants (Ioio *et al.,* 2008; Aloni *et al.,* 2005). Cytokinins influence many biological functions throughout the plant, such as plant cell division and growth. The CKs are produced in meristematic areas in roots (Werner *et al.,* 2003) and young shoot organs (Faiss *et al.,* 1997; Schmülling, 2002), thereafter being transported via the xylem stream to the shoot (McKenzie *et al.,* 1998; Emery and Atkins, 2002). CK levels are regulated by the feedback repression of isopentenyltransferase (*IPT*) gene expression in any tissue by the rates of CK synthesis, transport, and metabolism (Miyawaki *et al.,* 2004). Shoot CK level was dependent on CK delivery from the roots, deduced by comparing shoot CK levels in plants almost lacking transpiration (protected from any air movement in sterile translucent

polycarbonate/polypropylene boxes), and those grown under normal (transpiring) conditions. In addition, rapid up-regulation of the *IPT*-related genes expression was established in response to nitrate addition to N-starved plants, which adds more relevance to the regulatory role of root-sourced CKs transported to the shoot (Miyawaki *et al.,* 2004; Aloni *et al.,* 2005). Shoot CK synthesis may become more prominent during periods of stress (such as nitrogen deficiency), when roots cannot provide sufficient CKs (Miyawaki *et al.,* 2004; Takei *et al.,* 2004).

Cytokinin concentrations generally decrease as the soil dries, but normally return to pre-stress levels after re-watering (Bano *et al.,* 1993; Naqvi, 1995). Cytokinins enhance or have no effects on stomatal opening, depending on which CK and its concentration (Dodd, 2003). Historically, CKs have been considered ABA antagonists throughout the plant, therefore some drought-stress studies have emphasised the importance of ABA/CKs interactions in better explaining stomatal responses than ABA alone (Tran *et al.,* 2007; Zwack and Rashotte, 2015). However, CK-deficient Arabidopsis lines (*ipt1*, *3*, *5* and *7* mutants) maintained stomatal opening under well-watered conditions (Nishiyama *et al.*, 2011), suggesting a limited role for shoot CK biosynthesis in mediating stomatal responses.

Gibberellins

Gibberellins (GA) are a large group of tetracyclic diterpenoid carboxylic acids, with more than one hundred different chemical structures, although only some are biologically active and could influence plant growth, seed germination and stem and root development (Sun and Gubler, 2004; Taiz and Zeiger, 2010; Yamaguchi, 2008). Compared with other phytohormones, there is much less information on

how GA concentrations change as the soil dries (Pospíšilová, 2003; Yamaguchi, 2008; Colebrook *et al.,* 2014), and their role in mediating drought stress responses. There is also considerable evidence suggesting that stomatal aperture may be regulated through multiple signalling pathways or cross-talk between various plant hormones, including GA (Dodd, 2005; Coelho Filho *et al.,* 2013).

Although GA commonly acts as an ABA antagonist (Acharya and Assmann, 2009), certain GA-deficient *Arabidopsis* mutants (A_{70}) had similar transpiration rates compared to wild-type plants during drought stress (Cramer *et al.,* 1995), suggesting that GA had limited impacts on stomatal opening/closure. Transgenic tomato plants (overexpressing the *At*GAMT1 gene from Arabidopsis) with decreased foliar GA levels were tolerant to water-deficit, maintaining higher leaf water status and lower stomatal conductance than control plants when grown in drying soil (Nir *et al.,* 2014). The evidence for gibberellins regulating stomatal conductance is equivocal, and further work is required to establish their physiological significance in regulating stomatal responses to soil drying.

Ethylene

Ethylene, a gaseous plant hormone, is synthesized from the amino acid methionine to S-adenosyl-L-methionine (SAM) by the enzyme Met Adenosyltransferase. The following step, where it's converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS), is a key rate-limiting step for ethylene production (Kende, 1993; Wang *et al.,* 2002). Ethylene is involved in regulating seed germination, fruit ripening and leaf abscission.

Furthermore, soil drying induced both root ABA and ACC accumulation in plants with decreased xylem flow. Upon re-watering, some of these hormones were transported to the shoot via the xylem to act as a long-distance signal of drought, suggesting that ACC is involved in root-to-shoot signalling (Gómez-Cadenas *et al.,* 1996; Else and Jackson, 1998; Wilkinson and Davies, 2008). The ABA-deficient tomato mutant *flc* (which is impaired in the oxidation of ABA aldehyde to ABA), had increased ethylene production independently of plant water status (Sharp *et al.,* 2000; Sharp and LeNoble, 2002). Also, in ABA-deficient maize lines, the rate of ethylene production increased in the primary root as the soil dries (Spollen *et al.,* 2000; Voisin *et al.,* 2006). Thus endogenous ABA is required to maintain root elongation at low substrate water potential by limiting ethylene production, while ethylene accumulation can antagonize ABA-induced stomatal closure (Tanaka *et al.,* 2005; Wilkinson and Davies, 2010). Thus ethylene opposes stomatal closure under environmental conditions that stimulate ABA accumulation.

Salicylic acid

Salicylic acid (SA) is an endogenous plant phenolic hormone (lipophilic monohydroxybenzoic acid) that enhances various regulatory functions in plants, such as seed germination and plant growth (Aftab *et al.,* 2010; Hayat *et al.*, 2010). High SA levels, which could be produced locally and transported systemically within the plant (Raskin, 1992; Meuwly *et al.,* 1995), enhanced plant resistance to pathogen infection (Volt *et al.,* 2009). SA and JA increases are pathogen- and insect- (or wounding) induced, with SA signalling combatting biotrophic agents and JA signalling protecting against injuries (Glazebrook, 2005). The SA and JA response

pathways negatively interact, with one pathway repressing the other, with JA-induced by wounding inhibiting SA response (Doherty *et al.,* 1988; Bostock and Stermer, 1989; Doares *et al.,* 1995).

Drought-induced increases of SA concentrations *in planta* (roots and shoots) are proposed to confer osmotic stress tolerance to salinity or drought. (Horváth *et al.*, 2007). Exogenous SA (1 mM) sprays improved drought tolerance by increasing photosynthetic rate, leaf water potential and chlorophyll content in tomatoes (*Lycopersicon esculentum*) and *Artemisia annua* L. (Hayat *et al.*, 2008; Aftab *et al.,* 2010). In addition, similar increases in transpiration rate and stomatal conductance were found in soybean and maize when leaves were sprayed (0.1 mM) with SA solution (Khan *et al.,* 2003). However, under drought stress, simulated by polyethylene glycol (PEG)-6000, *Arabidopsis* mutants (*snc1* overexpressing SA level) stomatal conductance decreased in comparison to the wild type, suggesting greater drought tolerance (He *et al.,* 2014), and also the transpiration rate decreased significantly in *Phaseolus vulgaris* and *Commelina communis* after exogenous SA application, likely reflecting stomatal closure (Larque-Saavedra, 1979). Thus SA changes could be involved drought responses, perhaps via cross-talk with other phytohormones such as JA.

1.2.3 Molecular studies of ABA and JA pathway

Plant functional genomics is among the most exploited techniques for determining gene function and the knowledge generated has been used to improve plant tolerance to drought stress (Shinozaki, 2007; Le *et al.*, 2012). Functional genomics is based on the study of various gene functions, where the genome sequencing of

soybeans and the development of detailed genetic and physical maps have revolutionized genomic research in this crop (Yamaguchi-Shinozaki and Shinozaki, 2006; Nakashima *et al.*, 2007). An important advance was the development of the total soybean sequence (975 Mb) (http://www.phytozome.net/soybean), where it has been possible to develop the genetic maps essential for genomic research. Phytozome (first released in 2008) is a comparative hub for plant genome and gene family data and analysis, at the level of sequence, gene structure, gene family and genome organization (Goodstein *et al.,* 2012).

Gene expression studies can indicate whether hormone-related genes are expressed at different stages of plant development, and in different tissues, when plants are under stress. Drought-responsive genes have been identified, such as the down-regulation of photosynthesis genes, hormonal changes and their function determined by overexpression and/or knockdown studies. Developing tolerant soybean genotypes requires an understanding of gene expression, biochemical and physiological responses, since drought responses depend on factors including plant genotype, developmental stage, frequency of drought events, and duration of water deficit (Cheong *et al.,* 2002; Mahajan and Tuteja 2005; Cattivelli *et al.*, 2008; Huang *et al.,* 2008; Le *et al.*, 2012; Gil-Quintana *et al.*, 2013).

ABA pathway

Biosynthesis of ABA in higher plants follows an indirect pathway that begins with isopentenyl pyrophosphate (IPP), the biological isoprene unit. Normally, the ABA biosynthesis pathway is studied from the conversion of zeaxanthin to *trans*-violaxanthin, including a two-step epoxidation process that is catalysed by zeaxanthin epoxidase (ZEP/ABA1) (Fig. 1.3; Marin *et al.*, 1996; Audran *et al.*, 2001). After that, *trans*-violaxanthin is catalysed by 9-cis epoxycarotenoid dioxygenase (NCED), which produces a 15C compound by the oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin, named as xanthoxin. This reaction is the last step of ABA biosynthesis that occurs in the plastids and is considered rate-limiting for ABA biosynthesis (Tan *et al.*, 2003). Thus, an upregulation of NCED expression in a detached or dehydrated leaf, especially the NCED3 gene, can be detected within 15–30 minutes (Qin and Zeevart, 2002; Tan *et al.,* 2003; Yen *et al.*, 2011). Moreover, NCED3 expression was increased in root tips, pericycle, and cortex cells at the base of lateral roots after these tissues had lost 15% of their fresh weight (Tan *et al.*, 2003). Following xanthoxin production, it is exported to the cytosol and converted to abscisic aldehyde by short-chain dehydrogenase/reductase (SDR/ABA2), then oxidized to ABA by aldehyde oxidase (AAO/MoCo) (Cheng *et al.*, 2002; Gonzaléz-Guzmán *et al.*, 2002; Seo *et al.*, 2004).

Vascular tissues have high activity of ABA biosynthetic enzymes (Kuromori *et al.*, 2014b). Bauer *et al.*, (2013) showed that guard cells could be autonomous in ABA biosynthesis (by transforming the ABA-deficient mutant *aba3-1* with guard cell-specific expression of the MoCo sulfurase ABA3 that is involved in the last step of

ABA biosynthesis), ensuring that leaves of the plants did not wilt when exposed to dry air. Thus guard cells may regulate their own ABA levels.

Compared with the ABA biosynthesis pathway, ABA catabolism is less complex. Abscisic acid can be hydroxylated in three different methyl groups such as C-7´, C-8´, and C-9´ (Zhou *et al.*, 2004), but the hydroxylation step does not reduce the biological activity of ABA. Of these hydroxylated products, only 8´-hydroxy ABA, which comes from the degradation of ABA by cytochrome p450 (CYP) can be transformed by cyclization to phaseic acid (PA) and then reduced to dihydrophaseic acid (DPA) (Miura *et al.*, 2009), which is the last product of ABA catabolism without any potential activity.

When soil drying increases ABA accumulation, the CYP family of genes is highly expressed to balance ABA biosynthesis and catabolism (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Seiler *et al.*, 2011). Apart from ABA biosynthesis and catabolism, glucosidation of ABA forms ABA glucosyl ester (ABA-GE), which can release active ABA in response to abiotic stresses (Lee *et al.*, 2006; Wasilewska *et al.*, 2008). Nevertheless, there is still some uncertainty as to which processes (biosynthesis, catabolism, or de/conjugation) primarily regulate ABA accumulation under stress.

In the intracellular ABA signalling network that ultimately leads to stomatal closure, ABA-binding proteins, including PYR1 (PYRABACTIN-RESISTANCE1) have been identified (Santiago *et al.,* 2009). PYR is one of the 14 homologues (PYL, PYRABACTIN RESISTANCE LIKE) present in Arabidopsis (Ma *et al.*, 2009; Park *et al.*, 2009; Nishimura et. al., 2010). The PYR/PYL/RCAR–ABA receptor complex binds ABA and forms ternary complexes with PP2C, inhibiting the ability of PP2C (Protein

phosphatase 2C) to dephosphorylate SnRK2 (serine/threonine kinases) compounds. Activating SnRK2 acts as a positive regulator of ABA signalling (Fujita *et al.*, 2009; Umezawa *et al.*, 2009; Vlad et. al., 2009; Li *et al.,* 2017). With increased ABA accumulation, the PYR/PYL–PP2C–SnRK2 complex activates a downstream cascade of ABA transcription factors, including the AREB/ABF (ABA-responsive cis-element binding protein/ABA-responsive cis-element binding factor). The AREB/ABF transcription factors have a bZIP domain and four phosphorylation sites contained by SnRK2. After SNRK is phosphorylated, the ABA-response is expressed in ABA signalling under drought stress conditions. Moreover, the expression of transcription factors (ABF genes) and the accumulation of endogenous ABF proteins were dramatically induced by ABA (Wang *et al.,* 2019). Thus ABA is one of the main molecules that makes use of that signal transduction during the drought stress response. Therefore, this transcription mediates a wide variety of genes involved in different processes, such as stomatal closure and metabolite (osmo-protectant) accumulation during water stress (Kuromori *et al.*, 2014a; 2014b).

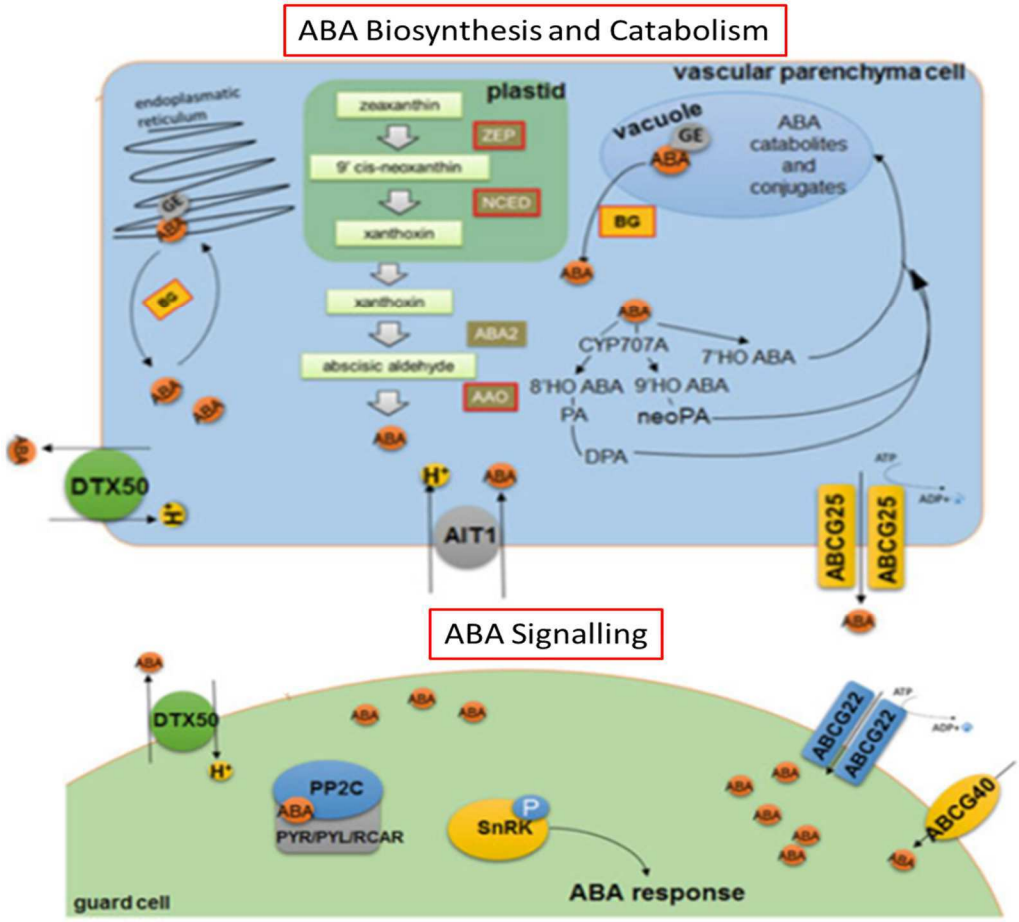


Figure. 1.3. ABA biosynthesis, catabolism, conjugation, de-conjugation, transport and signalling processes (redraw from Daszkowska-Golec, 2016).

JA pathway

Jasmonates (JAs), comprising JA and its derivatives, are lipid-derived signalling compounds (Fig. 1.4). Different branches of lipoxygenase pathway form JA from α-linolenic acid (α-LeA) in chloroplast membranes by oxidative processes. A sequence of a hydroperoxide cyclase (LOX), a reductase and ß-oxidation of the carboxylic acid occurs. In the LOX pathway, only the AOS branch leads to JA formation (Vick and Zimmerman, 1983; Feussner and Wasternack, 2002). Some LOX gene family members, which are 13-LOXs, are involved in wound-induced JA formation. In

addition, an exclusive LOX gene (LOX6) was suggested to allow root JA accumulation in response to abiotic or wounding stresses (Grebner *et al.*, 2013; Christensen *et al.*, 2015).

The enzyme of hydroperoxide cyclase undergoes a two-step reaction catalysed by, allene oxide synthase (AOS) and allene oxide cyclase (AOC) to 12-oxophytodienoic acid (OPDA). Also, the OPDA compound could be made by a spontaneous hydrolysis of the unstable epoxide to α- and γ-ketols and non-enzymatic cyclization (Brash *et al.*, 1988), with all enzymes of OPDA formation located in chloroplasts. As mentioned before, expression of the LOX6 gene could lead to an increase in the basal level of OPDA (Grebner *et al.*, 2013).

In second half of the JA biosynthesis, OPDA is reduced by an OPDA reductase and then it is followed by enzymes of ß-oxidation, the acyl-CoAoxidase (ACX) and the multifunctional proteins (MFPs). These reactions are important in JA biosynthesis to confer stereochemistry of intermediates and products. The AOC catalyzed step establishes the enantiomeric of 7R and 7S forms, which leads to cis-(+)-7-iso-JA. In addition, the next step that conjugates the 7R and 7S forms is one of the most recognised bioactive JA compounds, an isoleucine (+)-7-iso-JA-Ile, which is required for JA perception and signalling (Fonseca *et al.*, 2009). The majority of JA signalling is provided by homeostasis among different JA-Ile derivatives (Wasternack and Hause, 2013; Heitz *et al.*, 2016).

Jasmonic acid (JA) is also involved in facilitating signal transmission during stress (Kazan, 2015). In response to wounding, JA-Ile is rapidly synthesized in the plant tissue (Fonseca *et al.,* 2009; Wasternack and Kombrink, 2019). JA responses are

restrained by (JAZ), which interacts with COI1 (CORONATINE INSENSITIVE1). The complex is involved in the co-reception of biologically active JA-Ile, where JAZ seems to have a strong affinity with bHLH transcription factor such as MYC2 (Cheng *et al.,* 2011; Niu *et al.,* 2011). The target of JAZ proteins and MYC2 is one of the best studied complex considered as a master regulator of the JA signaling pathway (Hadiarto and Tran, 2011; Woldemariam *et al.,* 2011). To initiate transcription, MYC2 recruits the Mediator complex through physical interaction with the MED25 subunit of the plant Mediator complex (Çevik *et al.,* 2012; Chen *et al.,* 2012). Thus JAZ proteins and the transcription factor MYC2 have key regulatory roles during stress adaptation, since they are recognized as the main signalling centres of JA (Kazan, 2013; 2015).

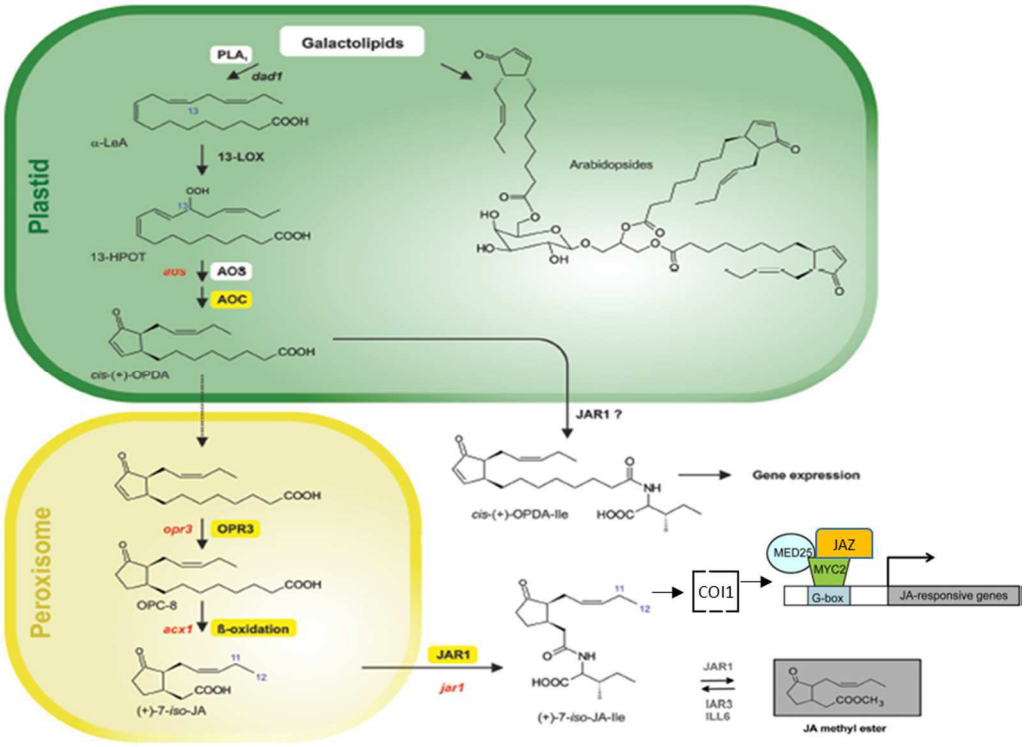


Figure 1.4. JA biosynthesis, catabolism, perception and signal transduction pathway inplant stress response (redraw from Wasternack and Song, 2017).

1.3 Integrating signalling and molecular hormone pathways to induce physiological responses

Maintaining a water potential gradient through the plant results in continued water movement from the soil to the plant. Root water potential must be lower than soil water potential to allow root water uptake (Serraj and Sinclair, 2002). Establishing and maintaining a well-developed root system has a high energy (carbohydrates consumption) requirement that could restrict yield (Bouman and Tuong, 2001; Blum, 2005). Furthermore, plant water use depends on leaf area and the stomatal conductance (Earl, 2003). By regulating water use, stomatal responses to soil drying could be used as a potential trait in selecting drought tolerant soybean genotypes (Vignes *et al.*, 1986; Manavalan *et al.*, 2009).

Plant responses to different environmental variables are carried out through complex and well-coordinated mechanisms. The various genetic and metabolic regulations are under the control of a signalling network that begins with the perception of stress until the final response or effect, such as soil drying (stress) and stomatal closure (effect). ABA and JA can be synthesized in different tissues (roots and leaves) and propagate through the xylem and phloem vessels, activating a number of responses, such as stomatal closure and root growth, and therefore mediate stress tolerance (Bauer *et al.*, 2013; Kuromori *et al.*, 2014b; Nakashima *et al.*, 2014; Yoshida *et al.*, 2015). Thus different techniques have been used to study both root and shoot communication and hormone transport between tissues defining the root-to-shoot and shoot-to-root signalling.

Root-to-shoot signalling requires that a compound moves acropetally in the plant via apoplastic (via xylem) or symplastic pathways, and influences a target organ (such as leaves) by inducing physiological responses. Therefore, xylem sap composition in response to various stresses, such as drought, have been quantified. Xylem sap ABA increases have been correlated with stomatal closure in drying soil (Davies and Zhang, 1990; Dodd, 2003). However, other studies propose that foliar sourced-ABA (synthesised in response to turgor loss) is required for decreasing stomatal conductance (Christmann *et al.,* 2007; McAdam *et al.,* 2016b; Sussmilch *et al.,* 2017).

Girdling is a practice that removes phloem tissue from the stem, disrupting the transport of assimilates, nutrients and, for this study hormones, from the leaves to the roots, while allowing xylem transport (root-to-shoot). By blocking downward phloem translocation, girdling increases ABA concentrations in leaves of citrus (Rivas *et al.*, 2011; Manzi *et al.*, 2015). In addition, carotenoids and xanthophyll compounds followed the same trend as the ABA. Thus, when shoot-to-root communication is impeded, different compounds, including hormones, cannot be transported to the roots. This also prevents the root "recycling" of those hormones into the xylem which allows transport upward through the transpiration stream.

Both ABA and JA have been described as long-distance signals of soil drying and wounding respectively, and both seem to accumulate in leaf and root tissues as the soil dries. Thus it makes sense to make a more detailed study of both hormones using different physiological, biochemical and molecular approaches, to better understand plant responses to soil drying.

Furthermore, since different analytical methods such as radioimmunoassay (RIA) or U-HPLC-MS have been used to quantify hormones, it is necessary to know which method is appropriate for the hormone analyses carried out in this thesis. Both techniques were compared for ABA analysis (Fig. 1.5) of samples from the experiment described in Chapter 4. Even if the absolute ABA concentrations differed between analytical methods, and were generally higher for RIA quantification, the values were highly correlated in both leaf (a, r^2=0.87) and root (b, r^2=0.89) tissues. Since U-HPLC-MS is a more expensive analytical technique, it was only employed in Chapter 4 whereas high throughput quantification of large numbers of samples by RIA was utilised in Chapters 2 and 3 of this thesis.

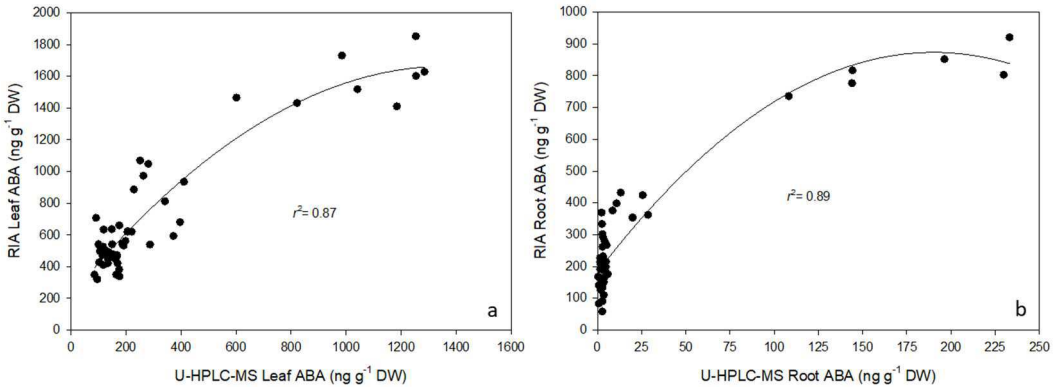


Figure 1.5. Comparing RIA and U-HPLC-MS quantification of ABA concentrations in leaf (a) and root (b). Each point is an individual samples and regressions fitted where significant.

1.4 Aims of the study

This research aimed to understand relationships between physiological (water status and stomatal conductance), biochemical (phytohormones) and gene expression changes and regulation throughout the plant, in response to drying soil. Soybean was chosen as a model species due to its high importance for human and animal nutrition, and the wide range of possibilities (such as plant breeding or genetic modification) to increase yield production and quality.

Initially, different soybean genotypes were used to study the genotypic variation in water relations and ABA accumulation (Chapter 2) to determine the regulation of stomatal conductance in this species. Since xylem sap ABA concentration was highly correlated with stomatal conductance, the role of root-shoot communication in mediating physiological and biochemical responses was investigated by exposing plants to a factorial combination of soil drying and stem girdling (Chapter 3). Since girdling decreased stomatal conductance compared to intact plants, potentially prior to significant ABA accumulation, other hormonal responses were explored following the same treatments (Chapter 4). Since ABA and JA accumulation was highly correlated with stomatal closure and soil water content after withholding water, the role of local hormone synthesis *versus* shoot-to-shoot signalling were studied by measuring the expression of biosynthesis, catabolism and signalling genes within both hormone pathways (Chapter 5).

The aims proposed for this thesis were:

1. To determine genotypic variation of water relations and ABA accumulation of soybean as the soil dries.
2. To study the role of ABA in root-shoot communication of soil drying by disrupting phloem transport via stem girdling just above the cotyledonary node.
3. To determine whether stem girdling and soil drying affect multiple plant hormones, and their role in stomatal closure.
4. To assess the co-ordination of root and shoot ABA and JA genes expression under girdling and drought conditions.

Chapter 2 - Genotypic variation in soybean stomatal conductance, water status and ABA accumulation upon soil drying

2.1 Introduction

Soybean production, as a predominantly rainfed crop, is normally affected by the variation in rainfall between different seasons and years, and is highly susceptible to drought (Pathan *et al.*, 2014; Fried *et al.*, 2019; da Silva *et al.*, 2019). Crop improvement has become essential to mitigate these yield losses, with one of the breeder's objectives to develop new genotypes that can withstand water scarcity and maintain high yields. In addition, genotypic variation can also be an effective tool for studying the regulation of crop stress responses (Alderfasi *et al.*, 2001; King *et al.*, 2009). Cultivar-specific responses to water stress reflect different underlying genetic, morphological, physiological, and biochemical mechanisms (Munns, 2002; Wang *et al.*, 2003; Lei *et al.*, 2006b) which contribute to avoiding yield losses. Early identification of genotypes capable of growing well when access to water is limited should be a breeder's priority. However, there is considerable uncertainty over whether (and which) physiological traits should be included in screening for drought tolerance (Bruce *et al.*, 2002; Manalavan *et al.*, 2009).

Roots can rapidly sense a decrease in soil moisture (Davies *et al.*, 1990; Tardieu and Davies 1993; Battisti and Sentelhas 2017) and rapidly send signals to the shoot (Davies and Zhang 1991) to limit water loss by inducing stomatal closure. These signals and / or their relationships have been classified as hydraulic when tissue

water potential changes (Comstock, 2002; Christmann *et al.*, 2007) and biochemical (Gowing *et al.*, 1993; Schachatman and Goodger, 2008), highlighted by increased abscisic acid (ABA) accumulation triggering stomatal closure. Decreased leaf water potential and stomatal conductance are directly related to yield losses in soybean, since they affect cellular turgor and leaf expansion (Bunce, 1977) and photosynthetic activity (Gilbert *et al.*, 2011). Different genotypes vary in their stomatal sensitivity to changes in soil water deficit (Hetherington and Woodward, 2003; Liu *et al.,* 2005c; Munns *et al.*, 2010; He *et al.,* 2016) and internal water relations (Hufstetler *et al.*, 2007). Thus drought resistance mechanisms to maintain high productivity need to be investigated by characterizing variation in stomatal closure as the soil dries.

ABA is produced in response to a loss of cellular turgor (McAdam and Brodribb, 2016), with root tissues accumulating less ABA in response to dehydration (Zhang *et al.,* 2018). In some species, stomatal conductance decreases in response to an increase or a re-distribution of ABA before any change in leaf water relations (Trejo and Davies, 1991; Ismail *et al.*, 2002). Although two old soybean cultivars (released before 1980) showed greater ABA accumulation as the soil dried, they maintained a higher stomatal conductance at the same level of leaf ABA accumulation than two new cultivars (He *et al.*, 2016). However, whether other soybean cultivars show genotypic variation in the relationships between ABA accumulation, stomatal conductance, leaf water potential and soil water content is still unknown.

To assess these questions, new soybean genotypes or accessions should be contrasted with the physiological responses of the genomic reference Williams 82

(Bernard and Lindahl, 1972) to determine if there is genetic variation in response to different stresses. Lam *et al.*, 2010 described the genotype Union (C08), which is popular in the USA, and Jindou 21 (C12) which is popular in China. C08 was described as a salt-susceptible genotype (Liu *et al.*, 2019). In addition, Hossain *et al.*, 2014 and 2015 described C08 and C012 as susceptible and tolerant genotypes to progressive soil drying respectively, since leaf water potential and stomatal conductance of C08 decreased faster than C12 as the soil dried. Furthermore, two other genotypes, Long Huang 1 (LH1) and Long Huang 2 (LH2), have been identified by farmers as drought tolerant (without being physiologically characterized) in the dry areas of northern China. Since new soybean cultivars had higher yields than old cultivars when water was withheld, and their stomata were seemingly less sensitive to closing stimuli (He *et al.*, 2016), it is necessary to investigate whether these classifications of differing stress sensitivity in soybean genotypes could be related to stomatal responses to soil drying.

Soybean plants of different genotypes were exposed to well-watered and soil drying conditions, with the reference genotype Williams 82 included. Stomatal conductance and water relations was measured in the first and second trifoliate leaf to evaluate their relationship with endogenous xylem sap and leaf tissue ABA concentrations as the soil dries. It was hypothesized that genotypic differences in endogenous xylem sap ABA concentrations better explained variation in stomatal closure in response to drying soil than leaf water relations, based on previous observations in a single soybean cultivar (Liu *et al.,* 2003a; 2005c).

2.2 Materials and methods

2.2.1 Plant materials and experiment design

Soybean (*Glycine max* (L.) Merr., genotypes Williams 82 (W82) (Bernard and Lindahl, 1972) and Jindou 21 (C12) and Union (C08) (Lam at al. 2010) and Long Huang 1 (LH1) and Long Huang 2 (LH2) (kindly supplied by Prof Hon-Ming Lam from Chinese University of Hong Kong)) seeds were sown directly in pots, which were filled with an organic loam (John Innes No. 2, J. Arthur Bowers, UK), and watered to the drip point. Holes (3 cm deep) were made in the soil surface using a bamboo stick, in which the seeds were gently placed and then covered with more substrate and then moistened. Two individual experiments were performed. Genotypes, pot volumes, daytime temperature (Tª), relative humidity (RH), supplementary light (PPFD) with maximum values measured at bench height with a quantum sensor, and photoperiod were as described in Table 2.1. Each environmental parameter was recorded hourly in the centre of the glasshouse using a Hortimax growing solutions Ektron II (Pijnacker, The Netherlands).

Table 2.1. Experimental design and environmental conditions for each experiment.

	Genotypes	Pot Volume (cm^3)	Tª (ºC)	RH%	PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	Photoperiod
Experiment 1	W82 - C12 - C08	762	28± 2	32-35	1200-1400	12 hours (0900-2100h)
Experiment 2	W82 - LH1 - LH2	1000	26 ± 2	30-35		

A commercial liquid fertilizer Miracle-Gro (24:8:16 N:P:K) was applied once (according to the manufacturer´s instructions of 15ml in 4.5 liters of water) to the

plants at the appearance of the first trifoliate leaf. All the plants were irrigated with tap water to drained capacity at 1600h daily (by replacing evapotranspirational losses, determined gravimetrically). During expansion of the second trifoliate leaf, the plants of each genotype were randomized into two treatments (WW: well-watered; Dr: droughted). Five plants from each treatment and genotype were harvested each day. Water was withheld on Day 0 when the experiment started.

2.2.2 Physiological measurements

Measurements were made on the first and second trifoliate leaf (when the second trifoliate leaf was completely expanded), numbering from the base of the plant, throughout the experiment. All the measurements were taken on each plant, only stomatal conductance (as the only non-destructive parameter) was measured on Day 0, since it was not possible to grow sufficient plants in the available glasshouse space to allow destructive sampling on all 6 days (including Day 0) of the experiments. First, the stomatal conductance (g_s) was measured on the central leaflet of both trifoliate leaves with a porometer (Model AP4, Delta-T Devices, Burwell, UK). Two measurements of each leaflet were sequentially made on each plant and averaged. After measuring the stomatal conductance, the central leaflet of the second leaf was excised and collected in Eppendorf vials, which were immediately frozen in liquid nitrogen. Then the remaining two leaflets were excised at the petiole junction with the stem, to measure their leaf water potential (Ψ_{leaf}) with a Scholander-style pressure chamber (Soil Moisture Equipment Crop., Santa Barbara, CA, USA).

The entire first trifoliate leaf was excised at the petiole junction with the stem and Ψ_{leaf} was measured. For all water potential measurements, the chamber was gradually pressurized at 0.03 MPa s^{-1} until the meniscus of the sap appeared, at which time the pressure was recorded. After the first trifoliate Ψ_{leaf} was measured, xylem sap was collected at 0.3 MPa overpressure (Dodd, 2007) above the balancing pressure into Eppendorf vials and immediately frozen in liquid nitrogen. Both leaf tissue and xylem sap were stored at -80ºC for further analysis.

After collecting xylem sap, the entire soil volume was removed from the pot, weighed and then placed in a drying oven until constant weight, to calculate gravimetric soil water content (θ) with the following relationship:

Soil Water Content (θ) = (Fresh soil weight – Dry soil weight) / Dry soil weight

ABA was determined using a radioimmunoassay using the monoclonal antibody MAC252 (Quarrie *et al.*, 1988). Since Liu *et al.,* (2003a) observed no cross-reaction of this antibody with other compounds when crude deionised water extracts or xylem sap were measured, no correction was made to the determined ABA concentrations. While the sap samples were measured without further purification, the leaf tissue samples were lyophilized and finely ground. Deionized water was added (1:25 weight ratio), the sample incubated on a shaker at 4°C overnight, then centrifuged to collect the aqueous extract.

2.2.3 Statistical analysis

Two experiments were done as described above, with the Williams 82 genotype grown in each as a widely used genotypic reference (Table 2.1). Two-way analysis

of variance (ANOVA) determined the effects of water treatment, genotype and their interaction (Fig. 2.1-2.4). Heterogeneous groups were separated by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) to discriminate differences between treatment x genotype combinations (Fig. 2.1-2.4). Analysis of covariance (ANCOVA) and regression analyses determined whether different genotypes affected relationships between plant and soil variables (Fig. 2.5-2.10). To determine variation in the responses of Williams 82 between two independent experiments, two-way analysis of covariance (ANCOVA) determined the effects of water treatment, experiment and their interaction (Table 2.2).

Table 2.2. Comparing relationships between stomatal conductance, soil water content, leaf water potential, leaf xylem sap ABA accumulation and leaf tissue ABA accumulation in two sequential experiments with Williams 82. Relationships are presented as independent variable versus dependent (x-) variable. ANCOVA determined each main effect (*x*-variable and experiment) and their interaction, with *p*-values reported. All data (Days 0 to 5) above are analysed, with statistical differences in the interaction term (indicated in bold text below) observed only when including Day 5. Repeating the analysis with data from Days 0 to 4 resulted in no significant interaction terms, indicating comparable physiological responses in both experiments.

	ANCOVA p-value					
	Experiment		x-variable		Exp.*x-var	
	Days 0-4	Days 0-5	Days 0-4	Days 0-5	Days 0-4	Days 0-5
Stomatal Conductance vs Soil Water Content 1Leaf	0.920	0.685	<0.001	<0.001	0.132	**0.016**
Stomatal Conductance vs Soil Water Content 2Leaf	0.312	0.792	<0.001	<0.001	0.495	**0.019**
Stomatal Conductance vs Leaf Water Potential 1Leaf	0.081	0.184	<0.001	<0.001	0.218	0.514
Stomatal Conductance vs Leaf Water Potential 2Leaf	0.701	0.824	<0.001	<0.001	0.715	0.741
Leaf Water Potential vs Soil Water Content 1Leaf	0.089	0.760	<0.001	<0.001	0.401	0.216
Leaf Water Potential vs Soil Water Content 2Leaf	0.070	0.805	<0.001	<0.001	0.616	**0.010**
Stomatal Conductance vs Leaf xylem sap ABA	0.660	0.625	<0.001	<0.001	0.138	**<0.001**
Stomatal Conductance vs Leaf tissue ABA	0.080	0.400	<0.001	<0.001	0.070	0.900
Leaf Water Potential vs Leaf xylem sap ABA	0.320	0.281	<0.001	<0.001	0.073	**<0.001**
Leaf Water Potential vs Leaf Tissue ABA	0.192	0.338	<0.001	<0.001	0.057	0.438
Leaf xylem sap ABA vs Soil Water Content	0.802	0.002	<0.001	<0.001	0.866	**0.002**
Leaf Tissue ABA vs Soil Water Content	0.327	0.194	<0.001	<0.001	0.248	0.980

2.3 Results

2.3.1 Soil water status

In both experiments, soil water content of well-watered treatments remained between 0.8 and 1 g g^{-1} (Fig. 2.1). Withholding water for 5 days decreased soil water

content in all genotypes, with the reference genotype Williams 82 drying the soil more slowly in both experiments. In Experiment 1, genotype had no significant effect on soil water dynamics until Day 5, with greater drying in cultivars C12 and C08 (Fig. 2.1a).

In Experiment 2, genotypes LH1 and LH2 (Fig. 2.1b) dried the soil more rapidly throughout the experiment. Only on Day 3 of both experiments did the genotypes show a different response to the soil drying treatments (significant treatment x genotype interactions), largely due to the higher values of Williams 82 indicating a slower rate of soil drying.

In both experiments, Williams 82 dried the soil slower, since leaf area of the other genotypes was 10, 13, 22 and 12% higher in C12, C08, LH1 and LH2 respectively (Table 2.3).

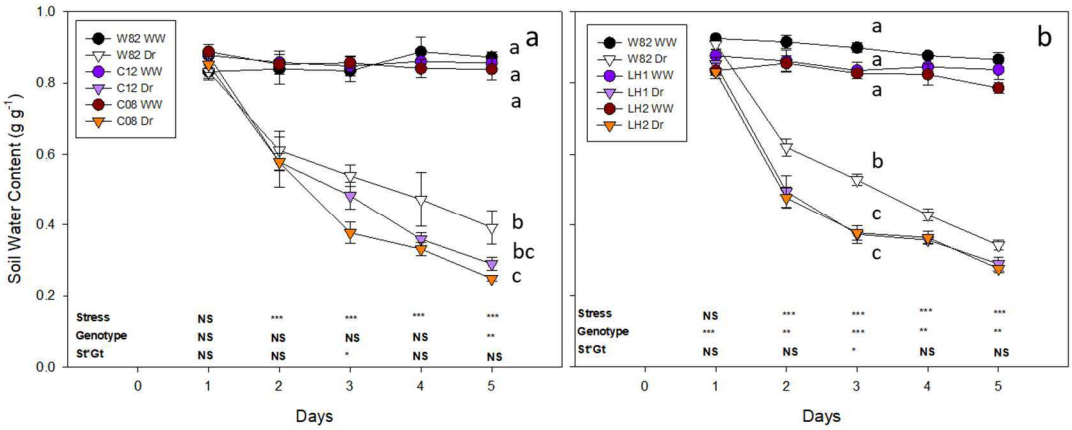


Figure 2.1. Soil Water Content during Experiments 1 (a) and 2 (b), with water withheld from droughted plants on Day 0. Circles and triangles represent well-watered (WW) and droughted (Dr) plants respectively. The reference genotype W82 is represented in black (WW) and white (Dr) colors in both experiments. Symbols indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on Days 3 (b) and 5 (a). Effects of watering treatment (Stress or St), genotype (Genotype or Gt) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Table 2.3. Total leaf area (cm^2) of well-watered and droughted plants after 5 days of withholding water. Values are the mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test, within an experiment (lower case and upper case letters represent Experiments 1 and 2 respectively). *P*-values from 2 way ANOVA of the effects of watering treatment (Stress or St), genotype (Genotype or Gt) and their interaction are indicated.

	Experiment 1			Experiment 2		
	W82	C12	C08	W82	LH1	LH2
Well-Watered	191.7 ± 22.5 ab	179. ± 16.2 ab	237.5 ± 5.7 a	223.8 ± 21.7 AB	245.9 ± 16.9 A	211 ± 10.9 ABC
Drought	146.7 ± 14.1 b	161.3 ± 8.5 b	167.2 ± 15.4 b	142.8 ± 22.6 C	182.5 ± 9.5 ABC	161.3 ± 9.2 BC
P-values from two-way ANOVA	Stress	<0.0012			<0.001	
	Genotype	0.0571			0.1395	
	St * Gt	0.2314			0.6631	

2.3.2 Effect of genotype and soil drying on plant responses

Stomatal conductance (g_s) was measured in the first and second trifoliate leaf in each experiment (Fig. 2.2). In Experiment 1, g_s of the first trifoliate leaf of well-watered leaf plants remained between 300 and 500 mmol m^{-2} s^{-1} (Fig. 2.2a). Soil drying significantly decreased g_s on Day 2, and g_s continued to decline to 20 mmol m^{-2} s^{-1} as a minimum value in C08 on Day 5. Genotype C12 maintained a higher g_s in both treatments on Days 1-3, then declined abruptly in the droughted treatment on Days 4 and 5. This decline resulted in a significant stress x genotype interaction from Day 4, with C12 having higher stomatal sensitivity to drying soil (Fig. 2.2a). Similar stomatal responses were seen in the second trifoliate leaf (Fig. 2.2b), with a significant stress x genotype interaction detected from Day 3. Again, C12 had the highest g_s in well-watered plants, with C08 having the lowest g_s in the drought treatments between Days 2 and 4. Generally, these differences could not be statistically attributed to differences in the rate of soil drying (cf. Fig 2.1a).

In Experiment 2, g_s of well-watered plants remained between 300 and 400 mmol m^{-2} s^{-1} in both first and second trifoliate leaves (Fig 2.2c, d), with limited genotypic differences. Soil drying decreased g_s from Day 2, with g_s declining to 30 mmol m^{-2} s^{-1} for all the genotypes by Day 5 (Fig. 2.2c). The rate of stomatal closure differed between genotypes, with Williams 82 maintaining a higher g_s on Days 2 and 3. Thus there was a significant genotype x treatment interaction on Day 3 (first trifoliate leaf) and Days 2 and 3 (second trifoliate leaf). Stomatal conductance of genotypes LH1 and LH2 was similar in both treatments. In this experiment, the less sensitive

stomatal response of Williams 82 was attributed to a slower rate of soil drying (cf. Fig. 2.1b).

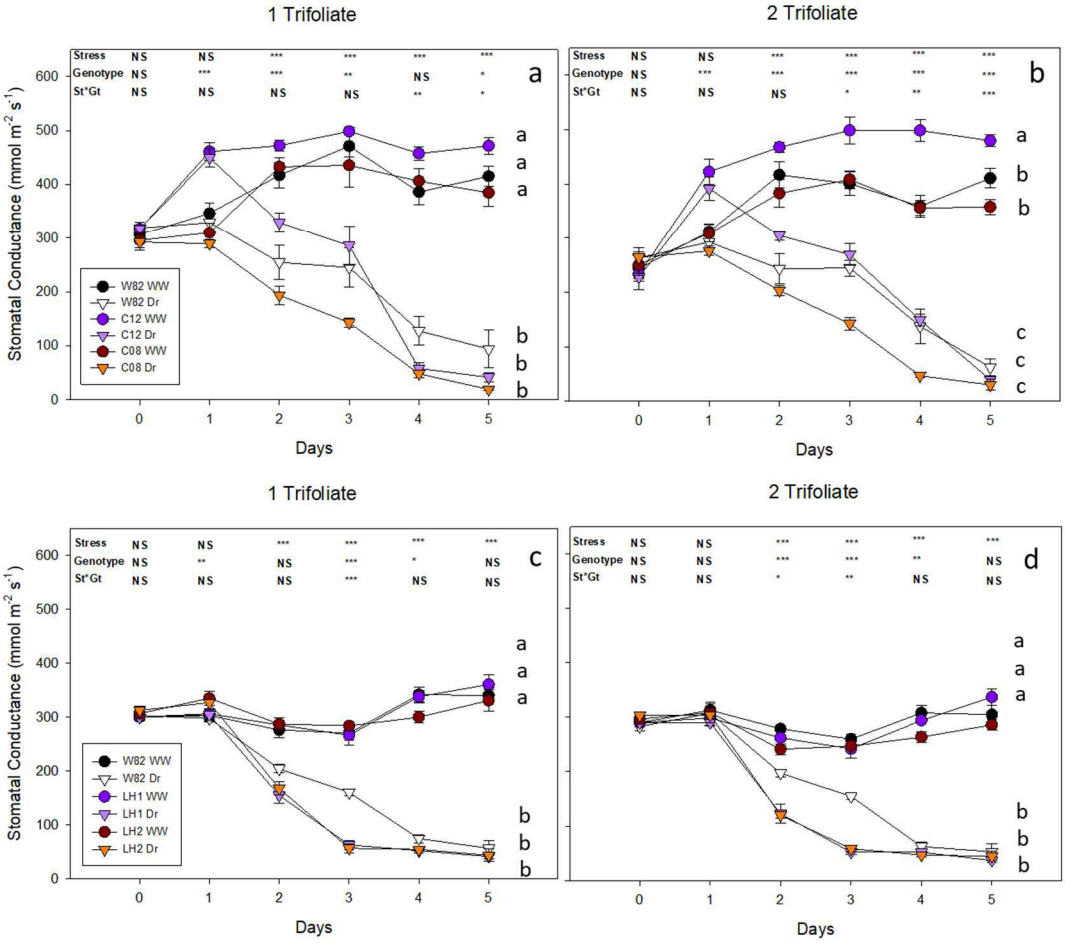


Figure 2.2. Stomatal conductance during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves, with water withheld from droughted plants on Day 0. Measurements on Day 0 were done before imposing stress treatment. Circles and triangles represent well-watered (WW) and droughted (Dr) plants respectively. The reference genotype W82 is represented in black (WW) and white (Dr) colors in both experiments. Symbols indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on Day 5. Effects of watering treatment (Stress or St), genotype (Genotype or Gt) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Soil drying decreased leaf water potential (Ψ_{leaf}) in both trifoliate leaves in both experiments, within 1 or 2 days of withholding water (Fig. 2.3). After withholding water in Experiment 1, Williams 82 had a higher Ψ_{leaf} than the other genotypes on Days 4 and 5 (Fig. 2.3a) and Days 3-5 (Fig. 2.3b) in the first and second trifoliate leaf respectively. On Day 5, C12 had a higher Ψ_{leaf} than C08 by 0.1 MPa in the first and 0.2 MPa in the second trifoliate leaf respectively (Fig. 2.3a, b). Genotypic differences in the sensitivity of Ψ_{leaf} to soil drying (significant genotype x treatment interaction) occurred in the second leaf on Days 3-5, likely due to a more rapid decline in Ψ_{leaf} in C08 than Williams 82.

In Experiment 2, LH1 and LH2 had similar response in both trifoliate leaves for well-watered plants throughout the experiment (Fig. 2.3c, d). Williams 82 had a higher Ψ_{leaf} than the other genotypes on Day 3-5 (Fig. 2.3c) and Days 2-5 (Fig. 2.3d) in the first and second trifoliate leaf respectively. Both LH1 and LH2 showed an accelerated decline in Ψ_{leaf} with soil drying, reaching values 0.4-0.5 MPa lower than Williams 82 on Day 5. Genotypic differences in the sensitivity of Ψ_{leaf} to soil drying (significant genotype x treatment interaction) occurred on Day 5 (first leaf – Fig. 2.3c) and on Days 3 and 5 (second leaf – Fig. 2.3d). Again, the relative insensitivity of Williams 82 Ψ_{leaf} to soil drying likely explained this variation.

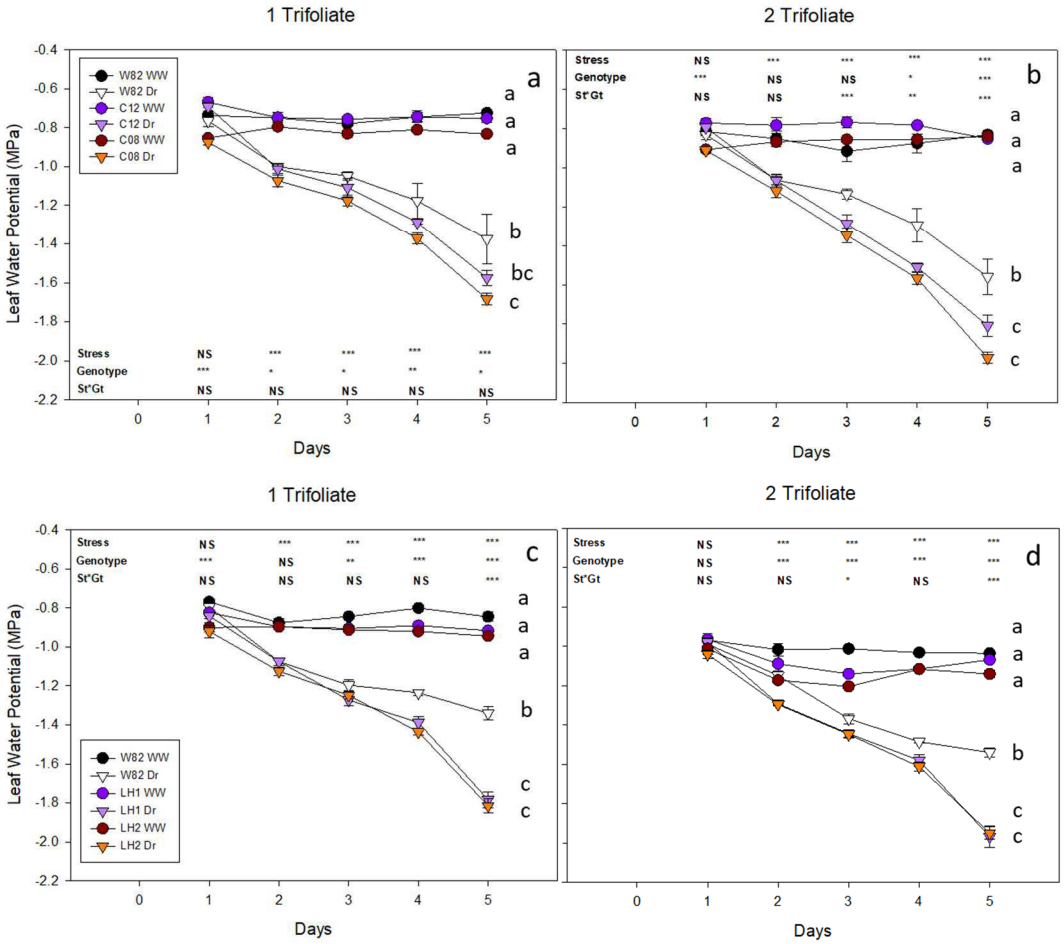


Figure 2.3. Leaf water potential during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves, with water withheld from droughted plants on Day 0. Circles and triangles represent well-watered (WW) and droughted (Dr) plants respectively. The reference genotype W82 is represented in black (WW) and white (Dr) colors in both experiments. Symbols indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on Day 5. Effects of watering treatment (Stress or St), genotype (Genotype or Gt) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Leaf xylem sap ABA ($[X\text{-}ABA]_{leaf}$) and leaf tissue ABA concentrations ($[ABA]_{leaf}$) increased in both experiments after withholding water (Fig. 2.4). In Experiment 1, in the first trifoliate leaf, $[X\text{-}ABA]_{leaf}$ of well-watered plants averaged 91, 82 and 112 nM for Williams 82, C12 and C08 respectively throughout the experiment (Fig. 2.4a). $[X\text{-}ABA]_{leaf}$ of droughted plants increased to 1841, 1161 and 2496 nM in Williams 82, C12 and C08 respectively by the end of the experiment, representing 20-, 14.5- and 22.3-fold increases compared to well-watered plants. Genotypic differences in the sensitivity of $[X\text{-}ABA]_{leaf}$ to soil drying (significant genotype x stress treatment interaction) occurred from Day 3, likely since C08 had higher $[X\text{-}ABA]_{leaf}$ values in drying soil. In the second trifoliate leaf, $[ABA]_{leaf}$ of well-watered plants averaged 928, 839 and 887 ng g^{-1} DW for Williams 82, C12 and C08 respectively throughout the experiment (Fig. 2.4b). $[ABA]_{leaf}$ of droughted plants increased to 3436, 3835 and 4167 ng g^{-1} DW in Williams 82, C12 and C08 respectively by the end of the experiment, representing 3.8-, 4.8- and 5.1-fold increases compared to well-watered plants. As occurred in the first leaf, genotypic differences in the sensitivity of $[ABA]_{leaf}$ to soil drying (significant genotype x stress treatment interaction) occurred from Day 2, likely since C08 had higher $[X\text{-}ABA]_{leaf}$ values in drying soil. Thus genotypic differences in the magnitude of ABA accumulation were consistent between different leaves, and irrespective of whether leaf tissue or xylem sap ABA concentrations were measured.

In Experiment 2, all genotypes had a similar xylem sap ABA concentration (120 nM) in well-watered plants (Fig. 2.4c). $[X\text{-}ABA]_{leaf}$ of droughted plants increased to 834, 1104 and 902 nM in Williams 82, LH1 and LH2 respectively by the end of the

experiment, representing 6.9-, 9.1- and 7.5-fold increases compared to well-watered plants. Genotypic differences in the sensitivity of $[X\text{-}ABA]_{leaf}$ to soil drying (significant genotype x stress treatment interaction) occurred on Days 2, 3 and 5, likely since LH1 had higher $[X\text{-}ABA]_{leaf}$ values. In the second trifoliate leaf, $[ABA]_{leaf}$ of well-watered plants averaged 1170, 835 and 1100 ng g^{-1} DW for Williams 82, C12 and C08 throughout the experiment (Fig. 2.4d). $[ABA]_{leaf}$ of droughted plants increased to 2843, 3554 and 4554 ng g^{-1} DW in Williams 82, LH1 and LH2 respectively by the end of the experiment, representing 2.4-, 4.3- and 4.1-fold increases compared to well-watered plants. In contrast to the first leaf, genotypic differences in the sensitivity of $[ABA]_{leaf}$ to soil drying (significant genotype x stress treatment interaction) in the second leaf occurred from Day 3, likely since LH2 had higher $[ABA]_{leaf}$ values. Although LH1 had the highest xylem ABA concentrations in response to drying soil, LH2 had the highest foliar ABA accumulation.

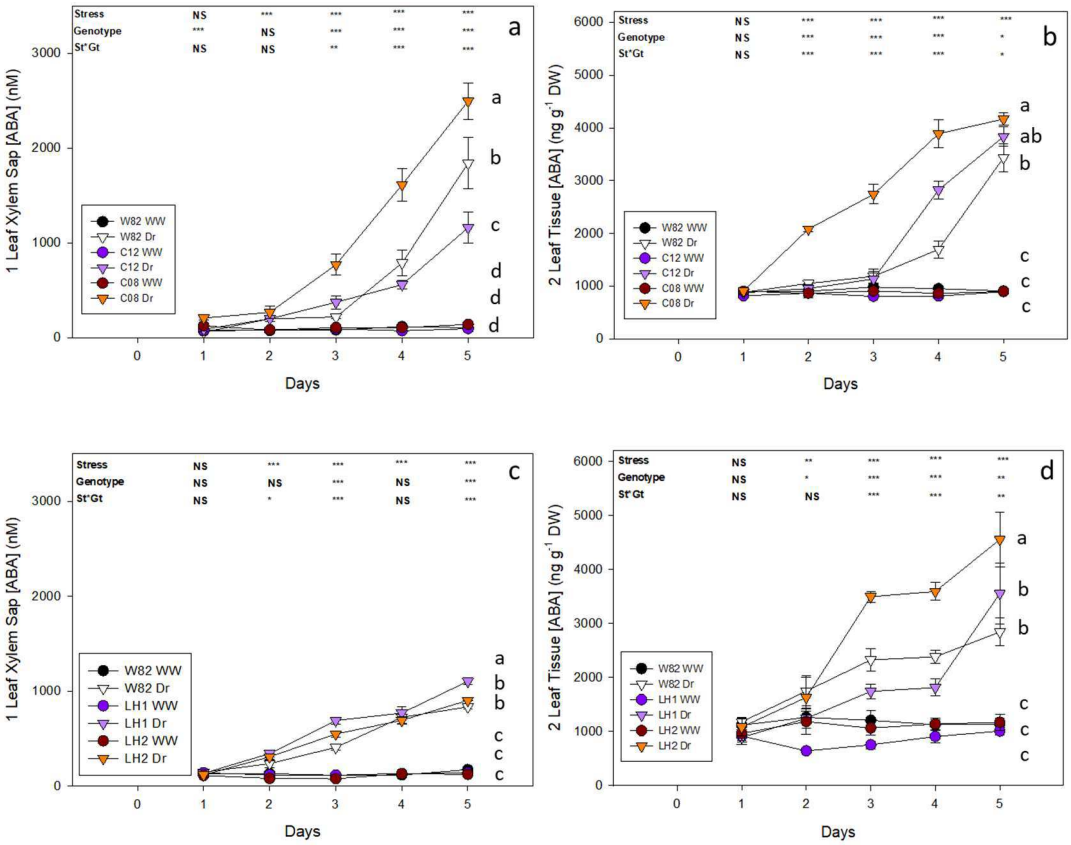


Figure 2.4. Leaf xylem sap ABA concentration (a, c) and leaf tissue ABA concentration (b, d) during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves, with water withheld from droughted plants on Day 0. Circles and triangles represent well-watered (WW) and droughted (Dr) plants respectively. The reference genotype W82 is represented in black (WW) and white (Dr) colors in both experiments. Symbols indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on Day 5. Effects of watering treatment (Stress or St), genotype (Genotype or Gt) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Overall, leaf xylem sap and leaf tissue ABA concentration measurements had a similar ability to discriminate significant genotype and stress effects in response to soil drying, as both ABA measurements increased at the same time. Furthermore, the relative magnitude of drought-induced changes in $[X\text{-}ABA]_{leaf}$ was greater than the changes in $[ABA]_{leaf}$, by 3- to 5- fold in Experiment 1 and 2- to 3-fold in Experiment 2. Ultimately, it is important to understand whether either ABA measurement can explain more of the variation in stomatal response, and the potential regulation of ABA status by variation in soil or plant water status.

2.3.3 Effect of soil drying on different variables

Stomatal conductance decreased as the soil water content decreased (Fig. 2.5). In Experiments 1 and 2, all genotypes had the same relationship between stomatal conductance and soil water content in the first (Fig. 2.5a, c) and the second (Fig. 2.5b, d) trifoliate leaves, as indicated by no significant genotype x soil water content interactions.

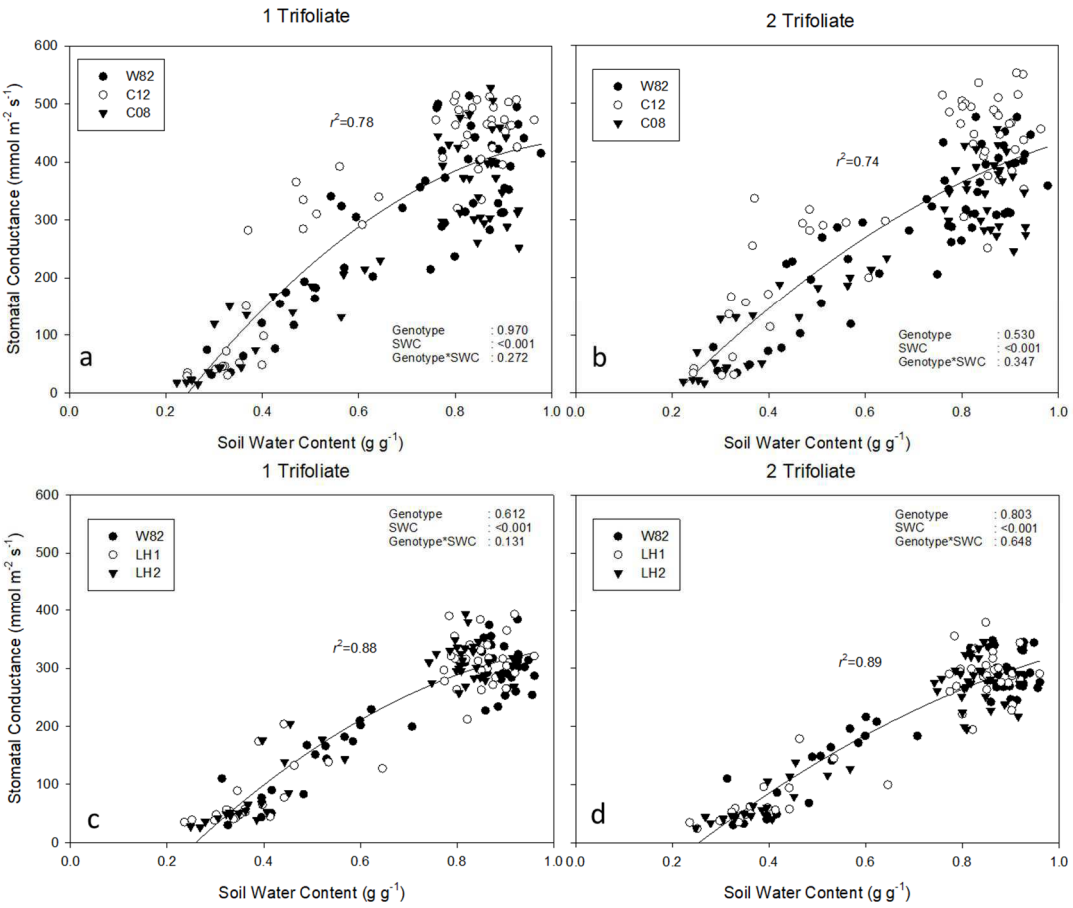


Figure 2.5. Relationships between stomatal conductance and soil water content during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (per experiment and trifoliate leaf) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and genotype) and their interaction are reported.

Leaf water potential decreased as the soil water content decreased (Fig. 2.6). As with stomatal conductance, all genotypes had the same relationship between leaf water potential and soil water content in the first (Fig. 2.6a, c) and the second (Fig. 2.6b, d) trifoliate leaves in both experiments, as indicated by no significant genotype x soil water content interactions.

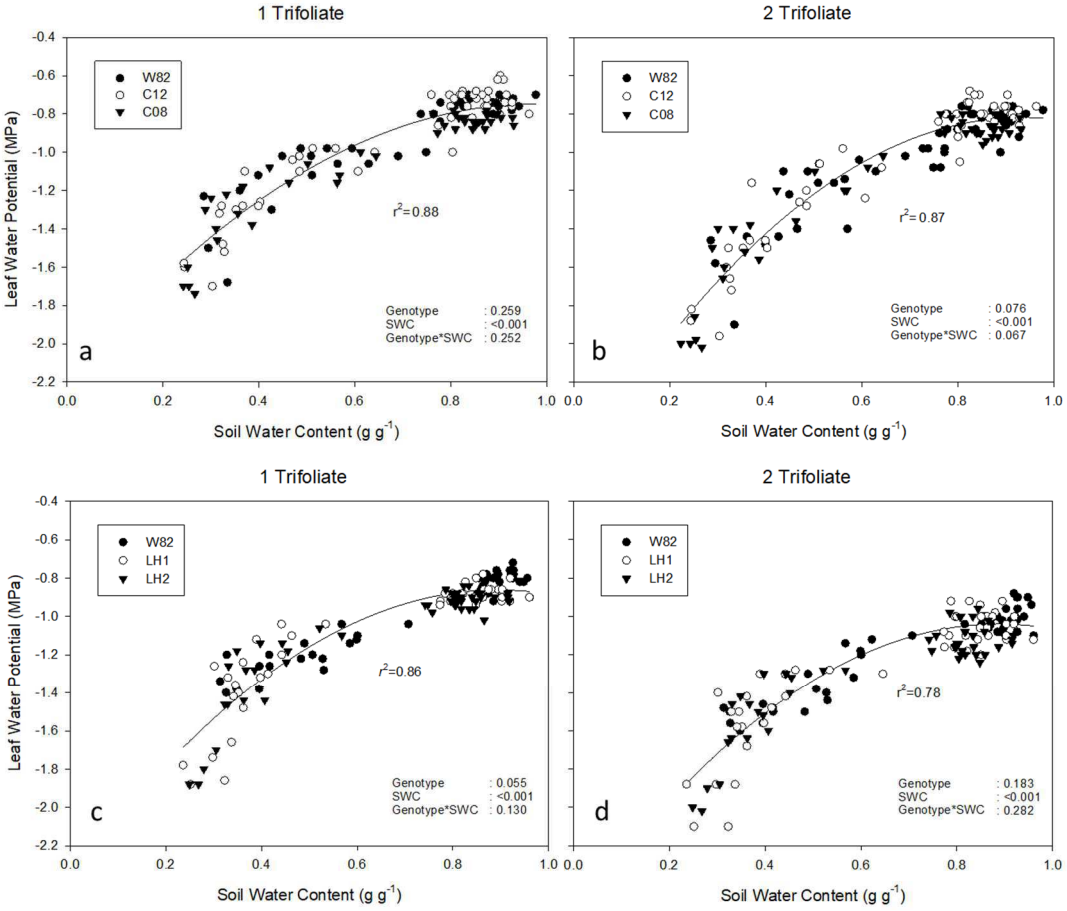


Figure 2.6. Relationships between leaf water potential and soil water content during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (per experiment and trifoliate leaf) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and genotype) and their interaction are reported.

There was genotypic variation in leaf xylem sap [ABA] and leaf tissue [ABA] responses to soil drying (as indicated by significant genotype x soil water content interactions (Fig. 2.7). In Experiment 1, leaf xylem sap [ABA] of the C12 genotype was less sensitive to changes in soil water content (Fig. 2.7a), while C08 showed enhanced foliar ABA accumulation (Fig. 2.7b). In Experiment 2, all genotypes had the same relationship between leaf xylem sap [ABA] and soil water content, as

indicated by no significant genotype x soil water content interaction (Fig. 2.7c). In the second trifoliate leaf, LH2 had higher ABA concentrations once soil water content declined below 0.4 g g^{-1} (Fig. 2.7d). Generally, leaf tissue [ABA] has greater genotypic variation upon soil drying, as in the C12, C08 and LH2 genotypes. On the other hand, W82 and LH1, variation in soil water content better explained leaf xylem sap [ABA] than leaf tissue [ABA].

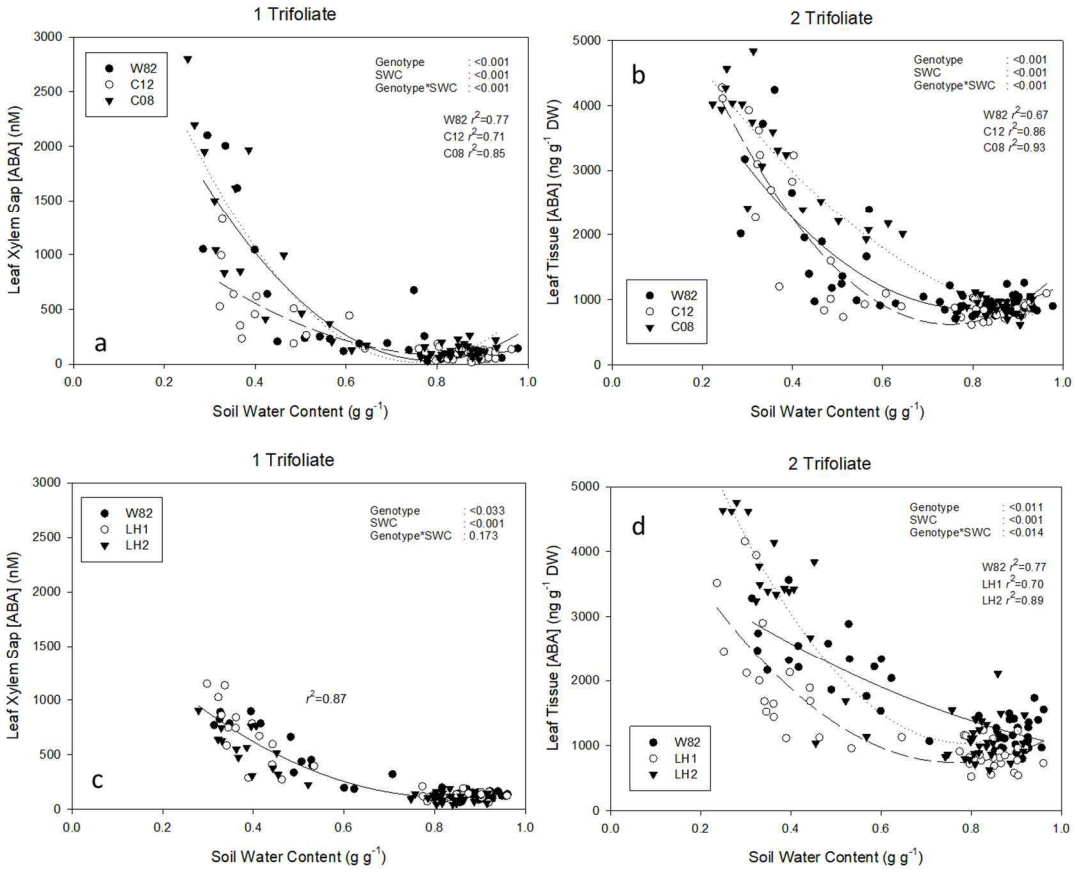


Figure 2.7. Relationships between leaf xylem sap ABA concentration (a, c), leaf tissue ABA concentration (b, d) and soil water content during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (c), solid line = W82, long dash = C12 and LH1 and dotted line = C08 and LH2 (a, b and d) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and genotype) and their interaction are reported.

2.3.4 Relationships between stomatal conductance, leaf water potential and ABA status

Stomatal conductance decreased as leaf xylem sap [ABA] and leaf tissue [ABA] increased in all genotypes (Fig. 2.8). In the first trifoliate leaf, C12 genotype differed from W82 and C08 in the relationship between g_s and leaf xylem sap [ABA] and in the second trifoliate leaf C08 differed from C12 in the relationship between g_s and leaf tissue [ABA]in Experiment 1 (Fig. 8a and b respectively). Stomatal conductance of the C12 and C08 genotypes was less sensitive to leaf tissue [ABA] (Fig. 2.8b). In Experiment 2, all genotypes had the same relationship between g_s and leaf xylem sap [ABA] in the first trifoliate leaf, as indicated by no significant genotype x [ABA] interaction (Fig. 2.8c). In the second trifoliate leaf, LH2 genotype differed from W82 genotype in the relationship between g_s and leaf tissue [ABA] (Fig. 2.8d). Generally, stomatal conductance was marginally better explained by variation in leaf xylem sap [ABA] than leaf tissue [ABA], as indicated by higher r^2 values in the correlations.

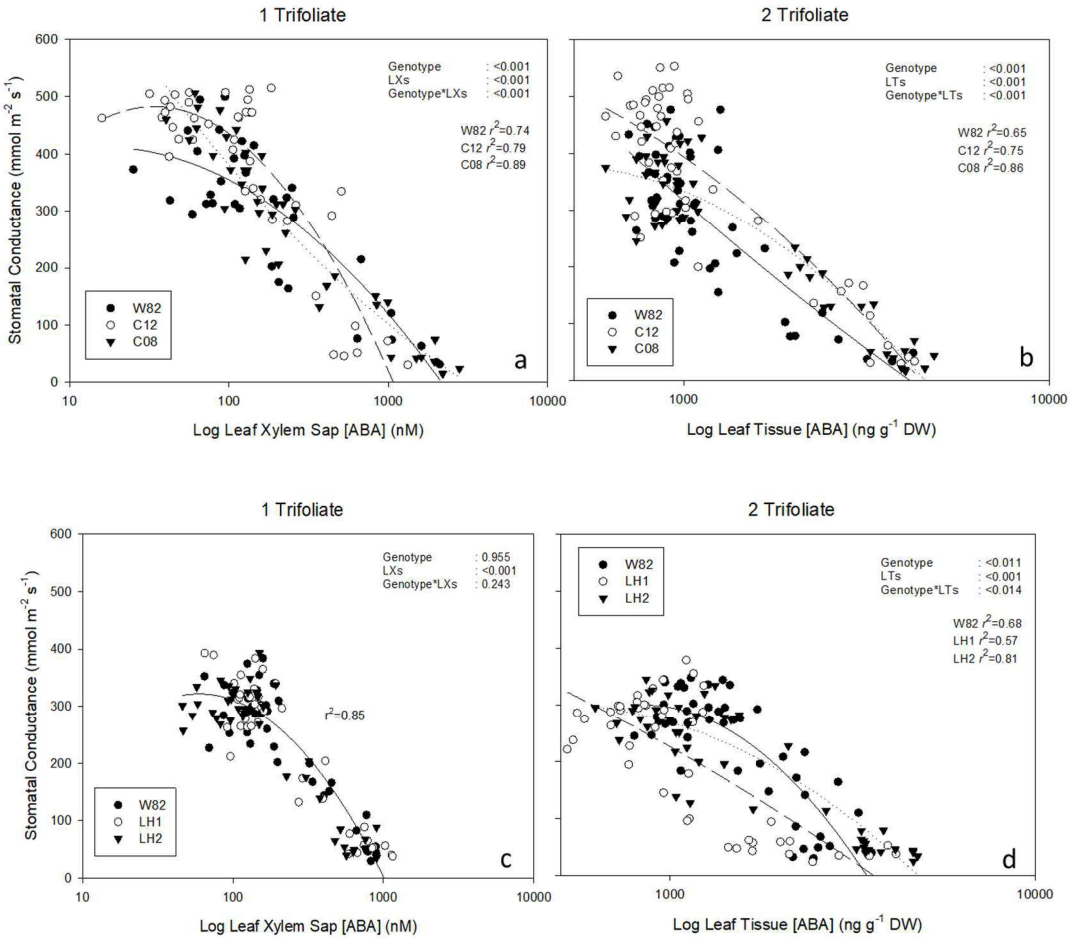


Figure 2.8. Relationships between leaf stomatal conductance and leaf xylem sap ABA accumulation (a, c) and leaf tissue ABA accumulation (b, d) during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (c), solid line = W82, long dash = C12 and LH1 and dotted line = C08 and LH2 (a, b and d) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect ([ABA] and genotype) and their interaction are reported.

The same pattern as mentioned above occurs in the relationships between first leaf xylem sap [ABA], second leaf tissue [ABA] and Ψ_{leaf} in Experiments 1 and 2 (Fig. 2.9). In the first trifoliate leaf, C12 genotype differed from W82 and C08 in the relationship between leaf xylem sap [ABA] and Ψ_{leaf}, but in the second trifoliate leaf

C08 differed from W82 and C12 genotype in the relationship between leaf tissue [ABA] and Ψ_{leaf} in Experiment 1 (Fig. 2.9a and b respectively). Leaf tissue [ABA] was more sensitive to changes in Ψ_{leaf} in the C08 genotype (Fig. 2.9b). In Experiment 2, all genotypes had the same relationship between leaf xylem sap [ABA] and Ψ_{leaf}, as indicated by no significant genotype x Ψ_{leaf} interaction (Fig. 2.9c). In the second trifoliate leaf, LH2 genotype was slightly different from LH1 in the relationship between leaf tissue [ABA] and Ψ_{leaf} (Fig. 2.9d). In Experiment 1, variation in Ψ_{leaf} better explained changes in leaf xylem sap [ABA] than leaf tissue [ABA] in the W82 genotype, whereas Ψ_{leaf} of C12 and C08 was more closely related to leaf tissue [ABA]. In Experiment 2, Ψ_{leaf} of LH1 and LH2 was more closely related to leaf tissue leaf xylem sap [ABA] than leaf tissue [ABA]. Generally, xylem sap [ABA] of the first trifoliate leaf was more tightly correlated with Ψ_{leaf} than leaf tissue [ABA] of the second trifoliate leaf was correlated with Ψ_{leaf}.

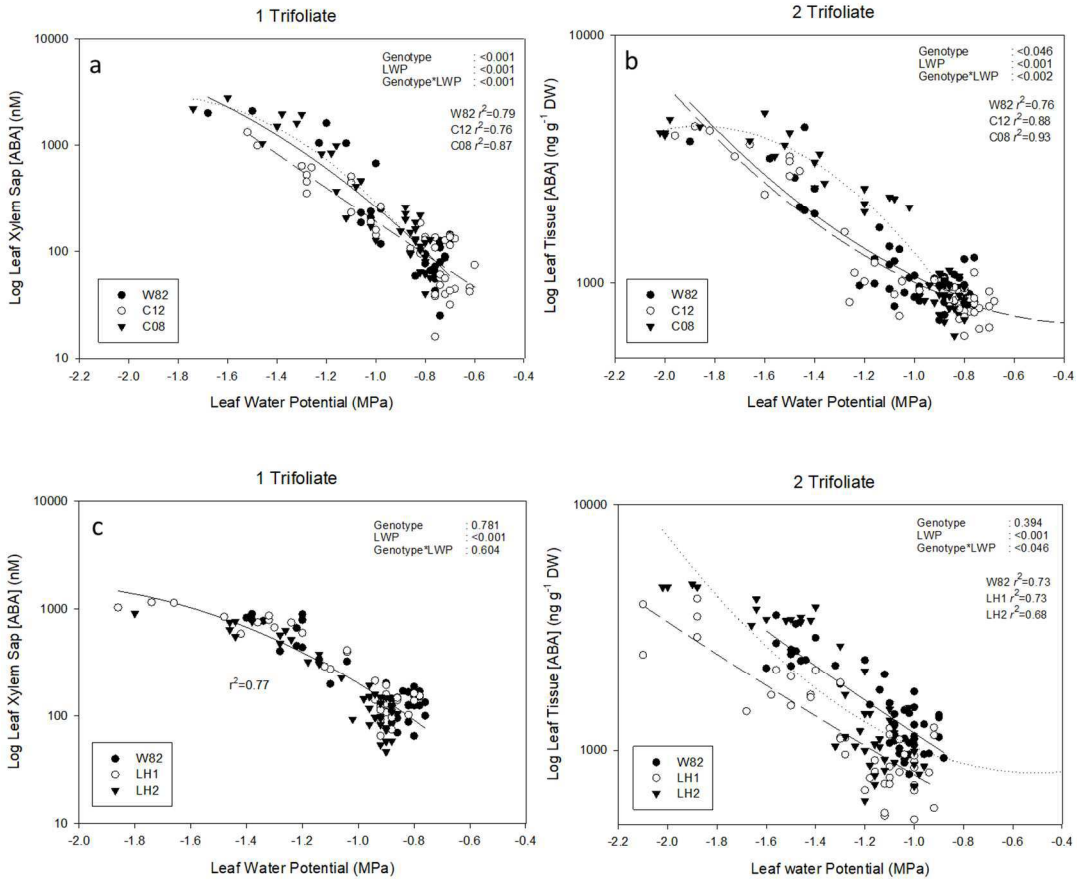


Figure 2.9. Relationships between leaf xylem sap ABA accumulation, leaf tissue ABA accumulation and leaf water potential during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (c), solid line = W82, long dash = C12 and LH1 and dotted line = C08 and LH2 (a, b and d) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and genotype) and their interaction are reported.

In addition, g_s decreased as the leaf water potential decreased (Fig. 2.10). In both experiments, all genotypes had the same relationship between g_s and Ψ_{leaf} in the first trifoliate leaf, as indicated by no significant genotype x Ψ_{leaf} interactions in both experiments (Fig. 2.10a, c). In contrast, the second trifoliate leaf showed genotypic variation in sensitivity of g_s to Ψ_{leaf} (significant genotype x leaf water potential

interaction) (Fig. 2.10b, d). In Experiment 1, C08 genotype differed from W82 and C12 in the relationship between g_s and Ψ_{leaf} (Fig. 2.10b). While in Experiment 2, LH1 and LH2 genotype differed from W82 in the relationship between g_s and Ψ_{leaf} (Fig. 2.10d). Generally, g_s of the second trifoliate leaf (rather than the first) was highly affected by genotypic variation in Ψ_{leaf}.

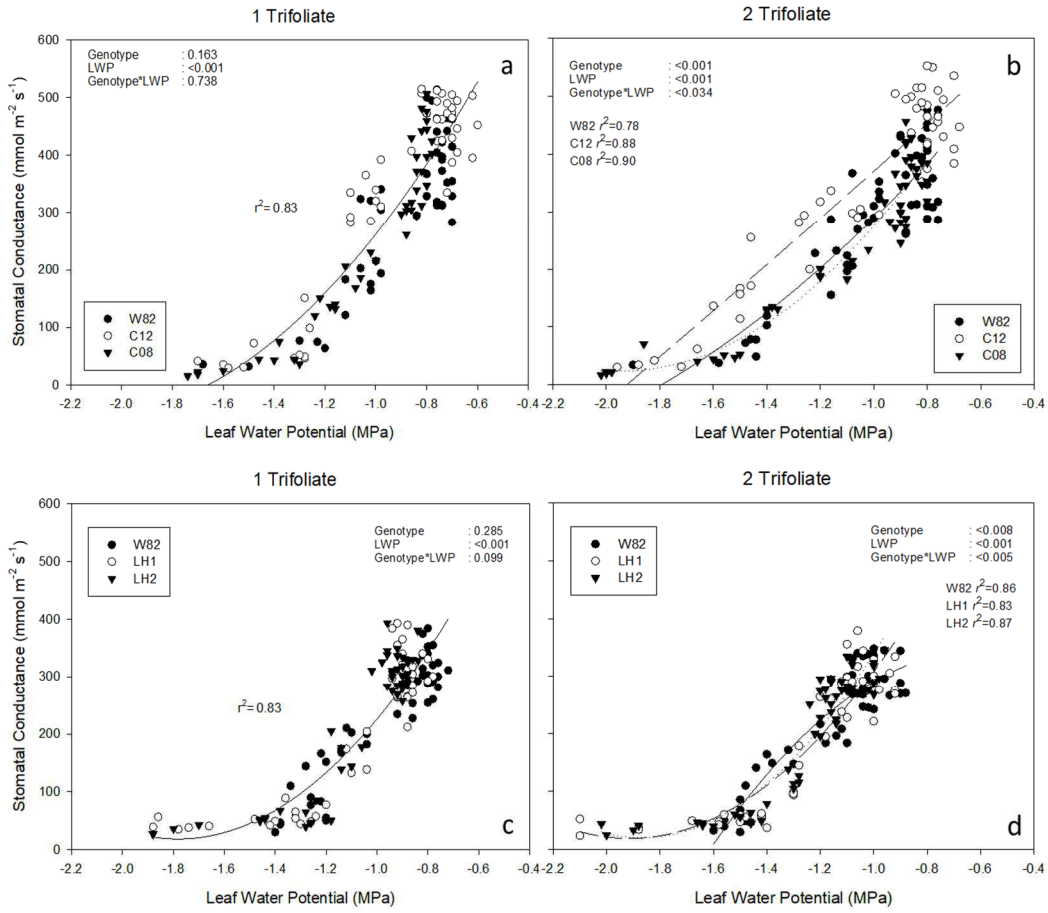


Figure 2.10. Relationships between leaf stomatal conductance and leaf water potential during Experiments 1 (a, b) and 2 (c, d) in the first (a, c) and second (b, d) trifoliate leaves. Filled circles represent W82 in both experiments as a reference. Hollow circles and filled triangles represent C12 and C08 respectively, and LH1 and LH2 respectively in each experiment. Each symbol is an individual plant and regression lines were fitted to all genotypes (c), solid line = W82, long dash = C12 and LH1 and dotted line = C08 and LH2 (a, b and d) where $P < 0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and genotype) and their interaction are reported.

2.4 Discussion

Drought tolerance in soybean has been emphasized by breeders with different strategies (such as yield, WUE and root morphology) to avoid yield losses (Fried *et al.*, 2019), while ABA relations have not been extensively investigated in this species. However, the numbers of cultivated and wild soybean genotypes make physiological screening for drought tolerance a labour-intensive exercise.

Since these experiments selected several genotypes with unknown drought tolerance, it was impractical to include all genotypes in a single experiment. Thus Williams 82 was selected as a reference genotype in two independent experiments conducted sequentially in the same growing space under similar environmental conditions (Table 2.1). Such an experimental strategy would be invalidated if the reference genotype showed contrasting physiological responses in independent experiments, suggesting a genotype x environment interaction commonly observed in breeder's trials. Nevertheless, correlating plant and soil variables between the 2 sets of Williams 82 data showed no significant experiment x x-variable interactions when Days 0 to 4 were considered in the analysis (Table 2.2), indicating comparable physiological responses of Williams 82 in both experiments. Only when the soil drying was prolonged (Days 0 to 5) did half the statistical analyses show significant experiment x x-variable interactions. This consistent response of Williams 82 to soil drying in both experiments (Table 2.2) validates the experimental strategy, yet it was not known whether this old genotype (released in 1972) is more or less sensitive to water deficit than more recently released varieties.

A recent study with two old and two new Chinese soybean cultivars indicated that stomatal conductance of the newer cultivars was more sensitive to soil drying (He et al. 2016), with stomatal closure initiated at higher soil moisture values. In contrast, leaf relative water content of the newer cultivars declined at much lower soil moisture values than the older cultivars, with stomatal closure occurring at higher soil moisture values than changes in leaf water status. This disparity suggests that stomatal closure in soybean was mediated by non-hydraulic factors, with leaf ABA accumulation increased at higher soil moisture values by a difference of 10% decline in soil water content.

In this study, Williams 82 dried the soil more slowly than the more modern genotypes (Fig. 2.1), even if significant genotypic differences were only detected on Day 5 in Experiment 1 (comparing against the C08 and C12 genotypes) and throughout Experiment 2 (comparing against the LH1 and LH2 genotypes). These genotypic differences were likely due to the lower leaf area of Williams 82 (Table 2.3) causing a slower rate of soil drying.

These responses could reflect lower sensitivity of Williams 82 to drought, since its stomatal conductance and leaf water potential were higher than the other genotypes in both experiments (Fig. 2.2 and 2.3). These responses were accentuated in the second trifoliate leaf, with higher values after two days (Experiment 1 - Fig. 2.3b and Experiment 2 – Fig. 2.3d) causing significant genotype x treatment interactions. However, in both experiments, all genotypes and both trifoliate leaves showed no variation in the relationships between the stomatal conductance or leaf water potential and soil water content (Fig. 2.5 and 2.6). Thus

all genotypes showed a similar sensitivity of stomatal conductance to soil drying (Fig 2.5), as indicated by no significant genotype x soil water content interactions. Temporal differences in stomatal responses of the genotypes as the soil dried (Fig. 2.2) were simply due to differences in the rate of soil drying (Fig. 2.1). Similarly, all genotypes showed a similar sensitivity of leaf water potential to soil drying (Fig 2.6), and minimal differences (only in Experiment 2) in the sensitivity of stomatal conductance to leaf water potential (Fig 2.10), suggesting that changes in leaf water status were the most parsimonious explanation for stomatal closure (cf. Liu *et al.,* 2003a). However, such correlations do not indicate causality, and maintaining leaf water status as the soil dried via root pressurisation did not prevent drought-induced stomatal closure (Gollan *et al.,* 1986).

Different signals causing stomatal closure have been described as hydraulic or biochemical, where it is supposed that a hydraulic signal (loss of leaf turgor) should stimulate ABA biosynthesis (Pierce and Raschke, 1980; Christmann *et al.*, 2007). On the other hand, previous studies suggest that ABA may be important in mediating stomatal closure of soybean before any decrease in leaf turgor (Liu *et al.,* 2003a; 2005c), so it is worth understanding whether leaf xylem sap or leaf tissue ABA measurement can explain better the stomatal responses. Under progressive soil drying conditions, leaf xylem sap [ABA] of some genotypes showed a lower fold-changed response than leaf tissue [ABA] (Fig. 2.4; 2.7), suggesting that the capacity to redistribute ABA between tissues is genotype-dependent. Taken together, leaf xylem sap ABA concentration better explained (higher regression values) variation in stomatal conductance than variation in foliar ABA accumulation when comparing

the two leaves analysed. This occurred since much of the ABA in the leaf was likely compartmentalised in mesophyll tissues, where it was inaccessible to the guard cells (Wilkinson *et al.,* 1997).

In conclusion, stomatal conductance was better explained by variation in $[X\text{-}ABA]_{leaf}$ than $[ABA]_{leaf}$, since the results suggest that leaf xylem sap ABA is highly correlated with stomatal conductance in all the genotypes studied (Fig. 2.8), which is physiologically important as stomatal closure limits soybean yields (Bunce, 1977). Since ABA in xylem sap can be derived from both root/apoplastic sources (Zhang and Davies, 1990) and leaf/symplastic sources (Borel and Simmoneau, 2002), the importance of root-to-shoot and shoot-to-root signalling in regulating xylem sap ABA concentration should be investigated.

Chapter 3 - Stem girdling uncouples soybean stomatal conductance from leaf water potential by enhancing leaf xylem ABA concentration

3.1 Introduction

Soybean is one of the most important crops in the world, but its production is often limited by drought (Doss *et al.*, 1974; Eck *et al.*, 1987; Liu *et al.*, 2003*b;* Pardo *et al.*, 2015). Soil water deficits developing during critical stages of reproductive development can limit seed set, induce pod abortion and decrease individual seed dry weight, thereby decreasing soybean yield (Liu *et al.*, 2003b; Pardo *et al.*, 2015). Understanding the physiological and molecular responses to drought offers opportunities to enhance soybean drought tolerance by overexpressing key regulatory genes, including those that determine plant hormone status (Manavalan *et al.*, 2009). Plant hormones control multiple physiological and developmental processes that determine crop yields (Morgan and King, 1984; Li *et al.*, 2013). Abscisic acid (ABA) is a key phytohormone involved in regulating plant water status by controlling stomatal aperture (Tardieu *et al.*, 1996; Schurr and Schulze, 1996; Wilkinson and Davies, 2002) and leaf and root hydraulic conductance (Pantin *et al.*, 2013; Dodd, 2013).

During water deficit, ABA concentrations increase throughout the plant, partially closing the stomata which acts to maintain leaf water status (Liu *et al.*, 2003a; 2005c), but there has been considerable debate as to which organ (roots *versus* shoots) is the first to perceive soil drying (cf. Kramer, 1988; Passioura, 1988). It was

proposed that ABA is primarily synthesized in the root, then transported in the xylem sap to the shoot where it accumulates in the leaf apoplast to initiate stomatal closure (Davies and Zhang, 1991), thus reducing transpiration. Root ABA concentration increases as soil water content and root water potential decreases (Zhang and Davies, 1989a; Puertolas *et al.*, 2013), suggesting that soil drying increases root ABA biosynthesis. Root ABA concentrations are linearly related to the concentrations of ABA detected in xylem sap, suggesting that roots are an important source of xylem ABA (Liang *et al.*, 1997). Moreover, the concentrations of ABA found in the leaf xylem sap are sufficient to close the stomata of species such as maize (Zhang and Davies, 1991) and pea (Rothwell *et al.*, 2015), as determined by experiments that measure the transpiration of detached leaves supplied with synthetic ABA via the xylem. Nevertheless, in some species, xylem sap ABA concentrations are insufficient to explain stomatal closure (Munns and King, 1988) and adding osmotica to the roots caused shoot ABA accumulation prior to any root ABA accumulation (Christmann *et al.*, 2005). Such observations have challenged the concept of root-to-shoot ABA signalling and prompted the search for other xylem-borne antitranspirants.

A further challenge to the concept of root-to-shoot ABA signalling comes from experiments that have suppressed shoot-to-root ABA transport by girdling (removal of stem phloem tissue at the root-shoot junction). Using this technique, different studies have demonstrated the importance of shoot-sourced ABA in explaining root ABA accumulation in response to water stress induced by chilling (Vernieri *et al.*, 2001) or drought (Liang *et al.*, 1997; Ikegami *et al.*, 2009; Manzi *et al.*, 2015). In

contrast, stem girdling had minimal effects on root ABA accumulation in both *Xanthium* and tomato, with dehydrated roots of stem-girdled plants showing 80% of the root ABA accumulation (averaged across both species) of intact plants (Cornish and Zeevaart, 1985), indicating root-autonomous ABA biosynthesis. These contrasting results demonstrate the need to further investigate the origin of the ABA accumulated in roots in response to drought.

Furthermore, the impact of obstructing the phloem flow on shoot ABA accumulation remains unclear. Early studies show that petiole girdling can stimulate ABA accumulation in leaf laminae and trigger stomatal closure (Setter *et al.*, 1980; Setter and Brun, 1981), while others show that stem girdling has no significant effect on leaf ABA accumulation (Vernieri *et al.*, 2001; Manzi *et al.*, 2015). In contrast, stem girdling stimulated pronounced (50% increase) foliar ABA accumulation in young vegetative tissues while ABA concentrations of mature leaves almost halved (Rivas *et al.,* 2011), indicating that the effect of girdling on ABA accumulation may intensify with distance from the wound site. This may be related to basipetal gradients in foliar ABA concentration (Mitchell *et al.,* 2016) and xylem ABA concentration (Soar *et al.,* 2004), which seem important in regulating stomatal responses. Root xylem ABA concentrations explained more of the variation in drought-induced stomatal closure than bulk leaf ABA concentration in soybean (Liu *et al.,* 2003a; b) and other species (Zhang and Davies, 1990). Nevertheless, the impact of stem girdling on leaf xylem ABA concentration has not yet been investigated.

To assess these questions, soybean plants were exposed to a factorial combination of soil drying and stem girdling. Stomatal conductance was measured daily and water relations / xylem ABA concentration measured in different parts of the plant (roots, shoots, leaves) to evaluate the dependence of ABA accumulation on tissue water relations. It was hypothesised that shoot to root ABA transport determines ABA distribution in the plant and thus stomatal responses to soil drying.

3.2 Materials and methods

3.2.1 Plant materials and experiment design

Soybean (*Glycine max* L. Merr. cv. Siverka) seeds were germinated in the dark on moistened filter paper for 3 days, then sown in pots which fitted perfectly inside a Scholander-type pressure chamber (Soil Moisture Equipment Crop., Santa Barbara, CA, USA). Pots were 6.5 cm in diameter and 23 cm in length (762 cm^3 in volume), with a steel mesh (0.7 mm aperture) base to allow drainage. Pots were filled with an organic loam (John Innes No. 2, J. Arthur Bowers, UK), watered to the drip point and then seedlings of uniform development (radical length 30-50 mm) transplanted.

Plants were grown in a naturally lit greenhouse with an average daytime temperature of 27 ± 2°C, with a relative humidity of 30-40% and supplementary lighting providing a PPFD at bench height of 250-400 µmol m^{-2} s^{-1} for a 13 h photoperiod (0700-2000h). A commercial liquid fertilizer Miracle-Gro (24:8:16 N:P:K) was applied once to the plants at the appearance of the first trifoliate leaf. All the plants were irrigated to drained capacity at 1600h daily (by replacing

evapotranspirational losses, determined gravimetrically). During expansion of the third trifoliate leaf, the plants were randomized into 4 groups, comprising the treatments applied: soil drying (WW: well-watered; DR: droughted) and girdling (NG: intact plants; G: Girdled plants) respectively. Five plants from each treatment were harvested each day. Girdling was achieved surgically (at 1400h on Day 0), when the third trifoliate leaf was completely expanded, by excising 10 mm of phloem tissue from the stem (at 100-110 mm above the soil surface) with a sharp razor blade. Plants were girdled between the cotyledonary node and the second node, where the unifoliate leaf was located. At this time, the cotyledons had either naturally abscised or were excised, to prevent them influencing root hormone concentrations (Waadt *et al.,* 2014). Water was withheld from half of the girdled and non-girdled plants after the girdling was complete on Day 0. Thus 20 hours elapsed between girdling and stomatal conductance measurements on the following day (Day 1).

3.2.2 Physiological measurements

Measurements were made on the third trifoliate leaf (when it was completely expanded) throughout the experiment. Stomatal conductance (g_s) was measured daily at 1000h (except on Day 0 that was at 1200h) on the central leaflet of the third trifoliate leaf with a porometer (Model AP4, Delta-T Devices, Burwell, UK). Two measurements were sequentially made on each plant and averaged.

Leaf, shoot and root water potential were measured with a Scholander-style pressure chamber (Soil Moisture Equipment Crop., Santa Barbara, CA, USA). After measuring stomatal conductance, the leaf was excised at the petiole junction with

the stem, then leaf water potential measured. Then the shoot was de-topped 6-7 cm from the stem base (in the middle of the girdled tissue to avoid phloem contamination of xylem sap samples) and placed in the pressure chamber to measure shoot water potential. Finally the entire pot was sealed in the chamber with sufficient stem protruding to measure root water potential. For all water potential measurements, the chamber was gradually pressurized at 0.03 MPa s^{-1} until the meniscus of the sap appeared, at which time the pressure was recorded.

Once the water potential of each organ was measured, xylem sap was collected at 0.3 MPa overpressure (Dodd, 2007) above the balancing pressure. Xylem sap was collected in Eppendorf vials and immediately frozen in liquid nitrogen, and stored at -80°C for further analysis. On the last day of harvest, when the soil volume was extracted from the pot, 15-20 mg (dry weight – determined retrospectively) of the root system was removed from the middle of the pot, briefly washed (to remove adhering soil debris), then frozen in liquid nitrogen. After measuring root water potential (and collecting root samples on the last day of the experiment), the entire soil volume was removed from the pot, weighed and then placed in a drying oven until constant weight, to calculate gravimetric soil water content (θ) with the following relationship:

Soil Water Content (θ) = (Fresh soil weight – Dry soil weight) / Dry soil weight

ABA was determined using a radioimmunoassay using the monoclonal antibody MAC252 (Quarrie *et al.*, 1988). While the sap samples were measured without further purification, the root tissue samples were lyophilized and finely ground.

Deionized water was added (1:50 weight ratio), the sample incubated on a shaker at 4°C overnight, then centrifuged to collect the aqueous extract.

3.2.3 Statistical analysis

The experiment was repeated twice with qualitatively similar results, thus data from a single experiment are presented. Two-way analysis of variance (ANOVA) determined the effects of water treatment, girdling and their interaction. Heterogeneous groups were separated by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) to discriminate differences between treatment x girdling combinations. Analysis of covariance (ANCOVA) and regression analyses determined whether girdling affected relationships between plant and soil variables (eg. Fig. 3.6; 3.7 and Table 3.1; 3.2 respectively).

3.3 Results

3.3.1 Soil water status

Soil water content of both well-watered treatments remained around 1 g g^{-1} during the experiment (Fig. 3.1). Withholding water for 5 days decreased soil water content similarly, by *circa* 60% compared to well-watered plants, in both droughted treatments. Girdling had no significant effect on soil water dynamics during the experiment, even if droughted–girdled plants dried the soil slightly slower.

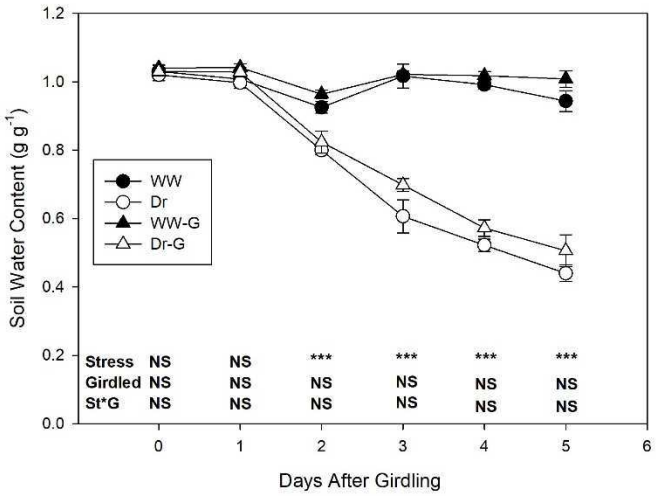


Figure 3.1. Soil Water Content during the experiment, with water withheld from droughted plants, and girdling on Day 0. Measurements on Day 0 were done before imposing treatments. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G) respectively. Symbols indicate mean ± s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

3.3.2 Effect of girdling and soil drying on plant responses

Stomatal conductance (g_s) of well-watered, intact plants remained between 130 and 150 mmol m^{-2} s^{-1} during the experiment, unlike the other treatments (Fig. 3.2). One day after girdling, g_s decreased by 15% (averaged across both water treatments). Girdling significantly decreased g_s of well-watered plants 4 days after girdling, and was almost half that of well-watered intact plants at the end of the experiment. Soil drying decreased g_s within 2 days of withholding water, and g_s steadily decreased during the experiment in both girdled and intact plants. Towards the end of the experiment, the effects of girdling on stomatal conductance depended on soil water status (significant girdling x treatment interaction), since

girdling substantially decreased g_s of well-watered plants but had no significant effect on plants in drying soil.

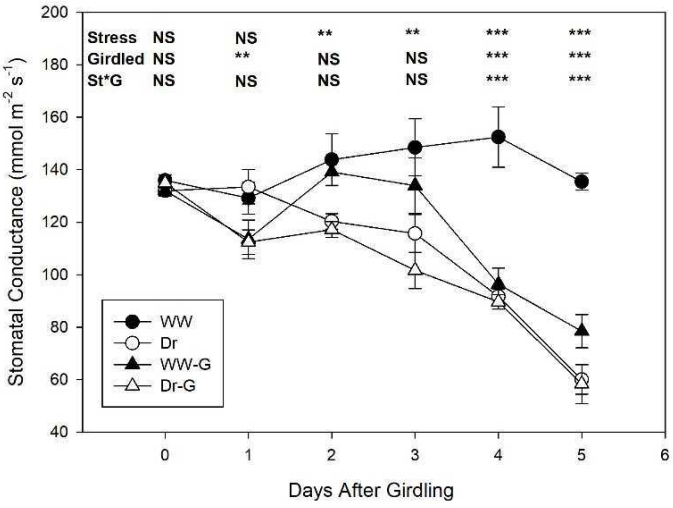


Figure 3.2. Stomatal conductance during the experiment, with water withheld from droughted plants, and girdling on Day 0. Measurements on Day 0 were done before imposing treatments. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G) respectively. Vertical bars indicate mean ± s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Soil drying decreased water potential of all tissues (Fig. 3.3). Soil drying decreased leaf water potential (Ψ_{leaf}) throughout the experiment, such that Ψ_{leaf} was 0.1 MPa and 0.2 MPa lower than well-watered plants for girdled and intact plants respectively (Fig. 3.3a). Girdling increased Ψ_{leaf} by 0.12 MPa (averaged across both water treatments) on Day 3 and increased Ψ_{leaf} of plants grown in drying soil on Day 5. On Day 5, the effects of girdling on Ψ_{leaf} depended on soil water status (significant

girdling x treatment interaction) since girdling had no effect on Ψ_{leaf} of well-watered plants but significantly increased Ψ_{leaf} of plants in drying soil.

Similarly, the effects of girdling on shoot water potential (Ψ_{shoot}) on Day 5 depended on soil water status, even though girdling had no significant effect throughout the experiment. Soil drying decreased Ψ_{shoot} by 0.15 MPa (intact plants) and 0.08 MPa (girdled plants) during the experiment.

Root water potential (Ψ_{root}) did not differ between the two groups of well-watered plants throughout the experiment. Soil drying significantly decreased Ψ_{root} on Days 2, 4 and 5 after withholding water. At the end of the experiment, soil drying decreased Ψ_{root} to -0.22 and -0.11 MPa in intact and girdled plants respectively (Fig. 3.3c). On the last two days of the experiment, the effect of soil drying on Ψ_{root} depended on girdling (significant girdling x treatment interaction) such that girdling decreased the Ψ_{root} of well-watered plants (by 0.04 MPa) but increased the Ψ_{root} of plants in drying soil (by 0.07 MPa). Taken together, soil drying decreased Ψ throughout the plant, but girdling mitigated this effect in all tissues, especially on the last day of measurements.

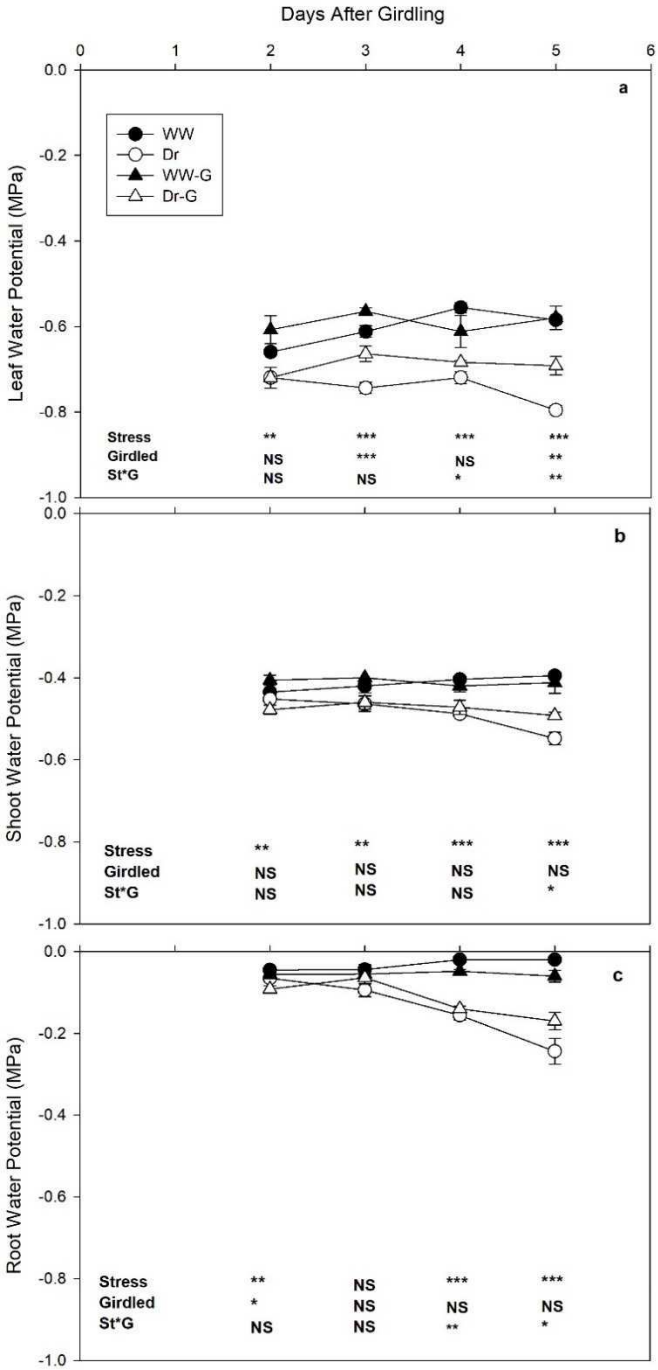


Figure 3.3. Leaf (a), Shoot (b) and Root (c) Water Potential during the experiment, with water withheld from droughted plants, and girdling on Day 0. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G) respectively. Vertical bars indicate mean ± s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

In well-watered intact plants, xylem sap ABA concentrations were stable throughout the experiment, averaging 126, 260 and 242 nM in samples collected from the roots, shoots and leaves respectively (Fig. 3.4). In well-watered plants, girdling increased leaf xylem sap ABA concentration (by 60% averaged over Days 3-5 of the experiment) (Fig. 3.4a), had no effect on shoot xylem ABA concentration (Fig. 3.4b) and decreased root xylem sap ABA concentration (by 66% averaged over the entire experiment) (Fig. 3.4c) compared with well-watered intact plants. Girdling decreased root xylem ABA concentration within two days.

In intact plants, soil drying increased root, shoot and leaf xylem ABA concentrations within 3-4 days of withholding water, with significant differences from well-watered plants first detected in root xylem ABA concentration. By the end of the experiment, soil drying increased root and shoot xylem ABA concentrations by 2.3-fold and in the leaf by 3-fold compared to well-watered intact plants. Girdling attenuated this soil-drying induced increase throughout the plant, such that at the end of the experiment, root, shoot and leaf xylem ABA concentrations were 84, 42 and 30% lower than in intact plants exposed to soil drying. Indeed, on Day 5, girdling resulted in well-watered plants and those exposed to drying soil having statistically similar xylem ABA concentrations throughout the plant.

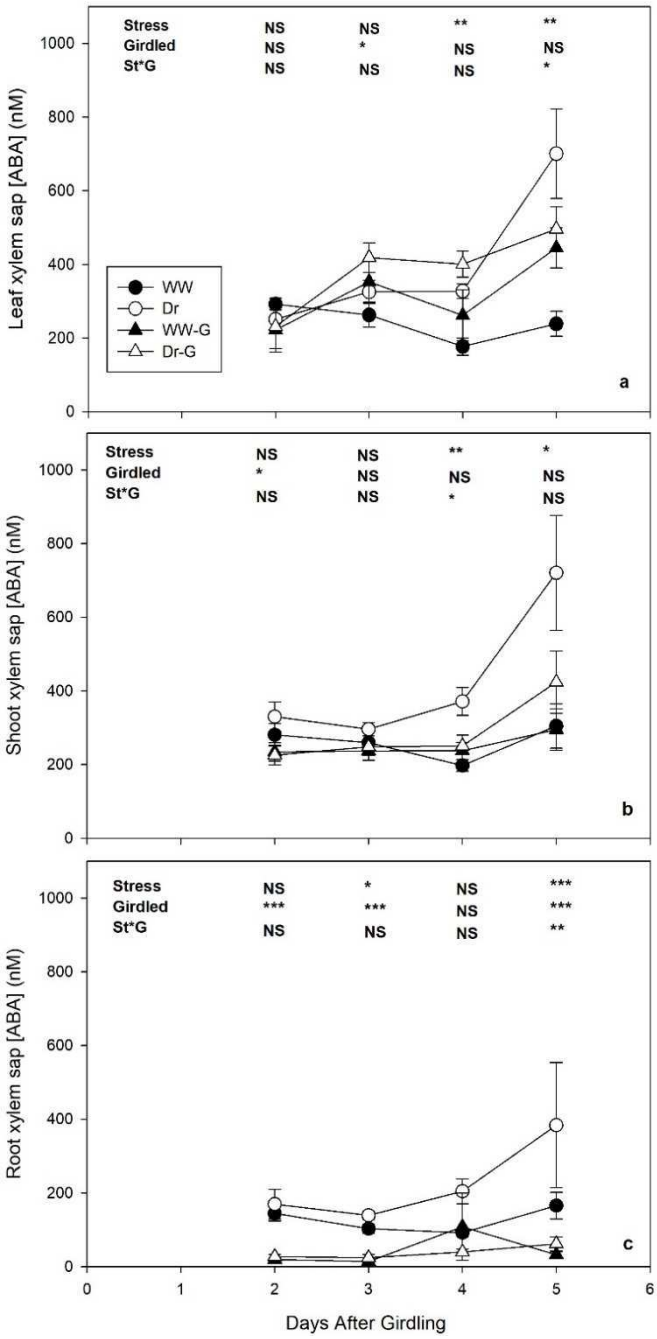


Figure 3.4. Leaf (a), Shoot (b) and Root (c) xylem sap ABA concentration during the experiment, with water withheld from droughted plants, and girdling on Day 0. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G) respectively. Vertical bars indicate mean ± s.e. (n=5). Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

Girdling decreased root ABA concentration by nearly 80% (compared to intact plants) within 20 hours (Day 1), a disparity that was maintained in well-watered plants on Day 3 (Fig. 3.5). In intact plants, 3 days of soil drying increased root ABA concentration by 4-fold compared to well-watered plants, but the magnitude of this increase was attenuated in girdled plants (3-fold increase). Thus well-watered intact plants and girdled plants exposed to drying soil had statistically similar root ABA concentrations on Day 3. Significant drought-induced root ABA accumulation occurred in intact plants also on Day 5, while in girdled plants an increase in root ABA concentration of well-watered plants resulted in no statistical differences from those exposed to soil drying. By Day 5, only intact plants exposed to soil drying had higher root ABA concentrations than the other treatments. Thus girdling decreased root ABA concentration of well-watered plants shortly after treatment (Days 1, 3), and attenuated drought-induced root ABA accumulation.

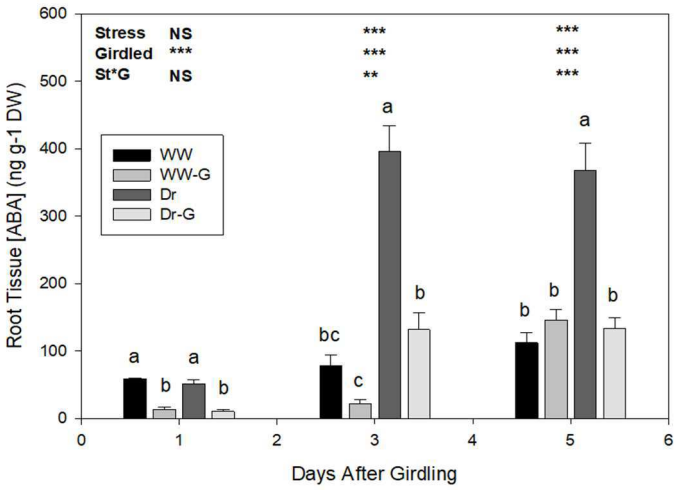


Figure 3.5. Root tissue ABA concentration of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) during the experiment. Vertical bars indicate mean ± s.e. (n=5). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each day. Effects of watering treatment (Stress or St), girdling (Girdled or G) and their interaction are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

3.3.3 Relationship of stomatal conductance to different variables

Stomatal conductance decreased as leaf water potential decreased in intact plants (Table 3.1), although girdling attenuated stomatal sensitivity to leaf water potential (significant girdling x Ψ_{leaf} interaction - Fig. 3.6a). In contrast, girdling did not affect the relationships between stomatal conductance and either leaf xylem ABA concentration (Fig. 3.6b) or soil water content (Fig. 3.6c). Stomatal conductance of individual well-watered plants varied 3-fold (with the lowest values in girdled plants), but was not related to soil water content, while soil drying below 0.6 g g^{-1} significantly decreased g_s. Thus girdling altered stomatal response to leaf water potential (Table 3.1), but not other putative regulatory variables.

	Girdled plants		**Intact plants**		**All plants**	
	p-value	r^2	*p*-value	r^2	*p*-value	r^2
g_s vs Ψ_{leaf}	0.348	0.03	<0.001	0.70		
g_s vs Leaf xylem [ABA]	0.001	0.28	0.001	0.28	<0.001	0.34
g_s vs θ	0.001	0.24	<0.001	0.64	<0.001	0.38

Table 3.1. Linear regression values (*p*-value and r^2) for the relationships between stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}), leaf xylem sap [ABA] and soil water content (θ) in girdled plants, intact plants and all plants. Each column represents all values from girdled plants, intact plants and all plants. Where a significant girdling x *x*-variable interaction exists (indicating that girdling affects the relationship), it is inappropriate to pool data for "all plants".

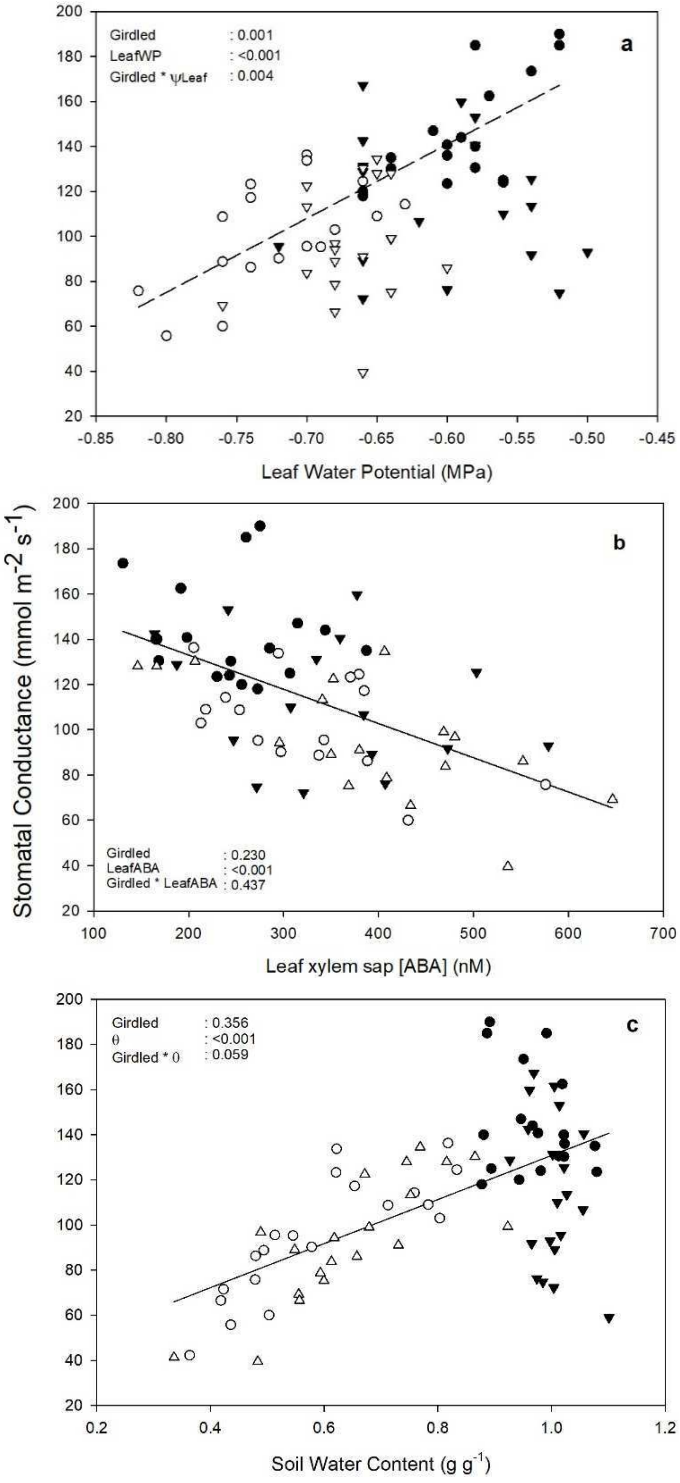


Figure 3.6. Relationships between stomatal conductance and leaf water potential (a), leaf xylem sap [ABA] (b) and soil water content (c). Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G), respectively. Each symbol is an individual plant and regression lines were fitted to intact plants (a) and all data (b, c) where $P<0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported.

3.3.4 Effect of soil drying on xylem ABA concentration

Girdling resulted in no significant relationships between tissue water status and xylem ABA concentrations of those tissues (Table 3.2). Although leaf xylem ABA concentration was not significantly related to leaf water potential in intact plants, shoot and root xylem ABA concentrations significantly increased as shoot and root water potentials decreased (Table 3.2). In all tissues, xylem ABA concentration increased as the soil water content decreased in intact plants (Table 3.2; Fig. 3.7). Although girdling did not significantly affect the relationships between leaf and shoot xylem ABA concentrations and soil water content (Fig. 3.7a, b), it attenuated the sensitivity of root xylem ABA concentration to the soil water content (significant girdling x soil water content interaction). Thus soil drying increased root xylem sap [ABA] to a greater extent (4.6-fold) in intact plants than girdled plants (Fig. 3.7c).

	Girdled plants		**Intact plants**		**All plants**	
	p-value	r^2	*p*-value	r^2	*p*-value	r^2
Leaf xylem [ABA] vs θ	0.043	0.12	<0.001	0.37	<0.001	0.17
Shoot xylem [ABA] vs θ	0.045	0.11	<0.001	0.42	<0.001	0.25
Root xylem [ABA] vs θ	<0.001	0. 28	<0.001	0.41		
Leaf xylem [ABA] vs Ψ_{leaf}	0.952	0.00	0.155	0.06	0.408	0.01
Shoot xylem [ABA] vs Ψ_{shoot}	0.854	0.00	0.045	0.12	0.106	0.04
Root xylem [ABA] vs Ψ_{root}	0.309	0.03	<0.001	0.34	0.004	0.12

Table 3.2. Linear regression values (*p*-value and r^2) for the relationships between leaf xylem sap [ABA], shoot xylem sap [ABA], root xylem sap [ABA] and soil water content (θ) and leaf / shoot / root water potential (Ψ_{leaf} / Ψ_{shoot} / Ψ_{root}). Each column represents all values from girdled plants, intact plants and all plants. Where a significant girdling x *x*-variable interaction exists (indicating that girdling affects the relationship), it is inappropriate to pool data for "all plants".

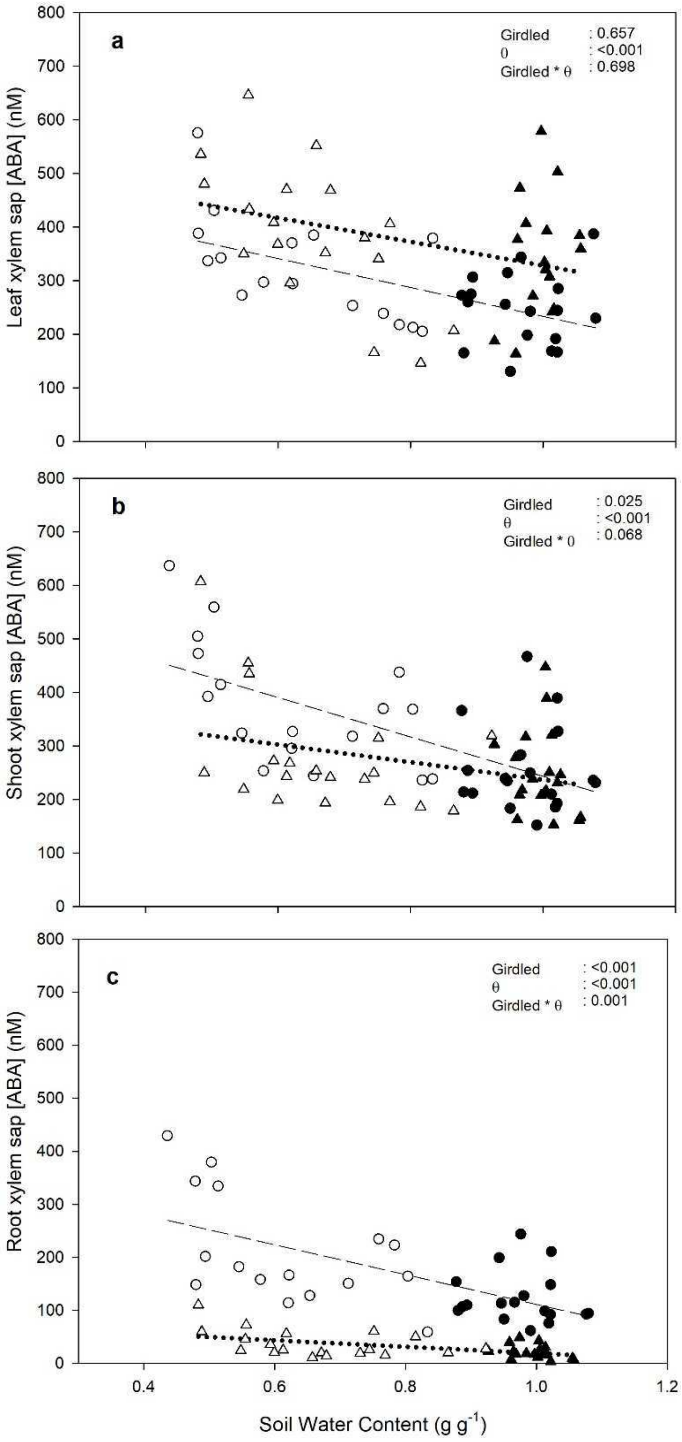


Figure 3.7. Relationships between leaf xylem sap [ABA] (a), shoot xylem sap [ABA] (b), root xylem sap [ABA] (c) and soil water content. Filled circles and filled triangles represent well-watered intact and girdled plants (WW and WW-G) respectively, while hollow circles and hollow triangles represent droughted intact and girdled plants (Dr and Dr-G), respectively. Each symbol is an individual plant and regression lines (dashed lines = intact plants; dotted lines = girdled plants) were fitted where $P<0.05$. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported.

3.4 Discussion

Recent studies emphasise the importance of foliar [ABA] in regulating stomatal conductance (Bauer *et al.,* 2013; McAdam and Brodribb, 2018). Increased foliar ABA levels have been correlated with decreased leaf water status (Sack *et al.,* 2018, Pierce and Raschke, 1980, McAdam and Brodribb, 2016). Our results show a unifying relationship between g_s and leaf xylem [ABA] irrespective of whether the plants were girdled (Fig. 3.6b), whereas g_s was only correlated with Ψ_{leaf} in intact plants (Fig. 3.6a), suggesting that foliar [ABA] regulates stomatal aperture regardless leaf water status when shoot to root ABA transport is interrupted. Similarly, frequent measurements of both variables as the soil dries demonstrated that leaf xylem ABA concentration increases prior to any change in Ψ_{leaf} (Liu *et al.*, 2005c) and better explained early stomatal closure (than leaf ABA levels) during the initial stages of soil drying (Liu *et al.,* 2003a, b). Moreover, girdling increased Ψ_{leaf} in both drying soil (Fig. 3.3a) and under well-watered conditions (Setter *et al.*, 1980; Mitchell *et al.,* 2016), while promoting ABA accumulation and stomatal closure, suggesting that ABA-mediated stomatal closure acted to maintain Ψ_{leaf}. Indeed, in other species, soil drying induced stomatal closure can be associated with increased Ψ_{leaf} (Kudoyarova *et al.*, 2007; Visentin *et al.*, 2016) suggesting that $\Psi_{leaf,}$ can be regulated by stomatal response. Taken together, these studies suggest that leaf ABA accumulation is not always associated with decreased leaf water status but in some situations can also be determined by ABA transport to and from the leaf.

Since ABA is an important stomatal regulator, it is necessary to understand where in the plant it is produced. By compromising communication between the aerial part of the plant and the roots via the phloem, stem girdling attenuated (Day 3) or eliminated (Day 5) root ABA accumulation in response to drying soil (Fig. 3.5). Similarly, girdled citrus plants showed attenuated root ABA accumulation following an initial (3 day) soil drying cycle, but following a 3 day recovery (re-watered soil) period, no drought-induced ABA accumulation during a subsequent drying cycle (Manzi *et al.,* 2015). Furthermore, stem girdling attenuated root hormone export to the shoot via the xylem as the soil dried (Fig. 3.7c). Drying soil increased xylem sap ABA concentrations irrespective of sampling position in intact plants, but girdling attenuated the increase in xylem ABA concentration as the soil dried (Fig. 3.4). This suggests that root ABA export partially depends on shoot-to-root ABA transport in the phloem (Slovik *et al.*, 1995). Recycling of ABA between phloem and xylem in the roots made a variable contribution to the root-to-shoot ABA signal depending on soil water status, comprising 45 and 72% of root ABA export under salinized and non-salinized conditions respectively (Wolf *et al.*, 1990). The remaining contribution originated from *de novo* root ABA biosynthesis, which was accentuated when roots were exposed to salinity. Taken together, *de novo* ABA synthesis in the roots makes a variable contribution to root ABA accumulation and xylem export, with clear impacts during the early stages of soil drying seemingly being abolished following more intense (Fig. 3.5) or repeated (Manzi *et al.,* 2015) soil drying, as time since girdling increased.

Similarly, girdling eliminated root ABA accumulation in bean plants exposed to chilling temperatures (Vernieri *et al.*, 2001) and when citrus plants were repeatedly exposed to soil drying (Manzi *et al.*, 2015), with girdling attenuating root ABA accumulation during an initial drying cycle. This temporal response was initially interpreted as being due to a limited supply of (unspecified) ABA precursors from the shoot (Ren *et al.*, 2007; Manzi *et al.*, 2015), but further studies in citrus did not find a direct relationship between carotenoid abundance and root ABA biosynthesis (Manzi *et al.*, 2016). The physiological significance of species differences in the ability of roots for *de novo* ABA synthesis in response to soil drying requires additional experiments to determine its local (eg. root hydraulic conductance) and long-distance (eg. stomatal conductance) physiological effects.

Leaf xylem ABA concentration increased even in well-watered, girdled plants (Fig. 3.4a) despite no significant root ABA export (Fig. 3.4c). It is therefore important to distinguish whether elevated leaf xylem ABA concentrations reflect *in situ* leaf ABA synthesis. Xylem sap collected by pressurising detached leaves (as conducted here) comes from both apoplastic and symplastic sources (Hartung *et al.*, 1988; Borel and Simonneau, 2002). Collecting large sap volumes (relative to apoplastic volume) from small leaves (which is often necessary to ensure sufficient sap volume for ABA analysis) increases the contribution of symplastic (membrane-filtered) sap, ensuring that leaf xylem sap ABA concentrations are closely related to leaf tissue ABA concentrations (Borel and Simonneau, 2002). Thus the higher shoot and leaf xylem ABA concentrations (Fig. 3.4) likely reflect relative tissue ABA concentrations, since leaves have much higher ABA concentrations than roots (Liang *et al.*, 1997;

Liu *et al.*, 2005c; Manzi *et al.*, 2015). Thus phloem transport of ABA to the roots not only determines root ABA accumulation (Manzi *et al.*, 2015; McAdam *et al.*, 2016b) but also leaf xylem ABA concentration, suggesting that much of the ABA in the xylem sap is actually shoot-sourced.

Alternatively, increased shoot ABA levels in well-watered girdled plants may represent a wound response (Hildmann *et al.*, 1992), even though wounding more commonly elicits the synthesis of other signalling hormones as jasmonic acid (JA) and its precursor the oxylipin 12-OPDA (Savchenko *et al.*, 2014). Since both xylem-borne ABA and JA act as antitranspirants (De Ollas *et al.*, 2018), synthesis of jasmonates in response to girdling may explain the lower stomatal conductance occurring one day after girdling (Fig. 3.2), likely prior to any xylem ABA accumulation (Fig. 3.3). Nevertheless, the sustained decrease in g_s of well-watered girdled plants after Day 3 coincides with increased leaf xylem ABA concentration (cf. Fig. 3.2, 3.4a). Moreover, the consistent relationship between leaf xylem ABA concentration and stomatal conductance independent of girdling (Fig. 3.6b) suggests that hormonal synthesis induced by girdling had no long-term influence on the regulation of stomatal conductance.

In conclusion, shoot-sourced ABA was necessary to allow root accumulation in response to soil drying (Fig. 3.5), and maintain root-to-shoot ABA signalling in response to soil drying (Fig. 3.7c) in soybean. Shoot to root ABA translocation also maintained high stomatal conductance by preventing increases in foliar ABA concentration under well-watered conditions.

Chapter 4 – Soil drying and girdling affect multiple plant hormones

4.1 Introduction

Phytohormones mediate plant growth, development and physiological responses to various internal and external stimuli, especially by functioning as chemical messengers to communicate cellular activities (Wolters and Jurgens, 2009; Peleg and Blumwald, 2011; Wani *et al.*, 2016). They play key roles in coordinating various signal transduction pathways in response to various abiotic stresses (Davies *et al.*, 1994; Kazan, 2015). Many studies have focused on how crop plants generate, transport and regulate short- and long-distance chemical signals, thereby affecting hormone concentrations at their site(s) of action (Dodd, 2005; Wilkinson *et al.*, 2012). Those phytohormones include cytokinins (CK), gibberellins (GA), auxins (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). While some studies have independently considered the impacts of soil drying (Acharya and Assmann, 2009) and girdling (Rivas *et al.,* 2011; Kong *et al.* 2012) on endogenous phytohormone concentrations, this study combined both treatments to evaluate the impact of shoot-to-root phytohormone transport on root and shoot phytohormone concentrations.

Girdling obstructs the downward phloem transport (shoot-to-root), therefore it could be used to test the relationships between plant water relations and foliar and root hormone concentrations. There is some evidence that interrupting phloem transport may diminish stomatal conductance and reduce leaf hydraulic

conductance (Sellin *et al.,* 2013). Some hormones (such as ABA and JA) tend to accumulate above the girdling site (Lopez *et al.,* 2015). Furthermore, girdling may alter endogenous hormone interactions, such as changes in ABA and CK concentrations affecting leaf senescence (Dai and Dong, 2011)

Soil-drying induced changes in phytohormone signalling can mediate changes in root and shoot biomass, and leaf water status and leaf gas exchange (Pospisilova, 2003; Albacete *et al.*, 2008; Valluru *et al.*, 2016). Although much attention has focused on drought-induced ABA synthesis as a key signalling component (Shinozaki and Yamaguchi-Shinozaki, 1997; 2007), soil drying changes the concentrations of other hormones (such as ABA, CKs, auxins) that may also be involved in regulating physiological responses (Masia *et al.*, 1994; Alvarez *et al.*, 2008). How these other hormones affect root and shoot functioning, and the role of long-distance signalling in regulating their concentrations throughout the plant, in particular under soil drying, are still unclear.

Soil drying limits leaf gas exchange by causing changes in stomatal opening and closure (Dodd, 2003; Schachtman and Goodger, 2008), in response to a balance of hormonal concentrations. Other plant hormones such as cytokinins, ethylene, auxins and gibberellins can alter stomatal response independently, and in concert, with ABA (Dodd 2003; Acharya and Assmann, 2009). However, many of these studies have determined stomatal responses to exogenous hormone applications to the leaves, instead of correlating stomatal conductance with endogenous hormone concentrations following soil drying (Anderson *et al.*, 1994; Dodd, 2003; Iqbal *et al.*, 2011).

In Chapter 3, girdling increased shoot ABA concentrations and decreased root ABA concentrations of well-watered plants, which could represent a wound response (Hildmann *et al.,* 1992). While the continued decrease in stomatal conductance of well-watered girdled plants coincides with increased foliar ABA concentrations (Castro *et al.*, 2019), the synthesis of other signalling hormones such as jasmonic acid (JA) (Savchenko *et al.,* 2014; De Ollas and Dodd, 2016; Per *et al.*, 2018) could influence ABA concentration and / or be involved in rapid stomatal closure within one day of girdling. Since both phytohormones can act as antitranspirants (De Ollas *et al.,* 2018), synthesis of jasmonic acid in response to the girdling may explain stomatal closure, independently of the response of the soil drying. Furthermore, JA accumulation could stimulate foliar ABA production (Savchenko *et al.,* 2014; Forster *et al.,* 2019) to cause stomatal closure. However, whether interactions and signalling of other phytohormones (such as cytokinins, ethylene and JA) play direct or indirect roles in modulating hormone concentrations and stomatal regulation during a period of drought (Mahouachi *et al.*, 2007; Arbona *et al.*, 2010; De Ollas *et al.*, 2013) remains unclear.

Determining whether many hormones may interact to induce stomatal closure first requires the ability to measure multiple hormones in a single same sample using liquid or gas chromatography in combination with mass spectrometry (Albacete *et al.,* 2008). Since some experiments suggest that other antitranspirants (than ABA) are needed to induce stomatal closure (eg. Munns and King 1988), this chapter aimed to determine whether stomatal responses to girdling and soil drying were

influenced by multiple plant hormones, and how these treatments affected root and shoot hormone balance.

4.2 Materials and methods

4.2.1 Plant materials and experimental design

Soybean (*Glycine max* L. Merr. cv. Siverka) seeds were sown directly in pots as described in Section 3.2.1. Soil, environmental conditions and the time of daily irrigation were as described in Experiment 1 in Section 2.2.1.

During expansion of the third trifoliate leaf, plants were randomized into 4 groups, comprising a factorial combination of the treatments applied: soil drying (WW: well-watered; DR: droughted) and girdling (NG: intact plants; G: Girdled plants) respectively. Measurements were made 0, 1, 2, 4, 24, 26, 48 and 96 hours after girdling and withholding water. Three to five plants from each treatment were harvested 0, 1, 2, 4, 24 and 26 hours after girdling and five plants per treatment at 48 and 96 hours. Girdling was achieved surgically at 0800h on Day 0, when the third trifoliate leaf was completely expanded, by excising 10 mm of phloem tissue from the stem (at 100-110 mm above the soil surface) with a sharp razor blade. Plants were girdled between the cotyledonary node and the second node, where the unifoliate leaf was located. At this time, the cotyledons had either naturally abscised or were excised, to prevent them influencing root hormone concentrations (Waadt *et al.,* 2014). Water was withheld from half of the girdled and non-girdled plants after the girdling was completed.

4.2.2 Physiological measurements

Measurements were made on the third trifoliate leaf of each plant throughout the experiment, once the girdling was achieved. Stomatal conductance (g_s) was measured on the central leaflet with a porometer (Model AP4, Delta-T Devices, Burwell, UK). Two measurements were sequentially made on each plant and averaged.

After measuring stomatal conductance, the entire leaf was excised and collected in Eppendorf vials, which were immediately frozen in liquid nitrogen. The shoot was de-topped 6-7 cm from the stem base (in the middle of the girdled stem) and placed in the pressure chamber to measure shoot water potential (Ψ_{shoot}). The chamber was gradually pressurized at 0.03 MPa s^{-1} until the meniscus of the sap appeared, at which time the pressure was recorded. After measuring Ψ_{shoot}, root water potential (Ψ_{root}) was measured with the entire pot sealed in the chamber with sufficient stem protruding including the other half of the girdled tissue.

After measuring root water potential, root tissue samples (80-100 mg dry weight – determined retrospectively) were collected from the middle of the pot. Samples were removed, briefly washed (to remove adhering soil debris) and then frozen in liquid nitrogen. Plant tissues were stored at -80°C for further analysis. Soil water content was calculated as described in Section 2.2.2.

4.2.3 Multi-hormone analysis

Phytohormones including cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR, and isopentenyl adenine, iP), gibberellic acids (GA1, 3, and 4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and the ethylene precursor 1-aminocyclopropane-1-carboxylic aid (ACC) were analysed in leaf and root tissues according to Albacete *et al.* (2008) with some modifications. Analyses were conducted by Dr Albacete at CEBAS-CSIC, Murcia, Spain. Freeze-dried leaf and root material (50 mg dry weight, DW) was extracted overnight at -20°C using a methanol/water/formic acid solution (15/4/1 by volume, pH 2.5). Then, 10 µL of internal standard mix, composed of deuterated phytohormones ($[^2H_5]$tZ, $[^2H_5]$tZR, $[^2H_6]$iP, $[^2H_2]$GA1, $[^2H_2]$GA3, $[^2H_2]$GA4, $[^2H_5]$IAA, $[^2H_6]$ABA, $[^2H_4]$SA, $[^2H_6]$JA, $[^2H_4]$ACC, Olchemim Ltd, Olomouc, Czech Republic) at a concentration of 1 $\mu g \cdot mL^{-1}$ each, was added to the extraction homogenate. Solids were then separated by centrifugation (20, 000 g) for 15 mins, and extracted again for 30 mins at 4°C in an additional 0.5 mL of the same extraction solution. The pooled supernatants were filtered through a Sep-Pak Plus C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and plant pigments, and evaporated at 40ºC under a vacuum either to near dryness or until organic solvent was removed. Any remaining residue was dissolved in 1 mL methanol/water (20/80, v/v) in an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore diameter nylon membrane (Millipore, Bedford, MA, USA).

Ten µL of filtered extract were injected into a U-HPLC-MS system comprising an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an

Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionisation (HESI) interface. Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA) was used to obtain mass spectra. To quantify the plant hormones, calibration curves were constructed for each analyzed component (1, 10, 50, and 100 $\mu g \cdot L^{-1}$) and corrected for 10 $\mu g \cdot L^{-1}$ deuterated internal standards. Recovery percentages ranged between 92 and 95%.

4.2.4 Statistical analysis

The experiment was a replicate of the one conducted in Chapter 3. Two-way analysis of variance (ANOVA) determined the effects of water treatment, girdling and their interaction (Fig. 4.1, 4.2). Analysis of covariance (ANCOVA) and regression analyses determined whether girdling affected relationships between phytohormones, water status and soil variables (Fig. 4.3-4.9).

Statistical analyses were conducted on all phytohormones that could be detected in at least 50% of analysed samples. Appropriate logarithmic transformations were applied to improve normality of residuals and are indicated where used.

Pearson's correlations were used to explore correlations between leaf and root phytohormone concentrations, plant water status and soil water content. The variables were log-transformed to improve normality of model residuals. Shoot and root water potentials were log-transformed from absolute (MPa) values. In scatter plots, Pearson's r coefficients were represented, with a trend line fitted when the *p*-values were statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ - Tables 4.1-5).

4.3 Results

4.3.1 Effect of girdling on plant water relations

Soil water content, Ψ_{root} and Ψ_{shoot} of well-watered plants was reasonably consistent throughout the experiment, remaining between 0.7 and 0.85 g g^{-1} for soil moisture, between -0.09 and -0.07 MPa for Ψ_{root} and between -0.7 and -0.6 MPa for Ψ_{shoot} (Fig. 4.1a, c, d). While girdling had no significant effect on soil water content in well-watered plants, it increased Ψ_{root} from 48 h (by 0.03 MPa) and Ψ_{shoot} from 96 h (by 0.035 MPa) compared to intact droughted plants. After withholding water, soil moisture declined to 0.58 g g^{-1} after 48 h and 0.25 g g^{-1} after 96 h (Fig. 4.1a), again with no significant effect of girdling. Soil drying did not significantly decrease Ψ_{root} until 48 h. At this time, Ψ_{root} was 0.02 MPa lower than in well-watered plants and by 96 h, Ψ_{root} was 0.29 MPa lower than in well-watered plants. Girdling attenuated the soil drying induced decline in Ψ_{root}, increasing Ψ_{root} by 0.05 MPa at 96 h (Fig. 4.1d). Soil drying decreased Ψ_{shoot} by 0.08 MPa at 48 h and by 0.29 MPa at 96 h compared to well-watered plants. Girdling attenuated the soil drying induced decline in Ψ_{shoot}, increasing Ψ_{shoot} by 0.04 MPa at 96 h (Fig. 4.1c). While soil drying substantially decreased soil moisture and tissue water potentials, girdling also measurably enhanced water status of plants exposed to drying soil.

Girdling significantly decreased g_s of well-watered plants within 24 hours, and g_s of these plants was half that of well-watered intact plants at 96 hours (Fig. 4.1b). After 48 hours of withholding water, g_s had declined similarly in both droughted treatments, and was 63% of well-watered plants. At the end of the experiment, soil drying had further decreased g_s, with a much greater response in intact plants, as

indicated by a significant stress x girdling interaction. Thus girdling caused stomatal closure of well-watered plants, but had no effect on the severity of soil-drying induced stomatal closure.

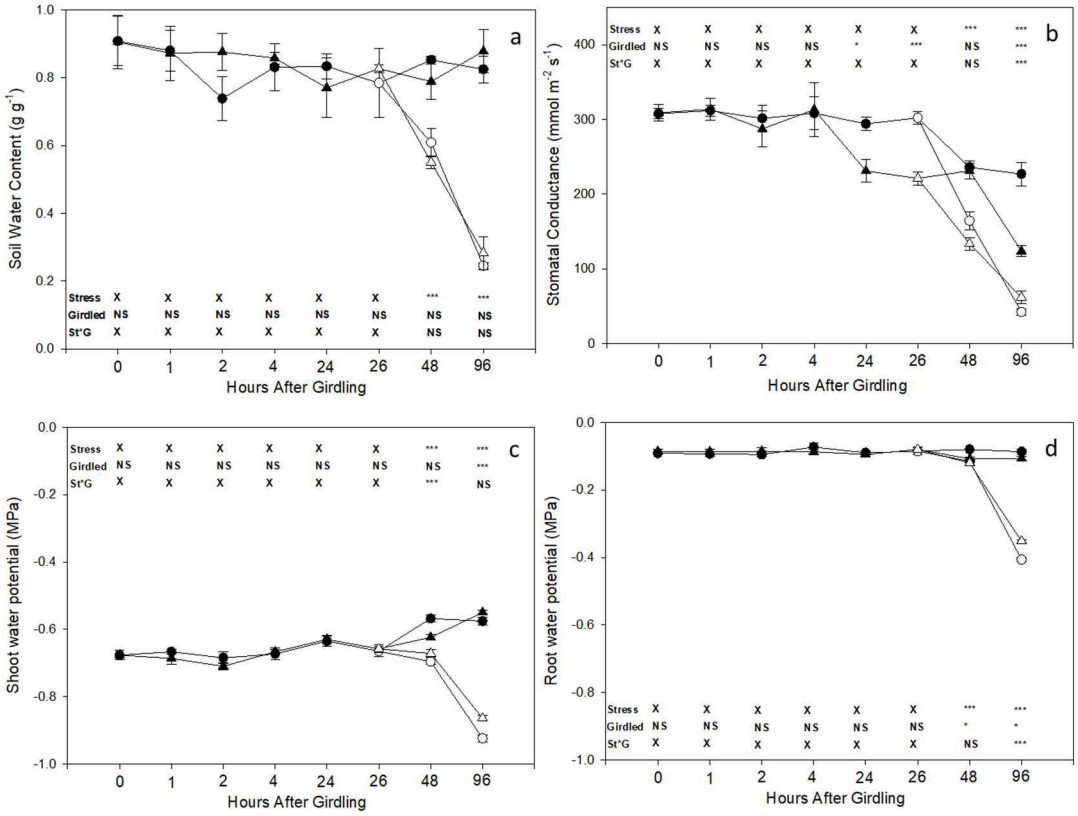


Figure 4.1. Soil water content (a), stomatal conductance in the third trifoliate leaf (b), shoot (c) and root (d) water potential at 0, 1, 2, 4, 24, 26, 48 and 96 hours after girdling. Measurements at 0 hours were done before girdling was applied. Filled and hollow symbols represent well-watered and droughted plants respectively, with intact and girdled plants indicated by circles and triangles respectively. Symbols indicate mean ± s.e. (n=5).Effects of soil drying (Stress), girdling treatment (Girdled) and their interaction (St x G) are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001. Since soil drying had no statistically significant effect on soil moisture within the first 26 h, the Stress and interaction factors are not analysed during this period, and are represented by the letter "X".

4.3.2 Plant hormone concentrations

Since soil water contents and plant water potentials did not significantly differ during the first 26 h of measurements (Fig 4.1), samples were pooled across watering treatments for data collected from 0 and 1 hours (0 h), 2 and 4 h (4 hours) and 24 and 26 h (26 h) in Figure 4.2, with effects of girdling separated. There were no systematic differences in hormone concentrations between the data collected 1-2 h apart (data not shown). At 48 and 96 h after imposing the treatments, samples were separated as well-watered intact (WW), droughted intact (Dr), well-watered girdled (WW-G) and droughted girdled (Dr-G) plants (Fig. 4.2). Tissue ZR and GA1, root iP and leaf ACC concentrations were not included in the data analysis since they were not detected in over 50% of samples analyzed. In contrast, tZ, GA3, GA4, ABA, JA and SA were detected over 50% of the tissue samples analyzed.

Although root iP concentrations could not routinely be quantified, soil drying significantly increased leaf iP concentrations of intact plants after 48 h, approximately doubling them (compared to well-watered plants) by the end of the experiment. Independently of soil drying, girdling decreased leaf iP concentrations throughout the experiment, with significant decreases noted within 4 h. The lowest iP concentrations (23% of well-watered intact plants) were detected 48 h after girdling well-watered plants, and 96 h after girdling the plants exposed to drying soil. Girdling suppressed drought-induced foliar iP accumulation, especially at the end of the experiment (Fig. 4.2a).

Although leaf ACC concentrations could not routinely be quantified, soil drying significantly decreased root ACC concentrations of intact plants by 25% after 48

hours, but there was no significant effect by the end of the experiment. Girdling transiently increased root ACC concentrations within the first 26 hours of the experiment, by 5.5-fold compared to intact plants after 4 hours and 10.8-fold after 26 hours. Thereafter, root ACC concentrations approximately halved in well-watered girdled plants for the remainder of the experiment compared to well-watered intact plants. In contrast, root ACC concentrations of droughted girdled plants increased by 2.8-fold compared to droughted intact plants by the end of the experiment. Thus, girdling increased drought-induced root ACC accumulation from 48 h (Fig. 4.2h), while over the same time, soil drying decreased root [ACC] of intact plants.

Soil drying and girdling did not affect root tZ concentrations throughout the experiment (Fig. 4.2i). In contrast, soil drying significantly increased leaf tZ concentrations of intact plants by 28% at the end of the experiment. Girdling significantly decreased leaf tZ concentrations by about 40% within the first 26 hours of the experiment, but these effects did not persist during the rest of the experiment. In addition, girdling had limited effects on drought-induced foliar tZ accumulation (Fig. 4.2b). Thus girdling transiently suppressed foliar tZ concentration, while prolonged soil drying increased leaf tZ concentration.

Soil drying significantly increased root gibberellin (GA3 and GA4) concentrations of intact plants by 18- and 2-fold respectively by the end of the experiment (Fig. 4.2 j, k). Independently of soil drying, girdling significantly decreased root GA3 and GA4 concentrations within 26 hours by 98% and 82% respectively at the end of the experiment. Girdling suppressed drought-induced root GA3 and GA4 accumulation,

especially at the end of the experiment. Thus root GA concentration seemed to depend on shoot-to-root gibberellin transport.

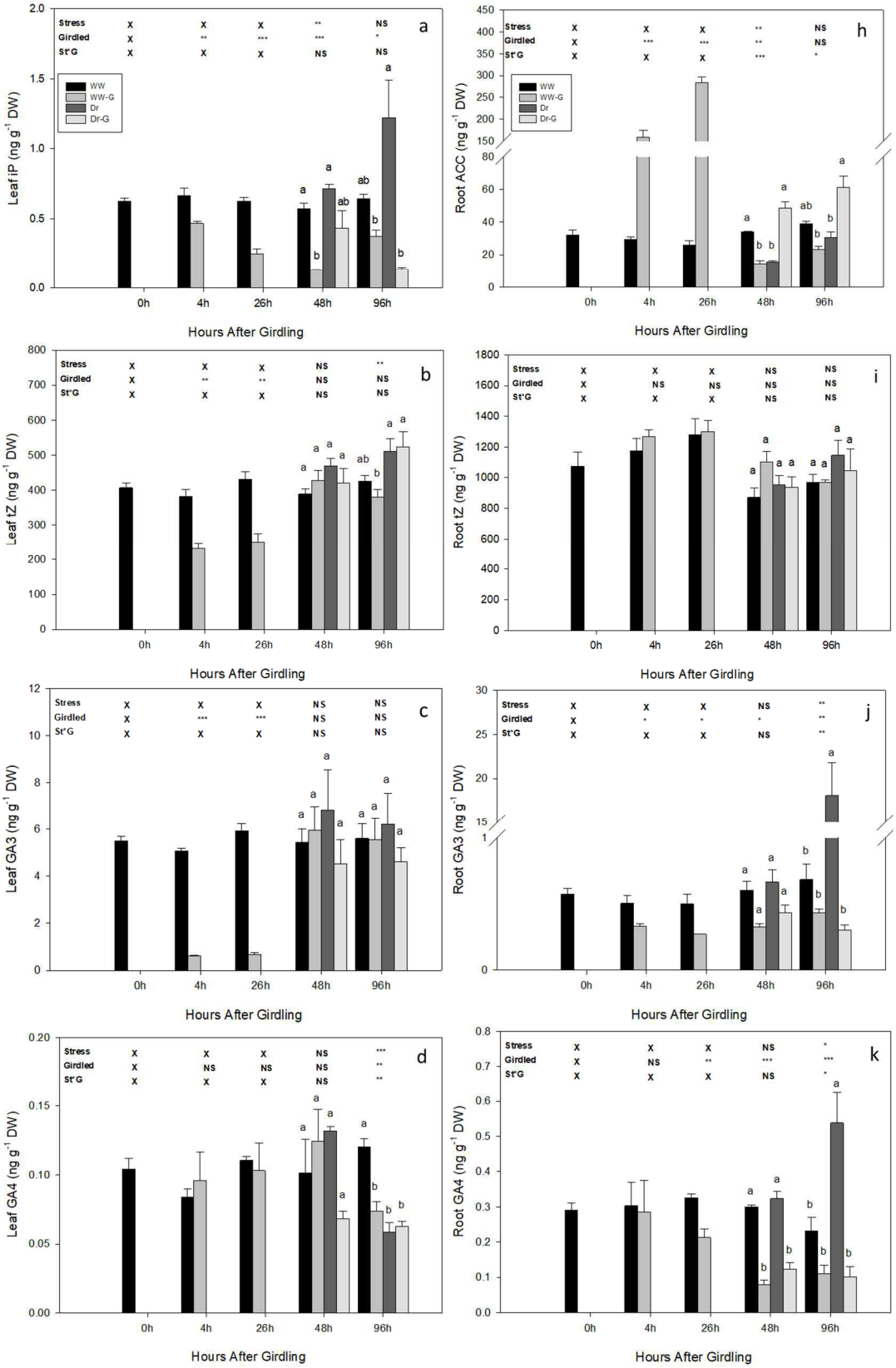
In contrast, soil drying only significantly decreased leaf GA4 by 50% compared with well-watered intact plants at the end of the experiment (Fig. 4.2d). In addition, girdling decreased leaf GA3 concentrations by approximately 90% compared to intact plants within the first 26 h (Fig. 4.2c). Also, girdling significantly decreased (approximately halved) leaf GA4 concentrations compared to well-watered intact plants at the end of the experiment (Fig. 4.2d), while girdling had no significant effect on these drought-induced changes in the leaves (Fig. 4.2 c, d).

Compared to well-watered intact plants, 48 h of soil drying significantly increased root ABA concentration by 6-fold in droughted intact plants, but not droughted girdled plants (Fig. 4.2l). After 96 h of soil drying, root ABA concentrations increased irrespectively of girdling, but root ABA concentrations were 46% higher in intact than girdled plants. In addition, leaf ABA concentrations significantly increased from 48 h (Fig. 4.2e), again with a greater response in intact than girdled plants. Girdling transiently decreased root ABA concentrations of well-watered plants by 18% and 60% at 26 and 48 h respectively, but significantly increased leaf ABA concentrations by 1.63-fold at 26 h, reaching a maximum difference of 1.9-fold at 96 h. Girdling affected soil drying induced leaf ABA accumulation only at 48 h (as indicated by a significant stress x girdling interaction), with no discernible effect of soil drying in girdled plants. Soil drying induced root ABA accumulation depended on girdling at both 48 h and 96 h (as indicated by significant stress x girdling

interactions), with girdling attenuating root ABA accumulation. These changes were broadly consistent with those determined in Chapter 3.

Soil drying significantly increased root JA concentrations in intact plants by 11.5- and 4-fold at 48 and 96 h respectively (Fig. 4.2m). Changes in leaf JA concentrations were more modest, with significant increases of 1.4- and 1.5-fold at 48 and 96 h respectively (Fig. 4.2f). Girdling transiently increased leaf JA concentrations within 4 to 26 hours (by 6-fold compared to intact plants), but this effect has reversed (48 h) or disappeared after 96 h (Fig 4.2f). In contrast, girdling significantly increased root JA concentrations of well-watered plants by 1.75-fold from 48 h (Fig. 4.2m). Girdling affected JA accumulation in response to drying soil, after 96 h in the leaves (Fig 4.2f) and 48 h in the roots (Fig. 4.2m), as indicated by significant stress x girdling interactions. In both tissues, girdling attenuated the soil-drying induced increase in JA accumulation.

Although soil drying significantly increased root SA concentrations by 26% compared to well-watered plants by the end of the experiment (Fig. 4.2n), leaf SA concentrations were not affected (Fig. 4.2g). While girdling did not affect root SA concentrations throughout the experiment, leaf SA concentrations steadily increased from 26 h, with 2.4-fold higher SA concentrations in well-watered girdled plants than well-watered intact plants by the end of the experiment. Soil drying attenuated this girdling-induced increase in leaf SA concentration at 48 h (as indicated by a significant soil drying x girdling interaction), but had no effect at 96 h. Thus girdling stimulated foliar SA accumulation, while prolonged soil drying enhanced root SA accumulation.


a
Stress X X X ** NS
Girdled X ** *** *** *
St*G X X X NS NS
WW
WW-G
Dr
Dr-G
Leaf iP (ng g-1 DW)
0h 4h 26h 48h 96h
Hours After Girdling
h
Root ACC (ng g-1 DW)
b
Leaf tZ (ng g-1 DW)
i
Root tZ (ng g-1 DW)
c
Leaf GA3 (ng g-1 DW)
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d
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k
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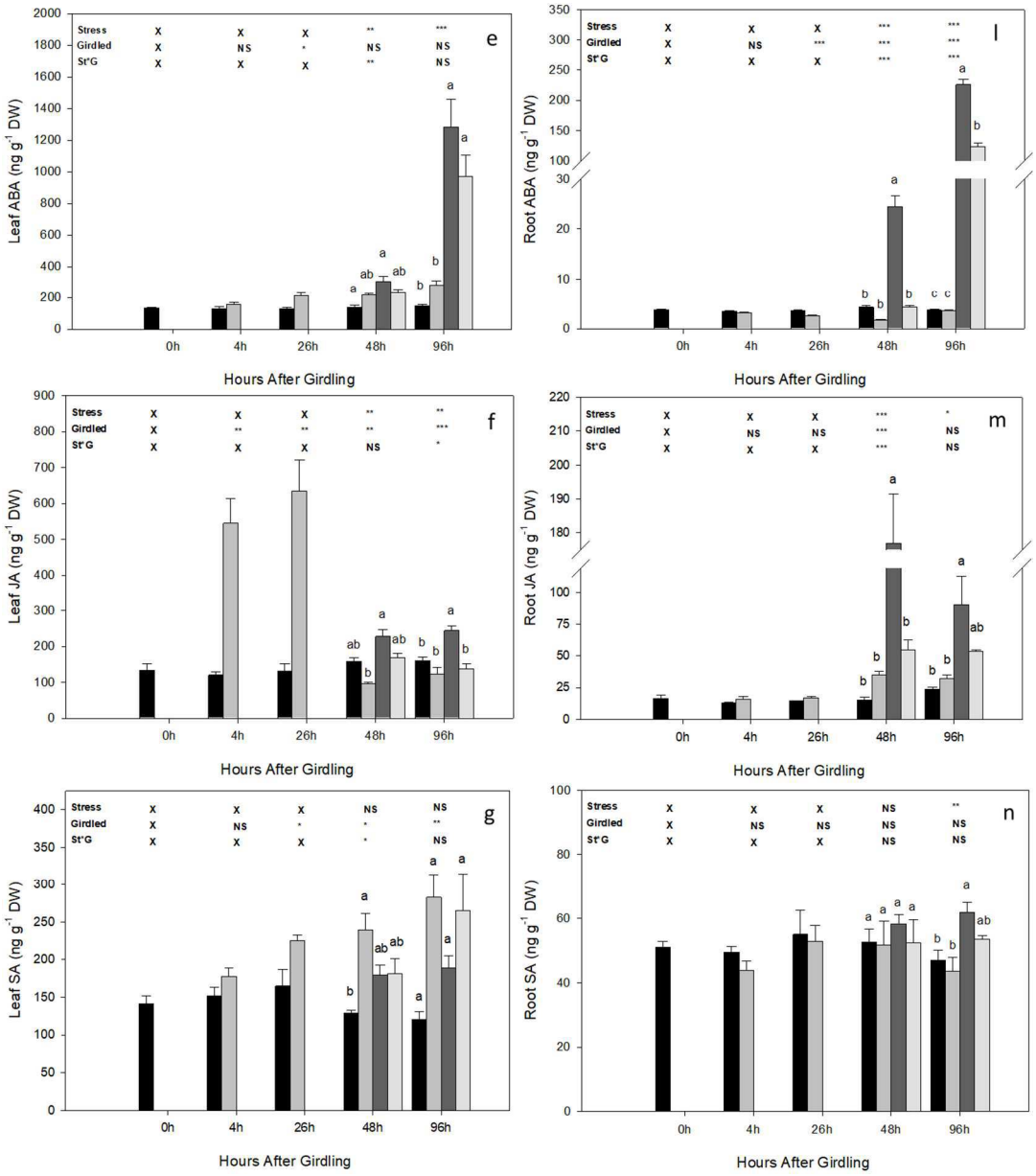


Figure 4.2. Phytohormone concentrations detected in leaf (a-g) and root (h-n) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) at 0, 4, 26, 48 and 96 hours after girdling. Vertical bars indicate mean ± s.e. (n=3-5). Effects of soil drying (Stress), girdling treatment (Girdled) and their interaction (St x G) are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001. Since soil drying had no statistically significant effect on soil moisture within the first 26 h, the Stress and interaction factors are not analysed during this period, and are represented by the letter "X". Different letters indicate significant differences (P < 0.05) according to the Tukey´s test at 48 and 96 h.

4.3.3 Correlations between phytohormones, water status and soil water content within 26 hours of girdling

Since girdling decreased g_s (but not Ψ_{shoot} and soil water content as shown before) within 24 hours, correlations between soil water content, plant water status and root (Table 4.1) and leaf (Table 4.2) phytohormone concentrations were determined during this time.

Table 4.1. Pearson's r correlation coefficients and trend lines between logarithmic values of root tissue phytohormone concentrations, root water potential (WProot) and soil water content (SWC). Significance of *p*-values reported thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant correlations involving WProot and SWC are highlighted with a red square.

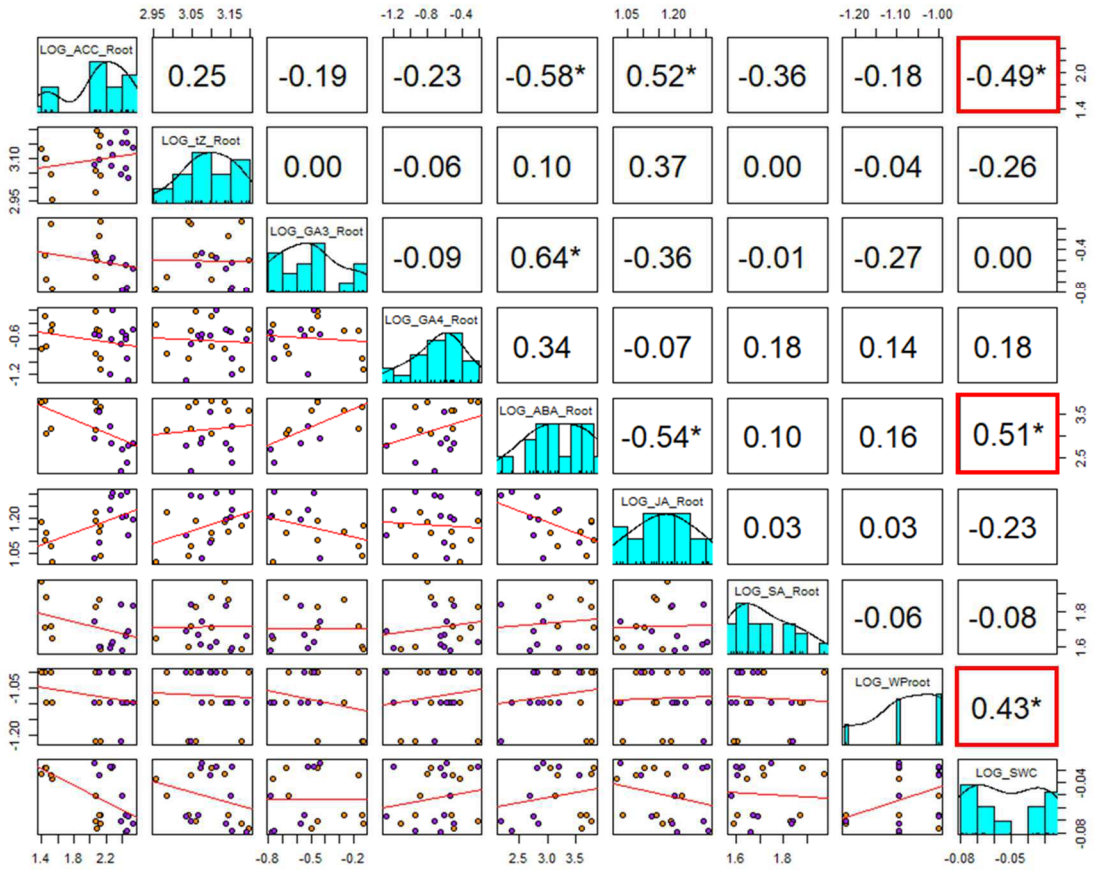
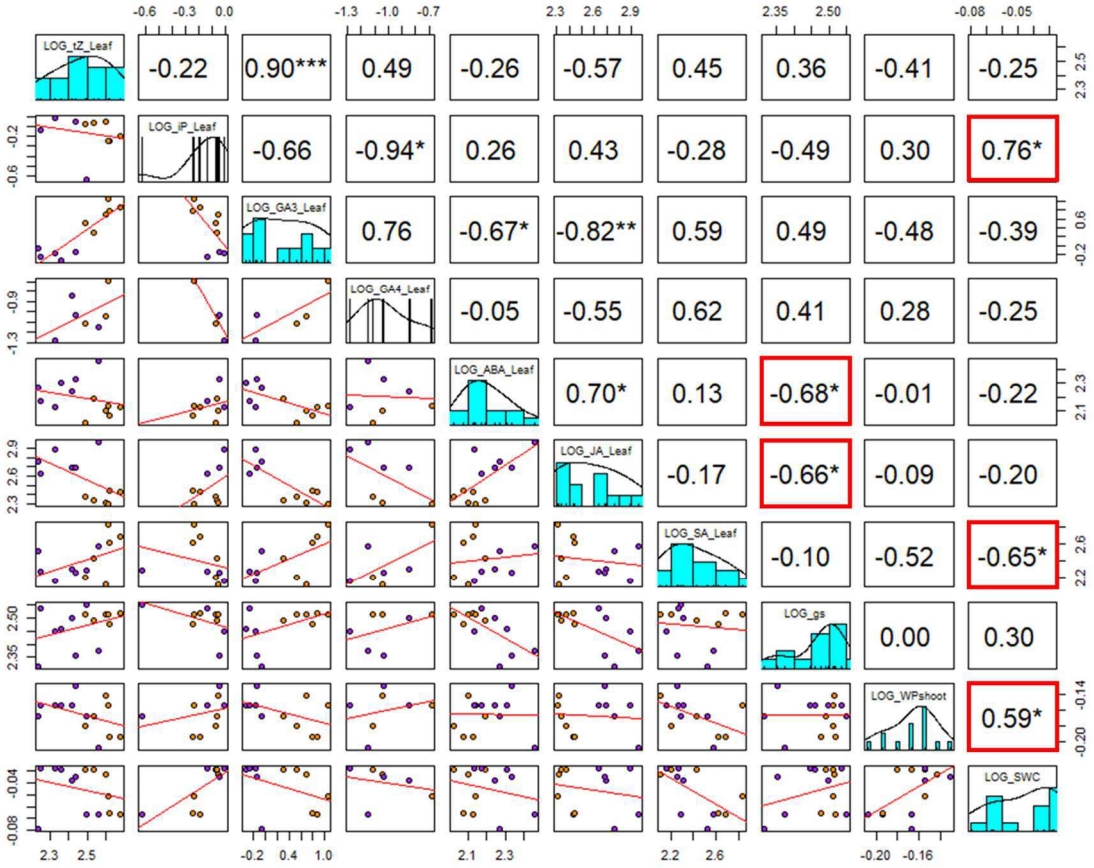


Table 4.2. Pearson's r correlation coefficients and trend lines between logarithmic values of leaf tissue phytohormone concentrations, stomatal conductance (*gs*), shoot water potential (WPshoot) and soil water content (SWC). Significance of *p*-values reported thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant correlations involving gs, WPshoot and SWC are highlighted with a red square.



Soil moisture was significantly ($P < 0.05$) positively correlated with root ABA concentration but significantly ($P < 0.05$) negatively correlated with root ACC concentration. Root water potential was significantly ($P < 0.05$) positively correlated with soil water content, but it was not significantly correlated with any of the root phytohormone concentrations.

Soil water content was significantly ($P < 0.05$) positively correlated with leaf iP concentration but significantly ($P < 0.05$) negatively correlated with leaf SA

concentration and Ψ_{shoot}. Shoot water potential was not significantly correlated with the concentration of any phytohormone. Stomatal conductance was significantly ($P < 0.05$), negatively correlated with leaf ABA and JA concentration.

Within the first 26 hours girdling affected the relationships between root ACC and ABA concentrations and soil water content (Fig. 4.3). Within the first 26 hours of the experiment, root ACC concentrations increased as soil moisture declined across a relatively restricted range (0.80-0.96 g g^{-1}). Although girdling did not affect the sensitivity of root ACC accumulation ($P=0.58$ for soil drying x girdling interaction), average root ACC concentrations were 2.8-fold higher in girdled plants (Fig. 4.3a). In contrast, girdling significantly ($P=0.012$) decreased root ABA concentrations (Fig. 4.3b) by 1.27-fold. Girdling affected the relationship between root ABA concentration and soil moisture ($P=0.022$ for soil drying x girdling interaction), with root ABA concentrations increasing with soil drying in intact plants, but decreasing in girdled plants. Thus limited soil drying affected both root ABA and ACC concentrations, as did girdling.

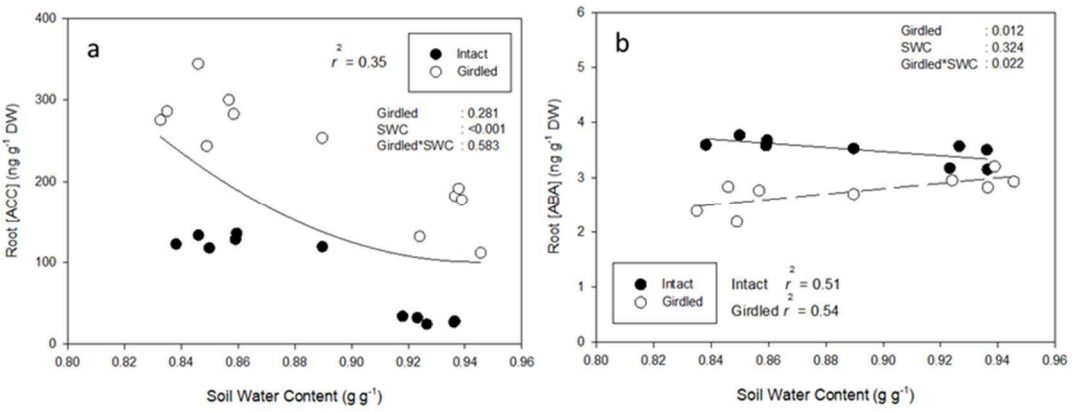


Figure 4.3. Relationships between root ACC (a) and ABA (b) concentration and soil water content during the first 26 hours of the experiment. Filled and hollow circles represent intact and girdled plants respectively. Each symbol is an individual plant and regression lines (P<0.05) were fitted to all data (a) where there was no significant girdling x SWC interaction and intact and girdled plants (b) where there was a significant girdling and *x*-variable interaction. *p-values* determined by ANCOVA for each main effect (soil water content and girdling) and their interaction are reported in each panel.

Furthermore, girdling affected the relationships between shoot water potential, the phythormones iP and SA and soil water content (Fig. 4.4). Shoot water potential tended to increase (P=0.058) as soil water content decreased within a restricted range, but there was no significant effect of girdling, and girdling did not affect the relationship between Ψ_{shoot} and soil water content as indicated by no significant girdled x soil water content interaction (Fig. 4.4a). Leaf iP concentrations decreased as the soil dried, but there was no significant effect of girdling, which did not affect the relationship between leaf [iP] and soil water content as indicated by no significant girdled x soil water content interaction (Fig. 4.4b). In addition, leaf SA increased as the soil dries, but there was no significant effect of girdling, which did not affect the relationship between leaf [SA] and soil water content as indicated by no significant girdled x soil water content interactions (Fig. 4.4c). Thus limited soil

drying increased Ψ_{shoot} and foliar SA concentrations, but decreased foliar iP concentrations.

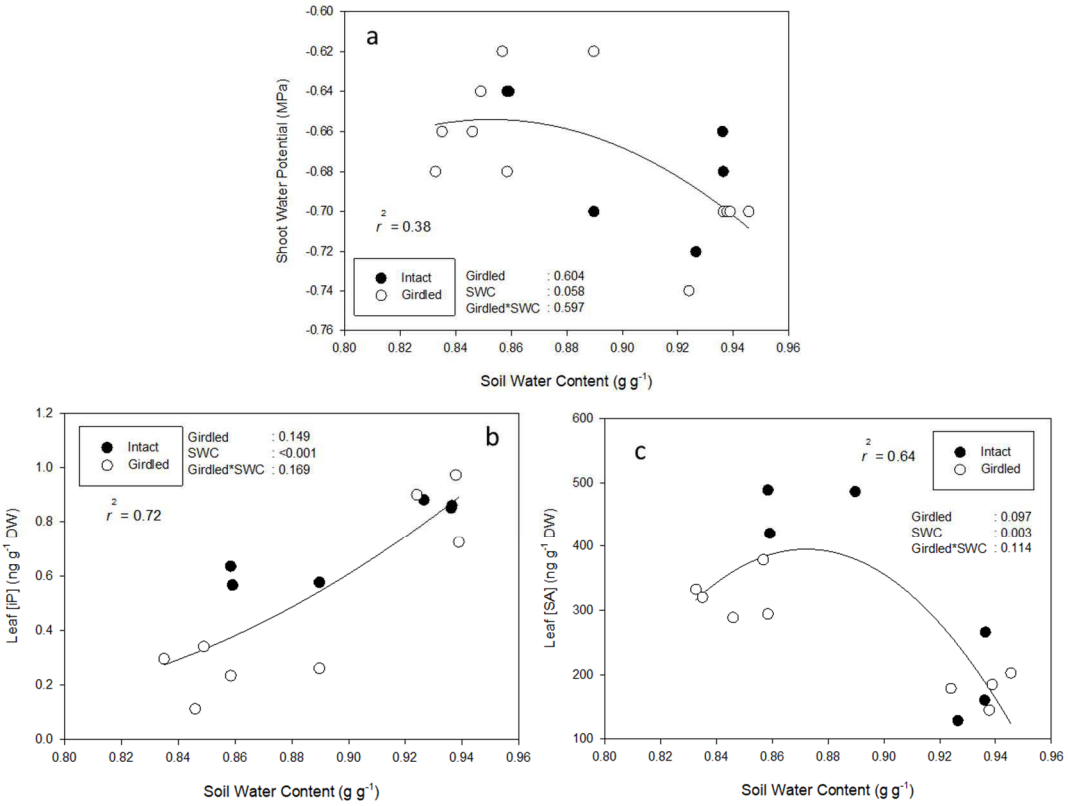


Figure 4.4. Relationships between shoot water potential (a), leaf iP (b) and SA (c) concentrations and soil water content during the first 26 hours of the experiment. Filled and hollow circles represent intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P < 0.05$) were fitted to all data when there was no significant girdling x SWC interaction. *p*-values determined by ANCOVA for each main effect (soil water content and girdling) and their interaction are reported in each panel.

Although there was limited soil drying within the first 26 hours of the experiment, stomatal conductance declined as leaf ABA (Fig 4.5a) and JA (Fig 4.5b) concentrations increased. Although girdling had no significant effects, and did not affect these relationships (as indicated by no significant girdled x leaf [hormone] interactions), higher hormone concentrations generally occurred in girdled plants.

Both ABA and JA explained a similar proportion (r2 = 0.49 for ABA, r2 = 0.45 for ABA) of the variation in stomatal conductance.

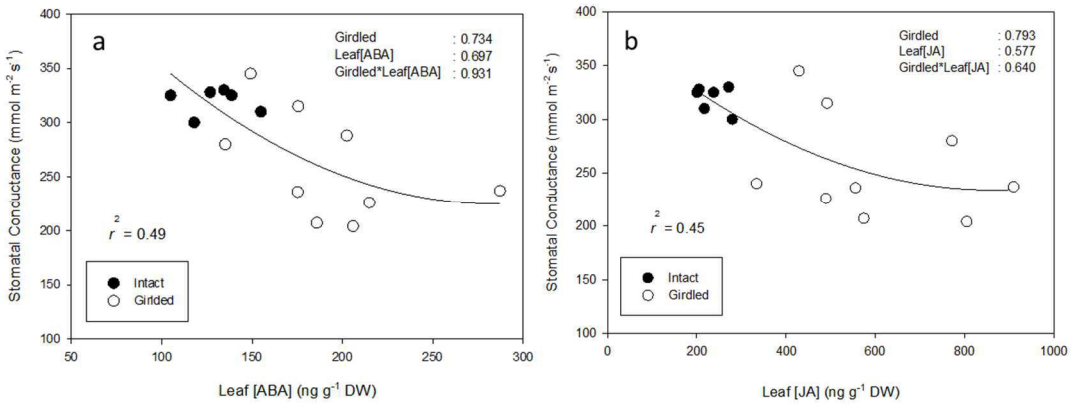


Figure 4.5. Relationships between stomatal conductance and lead ABA (a) and JA (b) concentrations during the first 26 hours of the experiment. Filled and hollow circles represent intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P < 0.05$) were fitted to all data when there was no significant girdling x [Hormone] interaction. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported in each panel.

4.3.4 Correlations between phytohormones, water status and soil water content during the entire experiment

Although soil drying had limited effects on plant water relations during the first 26 hours of the experiment, it is also necessary to understand correlations between root and leaf hormone concentrations and water status variables throughout the entire experiment (Table 4.3, 4.4).

Table 4.3. Pearson's r correlation coefficients and trend lines between logarithmic values of root tissue phytohormone concentrations, root water potential (WProot) and soil water content (SWC). Significance of *p*-values reported thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant correlations involving WProot and SWC are highlighted with a red square.

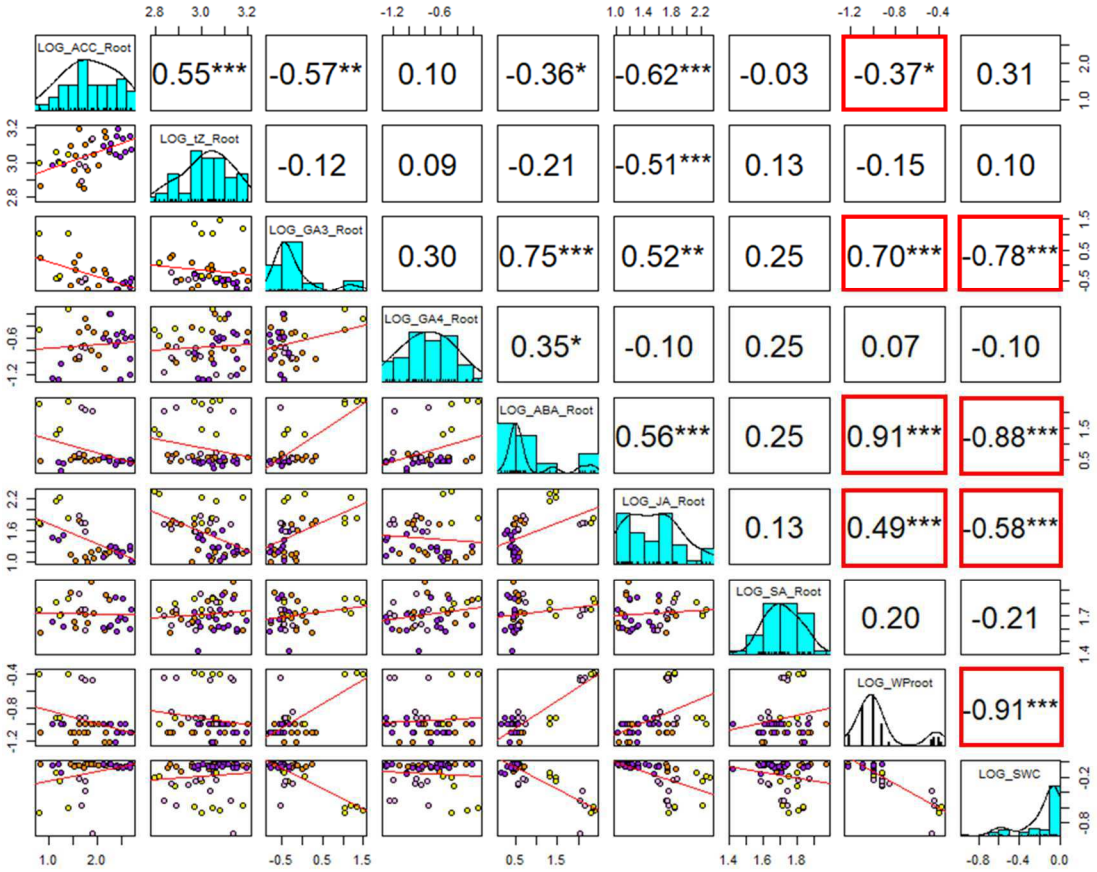
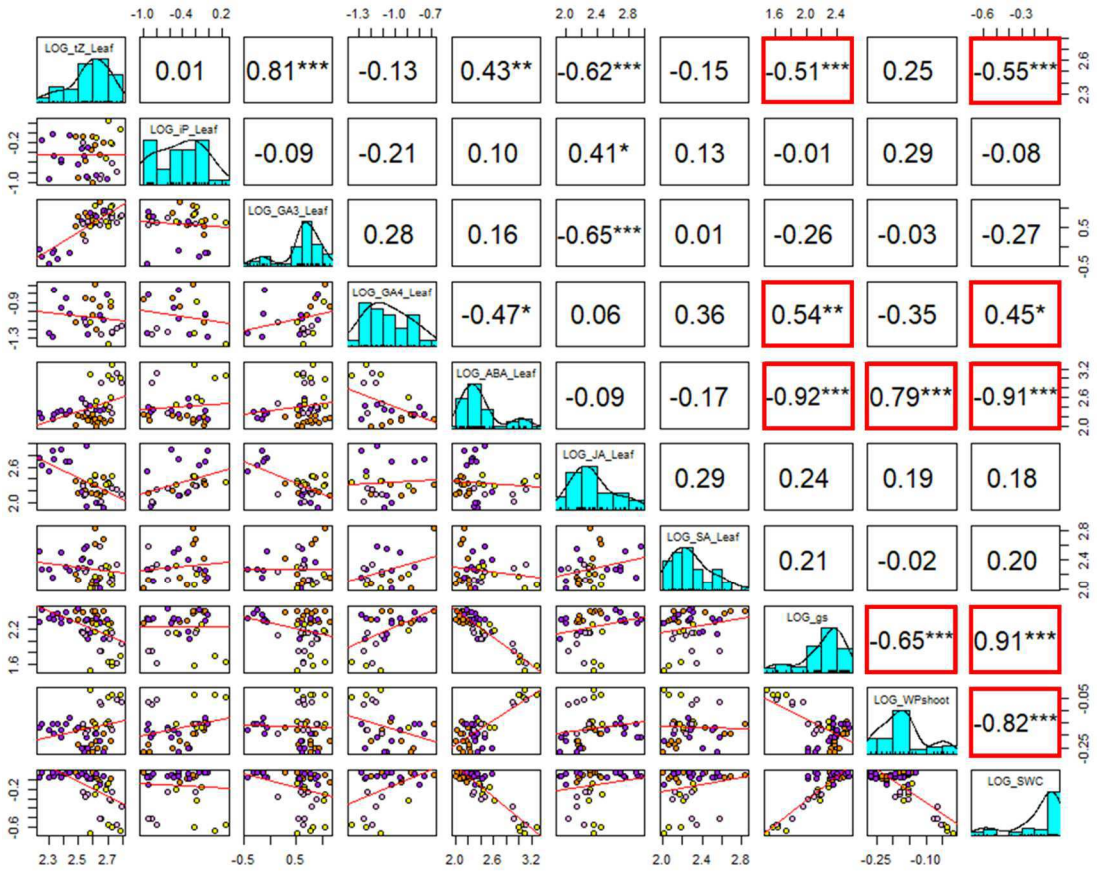


Table 4.4. Pearson's r correlation coefficients and trend lines between logarithmic values of leaf tissue phytohormone concentrations, stomatal conductance (gs), shoot water potential (WPshoot) and soil water content (SWC). Significance of *p*-values reported thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant correlations involving gs, WPshoot and SWC are highlighted with a red square.



Soil moisture was significantly ($P < 0.001$) negatively correlated with root GA3, ABA and JA concentrations but significantly ($P < 0.001$) positively correlated with Ψ_{root}. Root water potential was significantly ($P < 0.05$) positively correlated with root ACC concentration and significantly ($P < 0.001$) negatively correlated with root GA3, ABA and JA concentrations.

Soil water content was significantly ($P < 0.001$) negatively correlated with leaf tZ and ABA concentration but significantly ($P < 0.05$) positively correlated with leaf GA4 concentration and also significantly ($P < 0.001$) positively correlated with the stomatal conductance and Ψ_{shoot}. Shoot water potential was significantly ($P < 0.001$), positively correlated with ABA concentration but significantly ($P < 0.001$), negatively correlated with the stomatal conductance. Stomatal conductance was significantly ($P < 0.001$) negatively correlated with leaf tZ and ABA concentration but significantly ($P < 0.01$) positively correlated with leaf GA4 concentration.

Soil drying affected relationships between Ψ_{root}, soil water content and root [GA3], [ABA] and [JA] (Fig. 4.6). Root water potential declined as the soil dried, independently of girdling (Fig. 4.6a). Root GA3 concentrations increased as the soil dried in intact plants, but were not affected in girdled plants (Fig. 4.6b). Root ABA concentrations increased as the soil dried, although more sensitively in intact plants as indicated by a significant ($P<0.001$) girdled x soil water content interaction (Fig. 4.6c). Root JA concentrations increased as the soil dried, although to a greater extent in intact than girdled plants (Fig. 4.6d). Moreover, root JA concentrations seemed maximal at intermediate soil water contents (0.4-0.6 g g^{-1}) but declined with further soil drying (to 0.2 g g^{-1}). Thus soil drying stimulated root accumulation of multiple hormones (ABA, GA3 and JA), but this was attenuated in girdled plants.

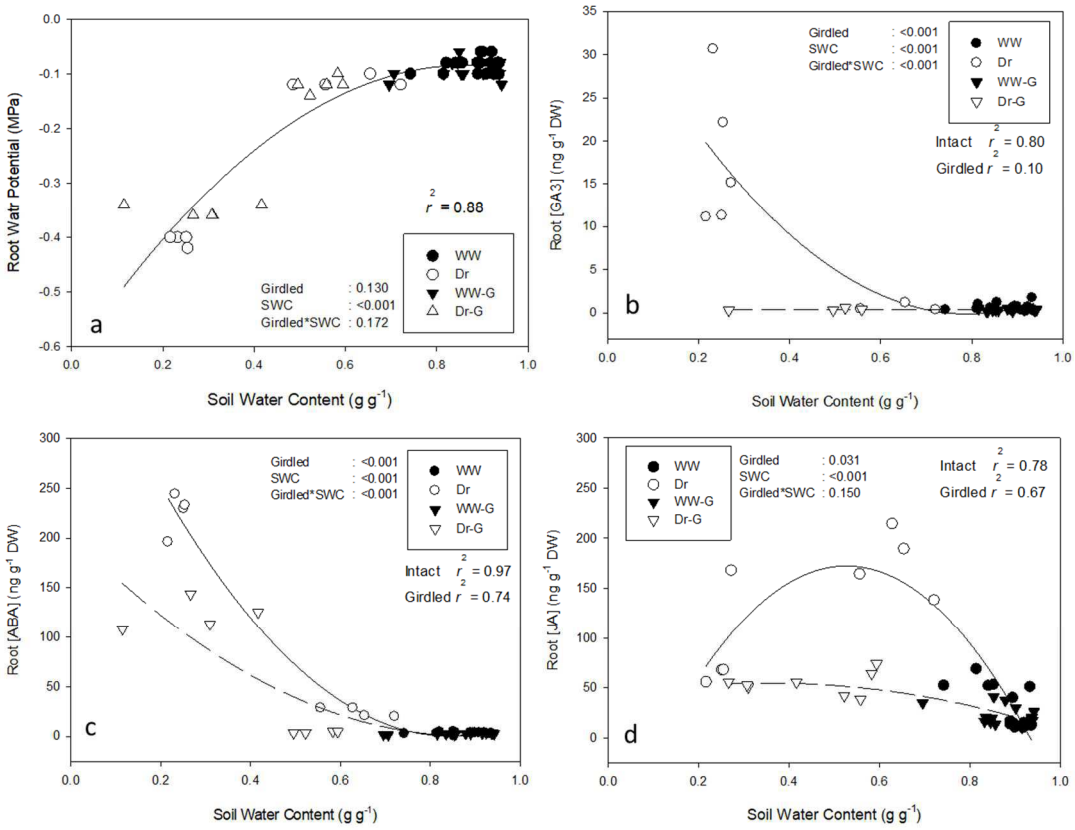


Figure 4.6. Relationship between root water potential (a), GA3 (b), ABA (c) and JA (d) concentrations and soil water content during the experiment. Filled circles and triangles represent well-watered intact and girdled plants respectively, and hollow circles and triangles represent drought intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P<0.05$) were fitted to all data (a) where there was no significant girdling x SWC interaction and to intact and girdled plants (b, c, d) where there was a significant girdling and *x*-variable interaction. *p*-values determined by ANCOVA for each main effect (soil water content and girdling) and their interaction are reported in each panel.

Soil drying also affected relationships between shoot water potential, stomatal conductance and leaf tZ, GA4 and ABA concentrations (Fig. 4.7). Thus Ψ_{shoot} decreased as the soil dried independently of girdled or intact plants, although a

significance girdled x soil water content interaction was detected (Fig. 4.7a), likely due to high Ψ_{shoot} values of well-watered girdled plants. Stomatal conductance decreased as the soil dried and shoot water potential declined respectively, but girdling did not affect these relationships (Fig. 4.7b, f). Leaf tZ concentrations increased as the soil dried, with a more sensitive response in girdled plants (as indicated by a significant girdled x soil water content interaction) as leaf tZ concentrations were lower in well-watered girdled plants (Fig. 4.7c). Leaf GA4 concentrations decreased as the soil dried, independent of girdling (Fig. 4.7d). Leaf ABA concentrations increased as the soil water dried, more sensitively in intact plants (Fig. 4.7e). The magnitude of these changes in leaf phytohormone concentrations with soil drying were 3.8-fold for ABA, 1.3-fold for tZ and 0.6-fold for GA4, with soil water content explaining 86%, 49% and 18% of the variation in ABA, tZ and GA4 concentrations respectively.

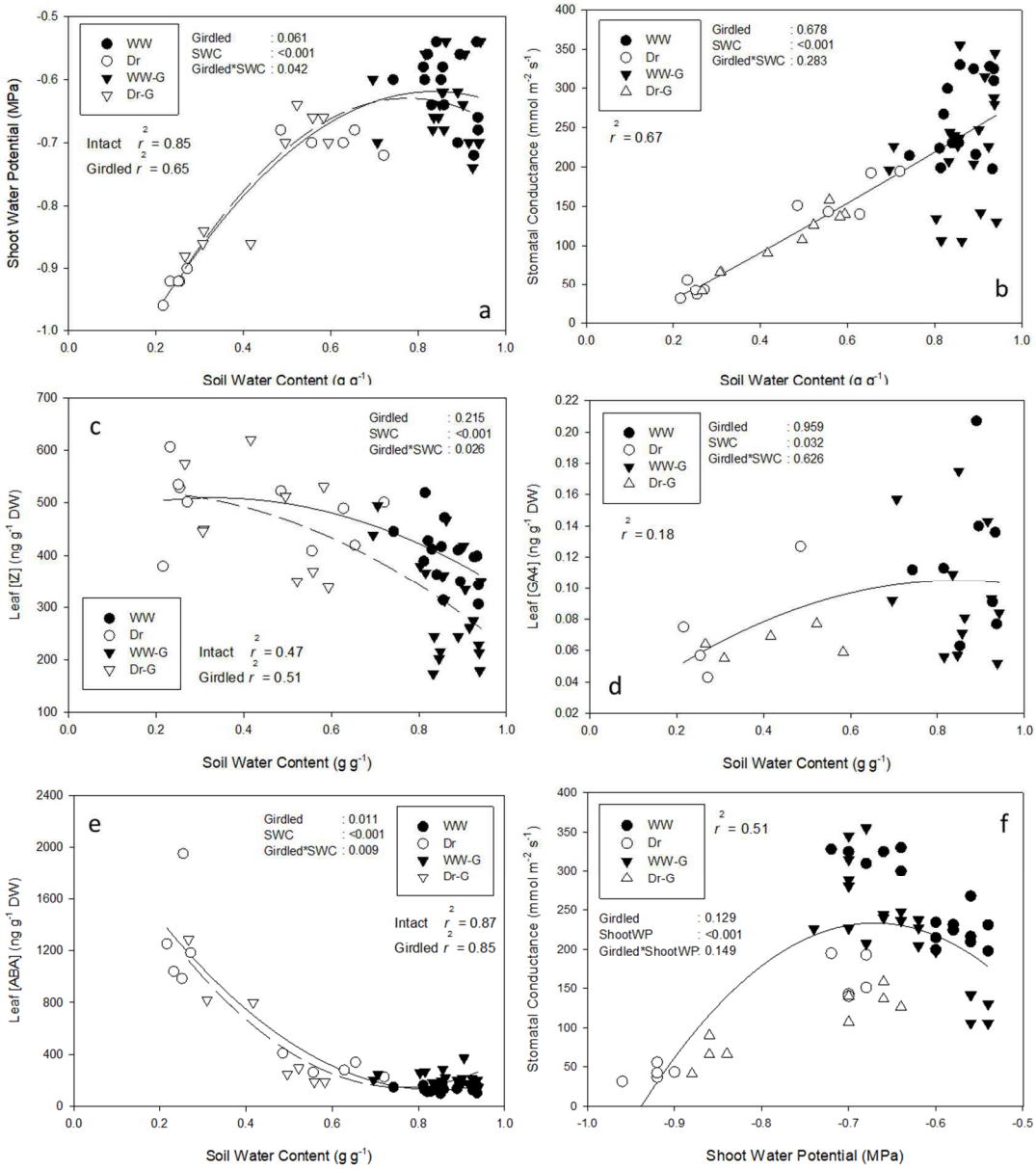


Figure 4.7. Relationship between shoot water potential (a), stomatal conductance (b), leaf tZ (c), GA4 (d) and ABA (e) concentrations and soil water content, and stomatal conductance and shoot water potential (f) during the experiment. Filled circles and triangles represent well-watered intact and girdled plants respectively, and hollow circles and triangles represent drought intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P < 0.05$) were fitted to intact and girdled plants (a, c, e) where there was a significant girdling and *x*-variable interaction and all data (b, d) whenthere was no significant girdling and *x*-variable interaction. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported in each panel.

Stomatal conductance was correlated with leaf tZ, GA4 and ABA concentrations (Fig. 4.8). Irrespective of girdling, gs declined as leaf tZ concentrations increased, even if leaf [tZ] explained relatively little of the variation in gs (r^2=0.33) (Fig. 4.8a). Irrespective of girdling, gs declined as leaf GA4 concentrations decreased, even if leaf [GA4] explained relatively little of the variation in gs (r^2=0.27) (Fig. 4.8b). Irrespective of girdling, gs declined as leaf ABA concentrations increased, with leaf [ABA] explaining much of the variation in gs (r^2=0.66) (Fig. 4.8c). Thus leaf ABA concentration was better correlated with gs than was shoot water potential (r^2=0.51).

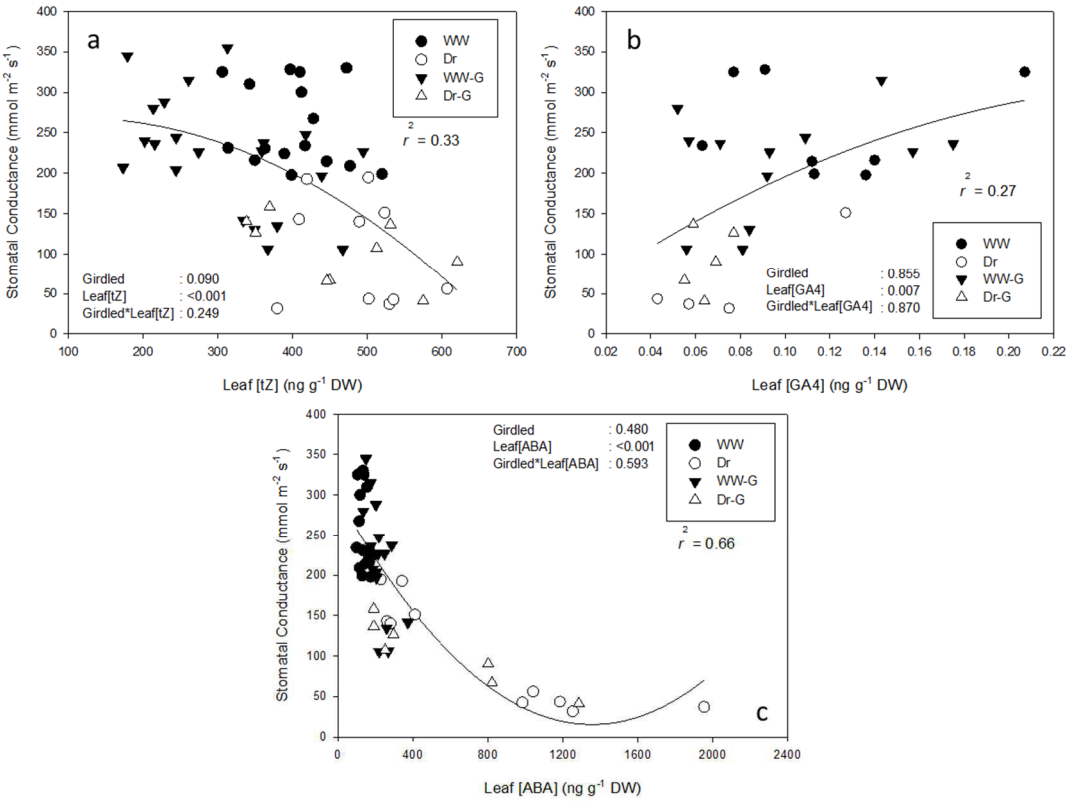


Figure 4.8. Relationship between stomatal conductance and leaf tZ (a), GA4 (b) and ABA (c) concentrations during the experiment. Filled circles and triangles represent well-watered intact and girdled plants respectively, and hollow circles and triangles represent drought intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P < 0.05$) were fitted to all data when there was no significant girdling x [Hormone] interaction. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported in each panel.

4.3.5 Correlations between leaf and root phytohormones during the experiment

Table 4.5. Pearson's r correlation coefficients and trend lines between logarithmic values of leaf and root tissue phytohormone concentrations. Significance of *p*- values reported thus: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significant correlations between leaf and root phytohormone concentrations are highlighted with a red square.

Finally, the possible inter-dependency of leaf and root hormone concentrations and their relationships between intact and girdled plants were analysed (Table 4.5 and Fig. 4.9). Leaf tZ concentrations were negatively correlated with root tZ concentrations, but only in girdled plants. In intact plants, leaf tZ concentrations were not correlated with root tZ concentrations (Fig. 4.9a). Likewise, leaf JA concentrations were negatively correlated with root JA concentrations, but only in

girdled plants. In intact plants, leaf JA concentrations were not correlated with root JA concentrations (Fig. 4.9b). Furthermore, leaf ABA concentrations were positively correlated with root ABA concentrations irrespective of girdling (Fig. 4.9c), although this relationship was more sensitive in intact plants due to the leaf ABA accumulation in well-watered girdled plants. Thus girdling affected the co-ordination of root and leaf phytohormone concentrations.

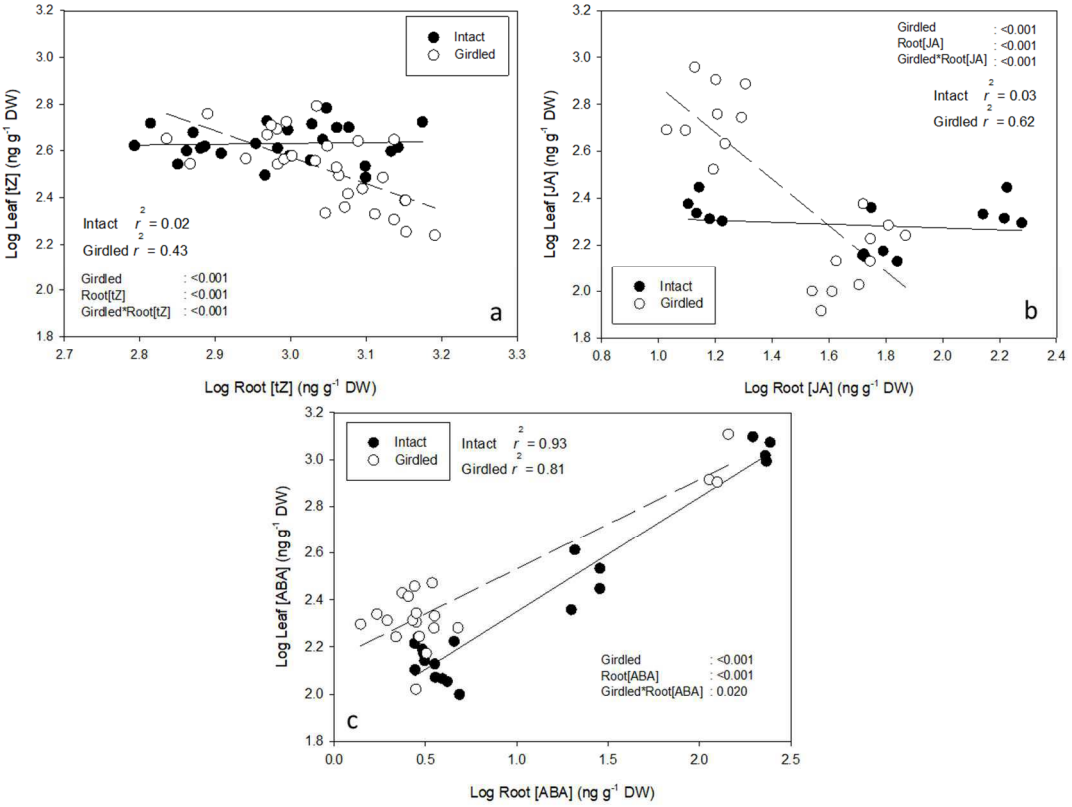


Figure 4.9. Relationships between the logarithm of root and leaf tZ (a), JA (b) and ABA (c) concentrations during the experiment. Filled and hollow circles represent intact and girdled plants respectively. Each symbol is an individual plant and regression lines ($P < 0.05$) were fitted to intact and girdled plants where there was a significant girdling and x [Hormone] interaction. *p*-values determined by ANCOVA for each main effect (*x*-variable and girdling) and their interaction are reported in each panel.

4.4 Discussion

Girdling decreased stomatal conductance within the first 26 hours, which might be caused by multiple changes in leaf hormone concentrations (iP, tZ, GA3, ABA, JA and SA- Fig. 4.2a-g) since shoot and root water potential was similar in intact and girdled plants during this time (Fig. 4.1c, d). Higher foliar SA and JA concentrations during this time (Fig. 4.2f, g) could reflect an early wounding response (Arbona and Gómez-Cadenas 2008; de Ollas *et al.,* 2013) that may induce partial stomatal closure but only JA accumulation was correlated with stomatal closure (Fig. 4.5b). As mentioned before, within 26 h of girdling, before any significant impact of soil drying on g_s was detected, plant-to-plant variation in stomatal conductance was significantly inversely correlated with both foliar ABA and JA concentrations (Table 4.2; Fig. 4.5). Since girdling increased leaf JA concentrations drastically within 2 h, this signal could interact with and/or stimulate ABA synthesis, leading to stomatal closure. Although girdled plants had higher leaf hormone concentrations (Fig. 4.2), girdling did not affect stomatal sensitivity to these hormones, as indicated by no significant stomatal conductance x [Hormone] interactions (Fig. 4.5). Thus girdling-induced stomatal closure was most probably caused by elevated foliar hormone (JA, SA, ABA) accumulation, without changing stomatal sensitivity to those hormones. Furthermore, leaf concentrations of several hormones (iP, tZ and GA3) known to promote stomatal opening (Dodd, 2003) were decreased during this time, even though their concentrations were not correlated with gs. Thus girdling induces multiple changes in foliar phytohormone concentrations that could induce stomatal closure.

Furthermore, girdling induced several changes in root phytohormone concentrations within 26 hours, with ACC concentrations increasing while GA3 and GA4 concentrations decreased (Fig. 4.2h-n). Of these, root ACC accumulation may enhance root-to-shoot ACC signalling to alter stomatal conductance by antagonising the action of ABA (Jackson, 2002; Tanaka *et al.,* 2005; Wilkinson *et al.,* 2012) or directly inducing stomatal closure (Gunderson and Taylor, 1991; Jackson, 2002; Acharya and Assman, 2009). Since ACC was not detected in leaf samples, it remains unclear whether root-to-shoot ACC signalling was involved in stomatal regulation. In addition, the decrease of root GA3 and GA4 concentrations may also diminish root-to-shoot gibberellin transport, which may explain the massive (6-fold) decreases in foliar GA3 concentrations within 26 hours of girdling. Further work should evaluate xylem sap composition of girdled plants within 26 hours of girdling, to determine whether root-to-shoot signalling may be involved in regulating stomatal conductance, potentially independently of the changes in foliar hormone accumulation discussed earlier. Although changes in root hormone status can alter root-to-shoot signalling (Dodd 2005), reciprocal grafting studies with hormone-deficient mutants can show relatively limited impacts of changes in root hormone status on tissue concentrations (e.g. for ABA – Li *et al.* 2018), but further studies are needed with a wider range of hormone-deficient mutants.

Hormonal regulation of stomatal conductance differed between the first 26 hours of the experiment (when there was limited soil drying) and during the entire experiment. Overall, stomatal conductance was significantly correlated with leaf tZ (negatively), GA4 (positively) and ABA (negatively) concentrations (Table 4.4). Soil

drying increased tZ and ABA concentrations independently of girdling (indicated by no Stress x Girdled interaction), while GA4 only decreased in intact plants by the end of the experiment (Fig. 4.2b, d, e). Soil-drying induced accumulation of [tZ] (Fig. 4.2b) may promote stomatal opening by decreasing stomatal sensitivity to ABA (Wilkinson and Davies, 2002; Hansen and Dorffling, 2003). In the absence of any consistent treatment effects on foliar GA accumulation, stomatal conductance may be regulated by the relative magnitude and effects of ABA/cytokinin balance.

Over the entire experiment, root GA3, ABA and JA concentrations were highly significantly correlated with soil water content (Table 4.3). While GA3 concentrations decreased, ABA and JA concentrations increased as the soil dries (Zhang and Davies, 1989b; Pandey *et al.,* 2004; Puértolas *et al.,* 2013; de Ollas *et al.,* 2018), which together could regulate root growth. Girdled plants showed less GA3 and JA accumulation in the roots as the soil dries, reflecting the importance of shoot-to-root GA3 and JA transport (Fig. 4.6b, d). For ABA, the correlation between soil water content and root ABA concentration might reflect localised ABA biosynthesis (Speirs *et al.,* 2013) or import of ABA from the shoots (McAdam *et al.* 2016b). Both mechanisms may operate simultaneously, since roots of girdled plants still accumulated some ABA as the soil dried, although much less than in intact plants (Fig. 4.6c). While the simplest explanation for decreased root ABA accumulation in girdled plants is the disruption of basipetal ABA transport (Manzi *et al.,* 2015), alternatively this may reflect enhanced root ABA export via the xylem or re-distribution of ABA within the roots to promote root growth.

Leaf tZ, GA4 and ABA concentrations were also significantly correlated with soil water content (Table 4.4). Soil drying decreased leaf GA4 concentration by the end of the experiment, while both leaf tZ and ABA concentrations increased as the soil dried, with girdling decreasing ABA accumulation compared to intact plants respectively (Fig. 4.7c, e). For ABA, the lower leaf ABA concentrations of girdled plants after prolonged soil drying might reflect decreased root export via the xylem and/or decreased foliar ABA synthesis as Ψ_{leaf} was higher (Castro *et at.*, 2019), with these effects overcoming the girdling-induced foliar ABA accumulation caused by disrupting basipetal shoot-to-root ABA transport. Similar explanations could apply to the regulation of foliar tZ concentrations, but in this case girdling did not affect soil-drying induced tZ accumulation, suggesting a more prominent role for root tZ export in foliar tZ homeostasis, as proposed for plants exposed to changes in substrate N status (Sakakibara *et al.,* 1998).

Overall, leaf and root ABA concentrations were the only hormones that were highly significantly correlated with each water relations parameter (stomatal conductance, shoot and root water potential and soil water content) during the entire experiment. Those results suggest the importance of shoot-to-root and root-to-shoot ABA signalling in modulating both root and leaf hormone concentrations under drought conditions (Castro *et at.*, 2019).

Finally, girdling altered correlations between root and leaf concentrations of tZ, ABA and JA during the entire experiment (Table 4.5; Fig. 4.9). Stability of leaf tZ and JA concentrations despite fluctuations in root hormone concentrations in intact plants suggests a limited role for root-to-shoot hormone signalling. However, in

girdled plants, leaf tZ and JA concentrations decreased as the root concentrations increased, which cannot be accounted by altered shoot-to-root hormone transport. Instead, this independence of root and shoot tZ and JA concentrations suggests local regulation of hormone biosynthesis in each tissue. However, girdling did not drastically affect ABA homeostasis, since soil drying increased leaf and root ABA concentrations in both intact and girdled plants, highlighting the importance of root-to-shoot ABA signalling and local synthesis. Rapid shoot JA accumulation in response to girdling (wounding) could trigger stomatal closure followed by increased ABA levels (Pinheiro *et at.*, 2011). Furthermore, as the soil dries leaf JA concentrations of girdled plants decrease, while ABA concentrations increase independently of girdling. Nevertheless, soil drying still significantly increased foliar JA concentrations in intact plants, perhaps mediated by root-to-shoot JA signalling (as soil drying increased root JA concentrations of intact plants to a much greater extent than in the leaves). These changes could directly or indirectly interact with ABA accumulation (mediated by biosynthesis, catabolism and signalling) to stimulate stomatal closure, thereby avoiding plant desiccation (de Ollas and Dodd, 2016; Per *et at.*, 2018).

In conclusion, root-to-shoot ABA and JA (Fig. 4.2) communication (Fig. 4.9b, c) seems important in regulating stomatal closure when girdling and drought conditions are applied (Table 4.3, 4.4). To further understand how the endogenous concentrations of these hormones are regulated, analysing the expression of genes involved in the biosynthesis, catabolism and signalling of both phytohormones may

be useful in determining relationships between transcription and the final hormone concentrations in leaf and root tissues (Chapter 5).

Chapter 5 – Co-ordination of root and shoot gene expression under girdling and drought conditions

5.1 Introduction

The previous chapter demonstrated the potential importance of changes in ABA and JA concentrations in roots and leaves for regulating stomatal conductance under drought and girdling conditions. Although ABA is primarily considered as a drought stress hormone, JA is associated with wounding responses, while both hormones can induce stomatal closure (Murata *et al.*, 2015). The physiological role(s) of both hormones (ABA and JA) in mediating drought and wounding responses have been studied using different mutants in their synthesis or degradation pathways (Iuchi *et al.*, 2001; Zhang *et al.*, 2006; Forster *et al.*, 2019).

De novo ABA biosynthesis originates from the carotenoids pathway by multi-enzymatic reactions, where the first committed step is regarded as the epoxidation of zeaxanthin and the ultimate step catalyses the conversion of ABA-aldehyde to ABA, as described in Chapter 1. Within this pathway, a key rate-limiting step in ABA biosynthesis is catalysed by expression of multiple NCED (9-cis-epoxycarotenoid dioxygenase) genes, with NCED2 and NCED3 highly expressed in roots and leaves, where NCED3 is considered the major contributor to *in planta* ABA increments in Arabidopsis (Tan *et al.*, 2003; Sussmilch *et al.*, 2017; Ma *et al.,* 2018). In contrast, ABA degradation seems less complex, as fewer enzymatic steps are involved (Figure 1.3), where the CYP genes family encodes an ABA 8'-hydroxylase which is

considered one of the primary catabolism genes in the pathway (Zheng *et al.,* 2012). Tissue de- and re-hydration decreases and increases CYP genes gradually and rapidly within the plant (Yang and Zeevart, 2006; Ma *et al.,* 2018).

There is increasing evidence that transcriptional expression of either ABA biosynthesis or catabolism genes plays an important role in ABA homeostasis. Tissue ABA concentrations are regulated by simultaneous changes in NCED3 and CYP gene expression during plant desiccation, where the expression of NCED3 goes up and CYP goes down (Liu *et al.,* 2014). Thus, a balance between both biosynthesis and catabolism gene expression is regulated by feedback loops regulating ABA status in leaves and roots, to elicit stomatal closure and maintain root growth respectively under drought conditions (Zhang *et al.,* 2006; Ma *et al.,* 2018). Following ABA accumulation, intracellular ABA signalling may alter hormone perception mechanisms within different tissues (Shinozaki and Yamaguchi-Shinozaki, 1997; Jia *et al.,* 2002; Chaves *et al.,* 2003).

ABA triggers a unique response by binding to its immediate protein receptors (PYR/PYL) to activate different signalling pathways (Miyakawa *et al.,* 2013). After being received from the membrane transporters (ABCG genes family) in the presence of ABA, ABA-bound PYR/PYL complex inactivates PP2C protein activity, which negatively regulates ABA signalling (Boursiac *et al.,* 2013; Merilo *et al.*, 2015). Then, formation of a second complex comprising PYR/PYL, ABA and PP2C, will phosphorylate the critical components in the ABA signalling pathway, SnRK2 and ABF proteins (Raghavendra *et al.,* 2010; Wang *et al.,* 2019). Thus such gene expression and accumulation of ABF proteins are induced by ABA signalling, where

those ABFs induce the expression of PP2C forming a loop that regulates ABA signalling (Fig. 5.1).

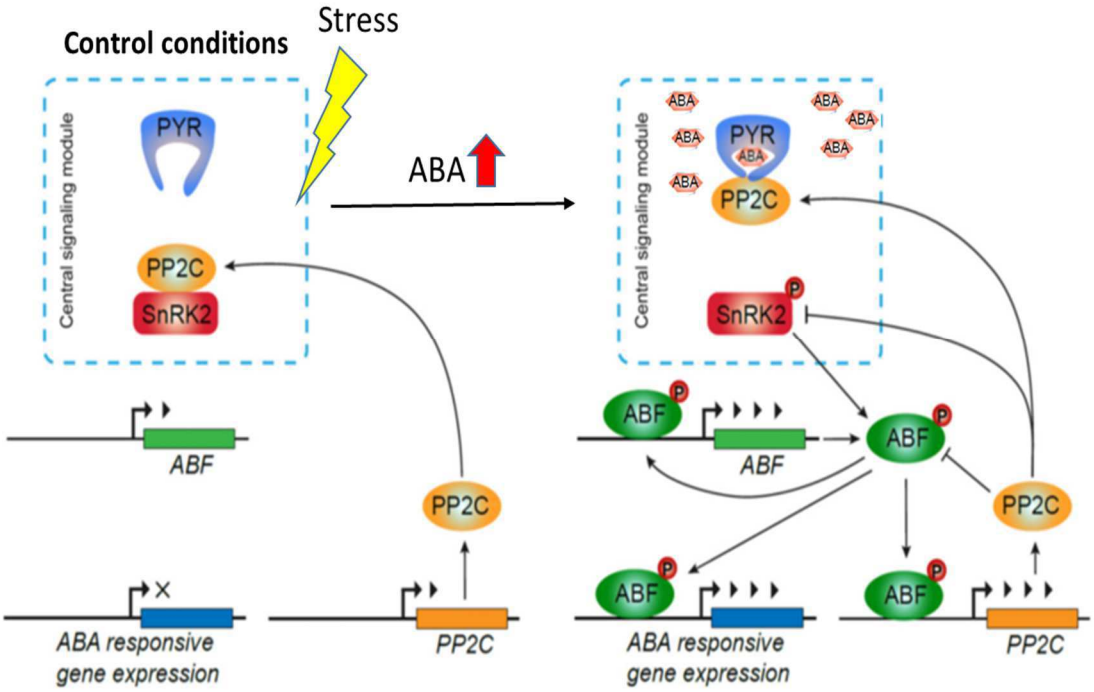


Figure 5.1. Model of ABA signalling pathway where ABFs and PP2C play a role in the feedback regulation of ABA homeostasis (redrawn from Wang *et al.,* 2019).

While ABA is a key hormone under water deficit, JA increases largely in response to wounding. Biosynthesis of jasmonates starts when α-linolenic acid is released from plastid membranes and ceases with the production of jasmonoyl-CoA (Figure 1.4). An important intermediate within JA biosynthesis is the production of 12-oxo-phytodienoic acid (OPDA), which seems to have additional biological functions, such as limiting transpiration (Dave and Graham, 2012; Roberts, 2016). The enzyme encoded by JAR1 catalyses the important reaction of conjugating the amino acid isoleucine to JA to form the main bioactive jasmonate, JA-isoleucine (Staswick and Tiryaki, 2004). In contrast to ABA, the methyl esterification of jasmonate and its

conjugation to the amino acid isoleucine is important in allowing biological activity leading to stomatal closure (Staswick and Tiryaki, 2004; De Ollas *et al.,* 2018). Furthermore, in response to wounding, JA levels can rapidly increase (within minutes to an hour - as shown in Chapter 4) to elicit physiological responses (Reymond *et al.,* 2000; Glauser *et al.,* 2008). Furthermore, feedback loops within the JA biosynthesis pathway, where many enzyme steps are positively regulated by their products thereby amplifying jasmonate concentration, lead to rapid increases in JA concentrations (Stratmann, 2003; Banerjee and Bose, 2011). Members of the CYP94 gene family (CYP94B3, CYP94C1) have been identified to hydroxylate JA-Ile and to carboxylate 12-OH-JA-Ile (CYP94C1) (Koo *et al.,* 2011; Heitz *et al.,* 2012), which hydroxylation and carboxylation contribute to JA catabolism. There is, however, a complex sustainment of JA-Ile homeostasis, in which hydroxylation and carboxylation remove the active JA-Ile (Heitz *et al.,* 2016).

JA also triggers a unique response between its immediate protein receptor COI1 to activate different signalling pathways (Yin *et al.,* 2016). The primary receptor of the JA signalling pathway is COI1, with JAZ proteins inhibiting jasmonate effects downstream of gene expression of JA responses (Pauwels and Goossens, 2011). Thus, the interaction between JA-Ile, COI1 and JAZ proteins releases MYC2 transcription factors, to initiate the expression of JA-responsive genes. This basic mechanism is conserved in different crops (Sheard *et al.,* 2010), where the transcription factor MYC2, which is central to jasmonate signalling, will act via JAZ proteins controlled by COI1 interacting with MYC2 activity and finally JA-responsive genes (Fig. 5.2).

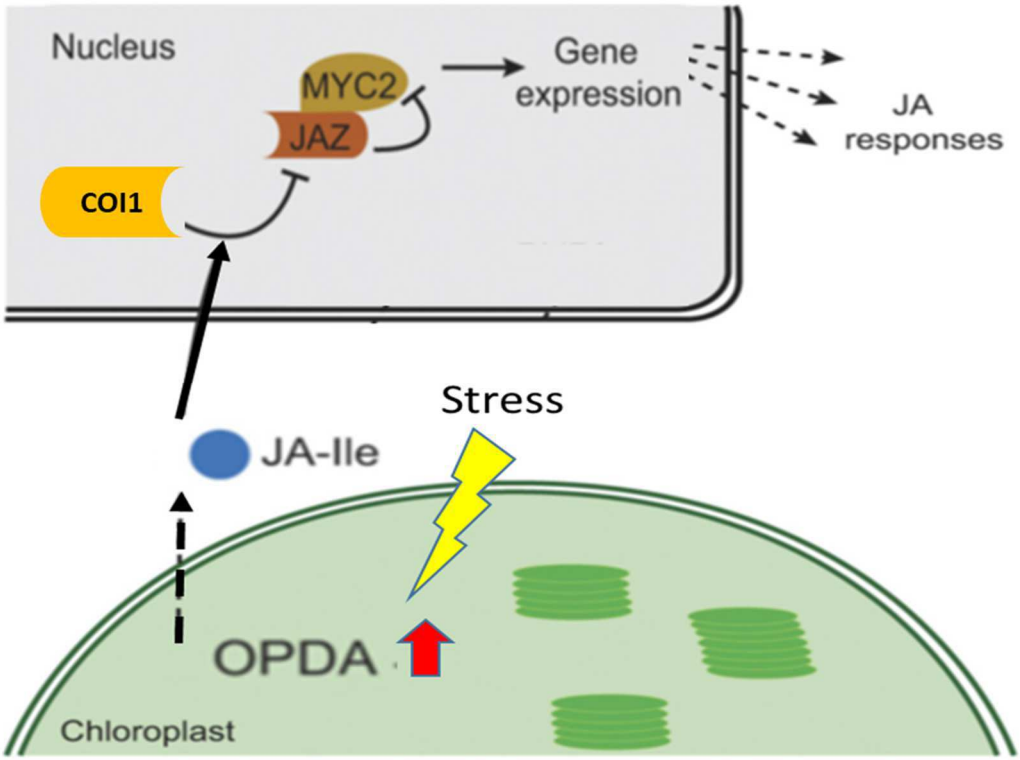


Figure 5.2. Model of JA signalling pathway where JAZ and MYC2 play a key role in the regulation of JA responses (redrawn from Mach, 2018).

Hormonal changes in response to girdling and soil drying (Chapter 4) could be attributed to local biosynthesis and/or degradation or long-distance signalling of these hormones. Therefore analysing gene expression within each hormone pathway, by RNA-sequencing and further qRT-PCR validation (Arbona *et al.,* 2010), would lead to a better understanding between the transcription level and the final hormone balance in each tissue and time. Moreover, measuring the expression of genes involved in downstream signal transduction of those hormones may provide some clues as the biological impact of those hormones.

5.2 Materials and methods

5.2.1 Plant materials and experimental design

Soybean (*Glycine max* L. Merr. Williams 82) seeds were sown, prepared and grown as described in Experiment 2, in Section 2.2.1. During expansion of the third trifoliate leaf, plants were randomized into 4 groups, comprising the treatments applied: soil drying (WW: well-watered; DR: droughted) and girdling (NG: intact plants; G: Girdled plants) respectively. As in previous chapters, since significant treatment effects were detected in the first 3 days, measurements were made from Days 0 to 3 after girdling. Leaf and root tissue of five plants per treatment were harvested from Days 1 to 3. Girdling was achieved surgically at 0800h on Day 0 when the third trifoliate leaf was completely expanded as described in Section 3.2.1. Water was withheld from half the girdled and non-girdled plants after the girdling was complete on Day 0.

5.2.2 Physiological measurements

Measurements were made on the third trifoliate leaf throughout the experiment, once the girdling was achieved. Each measurement comprised a different leaflet, described as left, central and right when viewed from the petiole junction. Stomatal conductance (g_s) was measured daily at 1000h, on the right leaflet with a porometer (Model AP4, Delta-T Devices, Burwell, UK). Two measurements were sequentially made on each plant and averaged. After measuring stomatal conductance, leaf discs (8 mm diameter) were punched from the left leaflet and immediately mounted on clean sample holders and wrapped in aluminum foil to prevent water

loss. Once all leaf discs had been collected, they were unwrapped and introduced into C52 chambers (Wescor Inc, Logan, UT, USA), for 2 hours of incubation. Then voltages were read by a microvolt meter (Model HR-33T, Wescor Inc, Logan, UT, USA) and converted into water potentials (Ψ) based on calibration with salt solutions of known osmotic potential. Once stomatal conductance and leaf water potential (Ψ_{leaf}) were measured, the remaining central leaflet was collected in Eppendorf vials and immediately frozen in liquid nitrogen. The whole root system was removed from the pot, briefly washed and collected in Eppendorf vials, then frozen in liquid nitrogen. Both tissues (leaf and root) from Days 1 to 3 were stored at -80 ºC for further transcriptomic and gene expression analyses. Soil water content was calculated as described in Section 2.2.2.

5.2.3 RNA extraction, sequencing and library construction

Leaf and root tissue, harvested 3 days after withholding water, were used for RNA-Seq analysis. Leaf and root tissues were ground in liquid nitrogen, with around 100 mg frozen/ground tissue used for total RNAs using a Plant RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA samples from three individual leaves and roots of each treatment were pooled together in equal amounts to generate one mixed sample. RNA was precipitated with ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80ºC. All RNA samples were examined for protein contamination (as indicated by the A260/A280 ratio) and reagent contamination (indicated by the A260/A230 ratio) with a Nanodrop ND1000 spectrophotometer (NanoDrop).

Total RNA purity and degradation were examined on a 1% agarose gel before proceeding. The mRNA was purified from 6 mg of total RNA using oligo(dT) magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under an elevated temperature (94ºC), and the cleaved RNA fragments were used for first-strand cDNA synthesis using reverse transcriptase and random primers. DNA polymerase I and RNase H were used to synthesize second-strand cDNA. Subsequently, short fragments were purified using a QiaQuick PCR extraction kit and resolved with elution buffer for end repair and poly(A) addition. Those fragments with a suitable range of lengths selected based on the results of agarose gel electrophoresis were used as templates for library amplification. The library quality was confirmed by the Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA Kit.

5.2.4 Analysis of RNA sequencing

The resulting cDNA library constructed from leaf and root RNA samples were used for paired-end (2 x 150 bp) sequencing on an Illumina HiSeq 4000 platform by Annoroad Gene Technology Co. Ltd (Beijing, China). Three replicates for each sample were trimmed to obtain clean reads for subsequent analysis. Raw image data generated by sequencing were transformed by base calling into sequence data, which are called raw data/raw reads, and were stored in fastq format (Liu et al., 2015).

The soybean reference genome annotation file (Glycine max Wm82.a2.v1) was downloaded from the Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html). Mapping of clean reads and

subsequent bioinformatic analysis were as described previously. Significant changes in differentially expressed genes (DEGs) were determined as log2FC >2 and *q*-value (false discovery rate, FDR <5%). Gene Ontology (GO) analysis (http://geneontology.org/) and Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.kegg.jp/) enrichment classification were carried out using the DEG data set.

5.2.5 Quantitative Real-Time PCR

For qRT-PCR, total RNA (~5 µg) from leaves and roots from Days 1 to 3 were reverse-transcribed into cDNA by using the Superscript First-Strand Synthesis System (Invitrogen, USA) following the manufacturer's instructions.

Transcript levels of selected genes were measured by CFX384 Touch™ Real-Time PCR Detection System (ThermoFisher Scientific China, Inc., Shanghai) with TransStart® Tip Green qPCR SuperMix (Transgen) according to manufacturer's protocols in a 12-µL reaction. ACT11 (cytoskeletal structural protein) was used as an internal standard (Neves-Borges *et al.,* 2012). The primers were designed according to significant changes in DEGs (fold-change in KEGG pathway, Fig. 5.5) originated from the RNA-sequencing to confirm their specificity. Primers selected and used for qRT-PCR are listed in Table 5.1. Relative expression of each gene was normalized against the internal reference gene (ACT11), and calculated according to the $2^{-\Delta\Delta CT}$ method as previously described (Schmittgen and Livak, 2008). Graphical representation for up- and down-regulation of each gene and time were represented as mean fold values relative to the expression level of each droughted and girdled comparison.

5.2.6 Statistical analysis

Two replicate experiments were conducted simultaneously, ensuring sufficient tissue to allow different assays, such as multi-hormone analysis (not presented here as data not yet available - but see Chapter 4). Day 3 samples of both experiments were analyzed comparing the expression of some selected genes by qRT-PCR (data not shown). Once it was seen that both experiments had similar responses (including plant water relations), samples from one experiment were chosen for the RNA-sequencing. Two-way analysis of variance (ANOVA) determined the effects of water treatment, girdling and their interaction on soil water content, stomatal conductance and leaf water potential (Fig. 5.3). Heterogeneous groups were separated by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) to discriminate differences between treatment x girdling combinations (Fig. 5.6-5.11).

5.3 Results

5.3.1 Effect of girdling and soil drying on plant water relations

Soil water content and Ψ_{leaf} of well-watered plants was, as observed in previous chapters, consistent throughout the experiment, remaining between 0.85 and 0.9 g g^{-1} for soil moisture and between -0.8 and -0.65 MPa for Ψ_{leaf} (Fig. 5.3a, c). Girdling had no significant effect on soil water content and Ψ_{leaf}. Withholding water decreased soil moisture to 0.72 g g^{-1} at Day 2 and 0.65 g g^{-1} at Day 3 (Fig. 5.3a). Soil drying did not significantly decrease Ψ_{leaf} until Day 2, when it was 0.4 MPa lower than in well-watered plants, and on Day 3 when it was 0.7 MPa lower (Fig. 5.3c).

Thus soil drying decreased soil water content and leaf water potential, but girdling had no significant effect during the experiment.

Girdling significantly decreased g_s of well-watered plants from Day 1 (Fig. 5.3b), and g_s of these plants remained 30% lower than intact plants on Day 3. After withholding water, g_s declined similarly in both droughted intact and girdled plants, approximately halving g_s compared to well-watered intact plants by the end of the experiment. Thus soil drying decreased g_s at the same rate independently of girdling, while girdling caused partial stomatal closure of well-watered plants.

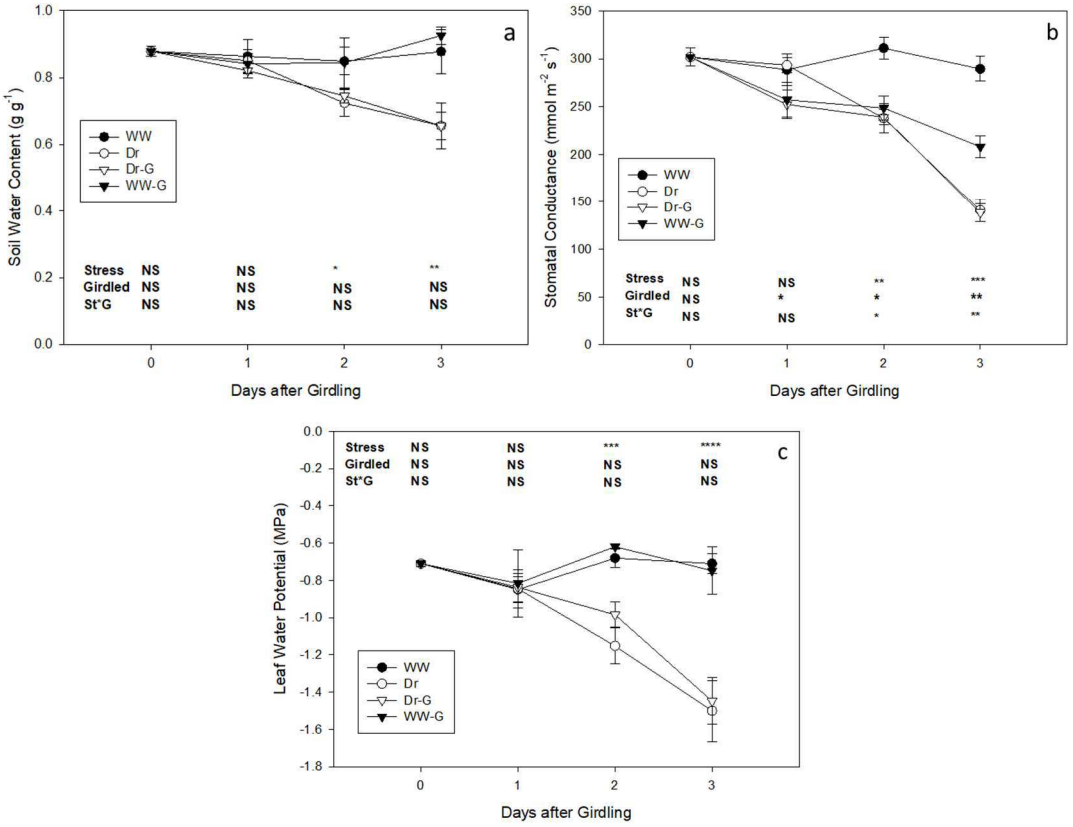


Figure 5.3. Soil water content (a), stomatal conductance (b), leaf water potential (c) during the experiment, with water withheld from droughted and girdled plants on Day 0. Measurements on Day 0 were done before imposing treatments. Filled and hollow symbols represent well-watered and droughted plants respectively, with intact and girdled plants indicated by circles and triangles respectively. Symbols indicate mean ± s.e. (n=5).Effects of soil drying (Stress), girdling treatment (Girdled) and their interaction (St x G) are indicated thus: NS, non-significant; * P <0.05; ** P <0.01; *** P <0.001.

5.3.2 Differential expression genes analysis and KEGG hormone pathway

The RNA-sequencing microarray analysis identified how many genes were up- and down-regulated (DEGs) 3 days after water was withheld and girdling was applied (Fig. 5.4). Soil drying up-regulated 3240 and 989 genes, and down-regulated 3900 and 286 genes in leaves and roots of intact plants, respectively. Girdling suppressed the number of genes up- (from 3240 to 498) and down- (from 3900 to 945)

regulated in leaves in response to soil drying. In contrast, girdling increased the number of genes up- (from 989 to 1056) and down- (from 286 to 1599) regulated in the roots in response to soil drying. Thus soil drying had greater effects on differential gene expression in the leaves than roots, with girdling seemingly decreasing the number of DEGs in the leaves while increasing the number of DEGs in the roots in response to soil drying.

In well-watered plants, girdling up-regulated 2097 and 7851 genes, and down-regulated 1621 and 11688 genes, in leaves and roots respectively. In addition, droughted girdled plants increased the number of up-regulated genes in leaves (from 2097 to 3545) but decreased the number of up-regulated genes in roots (from 7851 to 6676) compared to well-watered girdled plants. Similarly, soil drying increased the number of down-regulated genes in leaves of girdled plants (from 1621 to 2579) and decreased the number of down-regulated genes in roots (from 11688 to 10561) of girdled plants. Thus, girdling increased the number of DEGs in roots compared to leaves, independently of soil moisture.

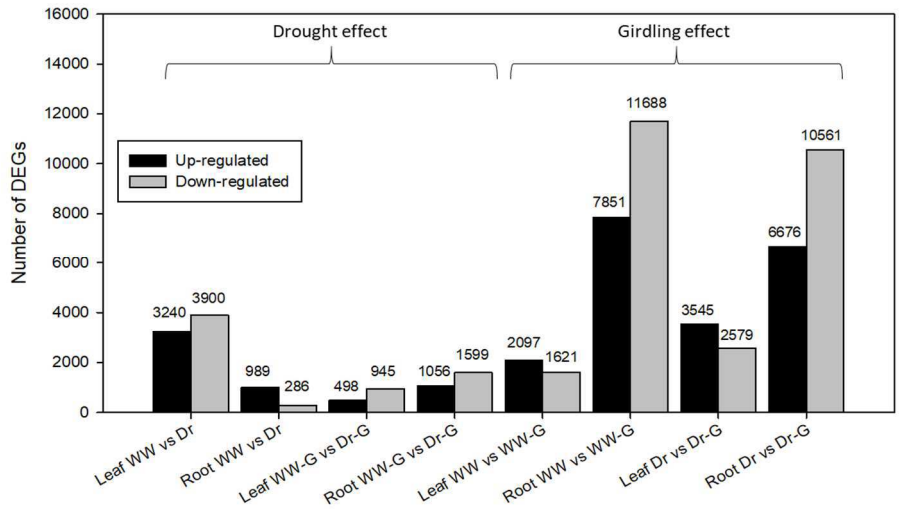


Figure 5.4. Number of differentially expressed genes (DEGs) up- and down-regulated (>Log2Fold-Change) in each comparison between leaf and root tissues of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G), 3 days after imposing treatments on Day 0.

Since changes in hormone concentrations, specifically ABA and JA, were involved in regulating stomatal conductance after applying both soil drying and girdling treatments (Chapter 4), the KEGG annotation pathways, from the RNA-sequencing, of the biosynthesis, catabolism and signalling of both hormones are shown (Fig. 5.5). Specific orthology (KOx) and enzymatic (EC x.x.x) steps followed for selecting the genes that were identified as DEGs are highlighted as red squares. For each step in the hormone pathways, (at least) one DEG that was up- or down-regulated (log2Fold-changed >1; *p*-value adjusted <0.01) in the RNA-sequencing was selected for qRT-PCR validation. Thus, the primer used of each gene for each KO or EC step is shown (Table 5.1).

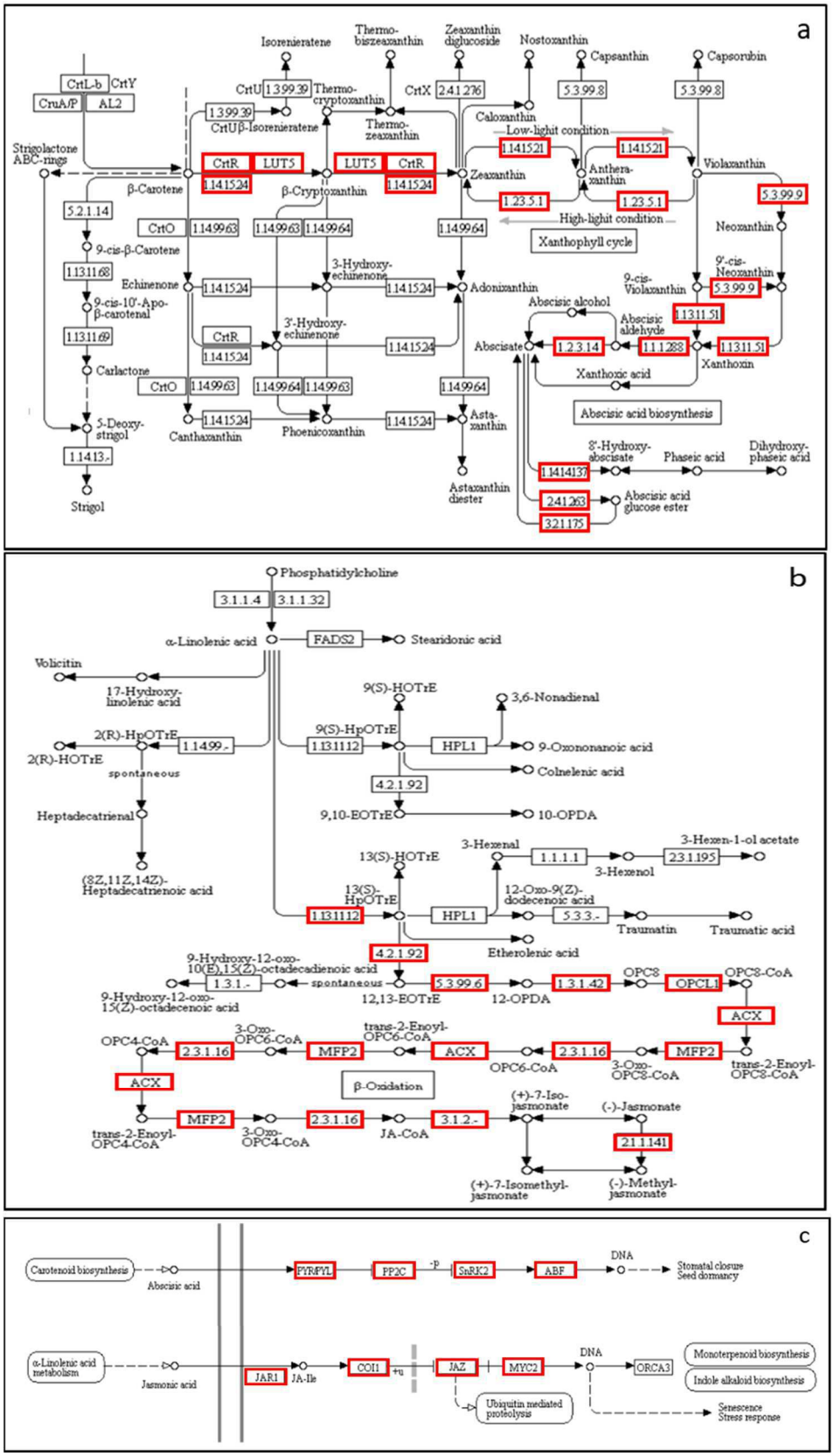


Figure 5.5. ABA (a) and JA (b) biosynthesis and catabolism pathways, and their signal transduction pathways (c) in plants mapped by KEGG annotation. Red squares indicate the orthologue and enzyme step of the specific route followed to identify each gene involved in the biosynthesis, catabolism and signalling from the RNA-sequencing microarray.

ABA - KEGG (KO) / (EC) - Annotation	Genes ID	Genes Annotation	Genes labelled for qRT-PCR	Oligonucleotide sequences (5´to 3`)	
CrtZ (KO15746) / (EC 1.14.13.129)	Glyma.20G162600	Beta-carotene 3-hydroxylase	CrtZ	TCCACCGCCGCAAACTTAAA	CTGGGTCGAACTCGAAGGA
LUT5 (KO15747) / (EC 1.14.13.90)	Glyma.09G000600	Zeaxanthin epoxidase	LUT5	GCACTTGTCAGGTTCAACTCC	TGAAAGAACCATCTCATTCCCACT
Zeaxanthin EC 1.14.1390 (KO9838)	Glyma.17G174500	Zeaxanthin epoxidase	ZEP/ABA1	TCTCTTAACCCTTCAACAACCGT	TGCTTCCTTGTTCTGCATCCT
	Glyma.19G251000	violaxanthin de-epoxidase	ZEP/VDE	TTATCCCATGGGGAAGGCAC	TGGCCAACACTTTGAGCACT
Violanxanthin EC 1.13.11.51 (KO9840)	Glyma.08G176300	9-cis-epoxycarotenoid dioxygenase	NCED3 (1)	ATGGCATCATCAGCAACAAACAC	CATGTGATGGTGTTGGAATTGGA
	Glyma.08G365700	Neoxanthin synthase	NSY (1)	TGCTTTTCCCATTCTCCATTGAC	TGCGTCCCTTACAAAGCTCA
	Glyma.15G250100	9-cis-epoxycarotenoid dioxygenase	NCED3 (2)	TCAGCAGCAGCAACAACAAC	GGCGTTGGAGTTGGAATTGG
	Glyma.19G047800	Neoxanthin synthase	NSY (2)	AAAGAGAAGCTCCAATTCCAAAGA	GCCCAATTTGCATCACAGCAT
Xanthoxin EC 1.1.1.288 (KO9841)	Glyma.02G078600	Short chain dehydrogenase	SDR	TGCACCTGCAACTTCAACTTC	AGGAAGCCAGCTTTGGTGAA
	Glyma.11G151400	Xanthoxin dehydrogenase	SDR/ABA2	TCTGGCTTCCACTCCAACAC	GGATATGGAAGAGGCGCACA
Abscisic aldehyde 1.2.3.14 (KO9842)	Glyma.02G272400	Abscisic-aldehyde oxidase	AAO3 (1)	ATGGAGCTGGAGCTGAAGAC	GCCGAGCTTGACACTCTTGA
	Glyma.14G045100	Abscisic-aldehyde oxidase	AAO3 (2)	TGGAACTGGAGAAGACACCA	CTTGAAACGAGTGCGAGTGC
	Glyma.09G002300	Molybdenum cofactor sulfurtransferase	MoCo/ABA3	TGGACGCTGCCAAAGAAGAAT	AGTTGCCCCAGCATGATCC
Abscisate EC 1.14.13.93 (KO9843)	Glyma.04G030200	Abscisic acid 8'-hydroxylase	CYP (1)	TTGTGGATGAGAATCGACAAGT	GGCATCTCATTAAGGGCCTC
	Glyma.16G076600	Abscisic acid 8'-hydroxylase	CYP (2)	TGTGCTTGTTTGCTTCTTTCCT	TAAGGCAAACCCATGGTCCC
PYR/PYL (KO14496)	Glyma.02G261900	Abscisic acid receptor PYR/PYL family	PYL5	CCCAGCAACCGATACATCCA	CGCTAAGGCTTACTTTCGCC
	Glyma.17G229900	Abscisic acid receptor PYR/PYL family	PYL2	CCTCGGAAACCCACCATCAT	GGTGCTTCAATCCGGTGTGT
PP2C (KO14497)	Glyma.02G250200	Protein phosphatase 2C	PP2C 3	AGTTGTTGGAGAATCCGGAGAG	CACGTCAGCAGCTATGTATCTC
	Glyma.14G162100	Protein phosphatase 2C	PP2C	TGAAGACGCCAAAACGCC	CGGATCTTCGTCTGGCAAGT
SnRK2 (KO14498)	Glyma.02G135500	Serine/threonine-protein kinase SRK2	SnRK2 3	TGAGTGTTGGGCCAGGAATG	CAGCAACAAGTTCCTCAGTATGT
	Glyma.07G209400	Serine/threonine-protein kinase SRK2	SnRK2	TGAACCGGAAGGATCGGTCT	CGGTGTGCTTGTCCCTCATA
ABF (KO14432)	Glyma.05G079800	ABA responsive element binding factor	ABF/ABRE	GCAAGAGCCAAAGACTACTACTAC	GCTTCCCCAAATTCCCAAGC
	Glyma.13G153200	ABA responsive element binding factor	ABF/ABI 5	ATGGCATTGGCGTTAGTGATAG	TCTCAGACTCAGGCACCACC
JA - KEGG (KO) / EC - Annotation	**Genes ID**	**Genes Annotation**	**Genes labelled for qRT-PCR**	**Oligonucleotide sequences (5´to 3`)**	
α-Linolenic Acid (KO00454) / (EC 1.13.1.112)	Glyma.12G054700	Linoleate 13S-lipoxygenase	a-Linolenic Acid (1)	TCACAGAACACCAACCAAATCC	CGTTGCGAGAAACCAGAACG
	Glyma.15G074300	Linoleate 13S-lipoxygenase	a-Linolenic Acid (2)	GAGCCAAAACTGAGCAACCC	GCTCCCGAGAACCCCAAATC
13(S)-HpOTrE (KO01723) / (EC 4.2.1.92)	Glyma.11G122700	hydroperoxide dehydratase	13(S)-HpOTrE	ATGGCTTCTTCCGACAGCAA	GCAAAGAACTTGTCGCGTCC
13,13-EOTrE (KO10525) / (EC 5.3.99.6)	Glyma.01G086900	allene oxide cyclase	13,13-EOTrE	TTCGTCCCTCAAACTCTCCC	ACTTGAGGGGTAGCTGAGAA
12-OPDA (KO05894) / (EC 1.3.1.42)	Glyma.06G016900	12-oxophytodienoic acid reductase	12-OPDA (1)	ATGGAAGAGAACGAGAAAGCGT	AGGCTGAGCCATGAAGTTGT
	Glyma.11G007600	12-oxophytodienoic acid reductase	12-OPDA (2)	GATGCTCCTCTTCTTACCCCA	GTTGGAAGTTCTCTGAGAGTAGTAG
	Glyma.13G109800	12-oxophytodienoic acid reductase	12-OPDA (3)	TCTCCATACAACAAGATGGGCA	CGGTGTTGATCTCTGAGCGT
	Glyma.17G209900	12-oxophytodienoic acid reductase	12-OPDA (4)	GGAAATTTTAATCTATCCCACAGCG	ATGCGGCTGAGGAACGAAG
OPCL1 (KO10526)	Glyma.20G192100	OPC-8:0 CoA ligase 1	OPCL1	GGAGAGCAAATCTTGTCATCCA	AAGATCAGGCCTTGTTGGGA
ACX (KO00232) / (EC 1.3.3.6)	Glyma.03G056400	acyl-CoA oxidase	OPC8-CoA	GCGATCAACTCTTCCAAGAATCC	AACGTTGATGCTGGTGTTGC
MFP2 (KO10527) / (EC 4.2.1.17)	Glyma.07G246300	enoyl-CoA hydratase	MFP2	ATGGGTAGCAGCAGAGGA	TCGCCTGATCAAAACTCTCC
3-Oxo-OPC8-CoA (KO07513) / (EC 2.3.1.16)	Glyma.07G180100	Acetyl-CoA C-acyltransferase	3-Oxo-OPC8-CoA	TGGAGAAAGCAATTCAAAGACAGAG	TGACCATGCGAAGAATTCCC
Jasmonate (KO08241) / (EC 2.1.1.141)	Glyma.14G072300	jasmonate O-methyltransferase	Jasmonate (1)	TGCAAACAGCTCGAAGGAAAC	CTGGCTCTGCGCAAGAGATT
	Glyma.18G238800	jasmonate O-methyltransferase	Jasmonate (2)	CCCTTCACATGAATGATGGCA	CACCTTCATGCAGCTAGGAGA
JAR1 (K14506)	Glyma.07G057900	jasmonic acid-amino synthetase	JAR1 (1)	TTAACACGGAGAGGATGATGG	CAGGATCCGTCCTCCCATT
	Glyma.19G254000	jasmonic acid-amino synthetase	JAR1 (2)	AGTGGGGGAGTTTAACATGGA	GCAAGTACTCTGCTGATGCG
COI1 (K13463)	Glyma.02G254300	coronatine-insensitive protein 1	COI1	GGCGAGGAGGTTATCGGATG	ACGTGTTTACGAGTGAGCGA
JAZ (K13464)	Glyma.17G043700	jasmonate ZIM domain-containing protein	JAZ	CAGGAGGGTGGAAGGCTTATC	GCATCACCGACCCATCATCA
MYC2 (K13422)	Glyma.08G271900	transcription factor MYC2	MYC2 (1)	GACCGAGTACCGGATGAACC	TTTTGCCGTGCCTGGAGTT
	Glyma.17G209000	transcription factor MYC3	MYC2 (2)	CTCCTCCCTGTGGCTGTCAA	ACCGCCTTTGTAGAGGAGAC

Table 5.1. List of genes analysed and used for qRT-PCR of ABA and JA biosynthesis, catabolism and signalling pathways.

5.3.3 Soil drying and girdling effects on ABA biosynthesis, catabolism and signalling related genes

Soil drying did not affect leaf and root gene expression in the ABA biosynthesis and catabolism pathways of intact and girdled plants on Day 1 (Fig. 5.6-7a, d). After two days of soil drying, the roots of intact plants up-regulated NCED3 (2) while the roots of girdled plants significantly up-regulated CrtZ, both NCED3s (1 and 2) and NSY (1), compared to their respective well-watered control plants. At this time, the roots of both intact and girdled plants down-regulated the NSY (2) gene compared to their respective well-watered controls (Fig. 5.6-7e). After three days of soil drying, the roots of both intact and girdled plants up-regulated NCED3s (1 and 2) and NSY (1), while the SDR and SDR/ABA2 genes were up-regulated only in intact plants (Fig. 5.6-7f). Thus continued soil drying upregulated some genes in the ABA biosynthesis pathway in roots of both intact and girdled plants.

After two days of soil drying, the leaves of intact plants significantly up-regulated SDR, SDR/ABA2 and CYP (2) genes, while both NCED3s were significantly down-regulated compared to well-watered intact plants. Soil drying up-regulated NCED3 (1 and 2) and SDR in leaves of girdled plants compared to well-watered girdled plants (Fig. 5.6-7b). After three days of soil drying, leaves of intact plants up-regulated LUT5, NCED3s (1 and 2), NSY (1 and 2), SDR, SDR/ABA2, AAO3 (2), MoCo/ABA3, CYP (1 and 2). In contrast, leaves of girdled plants down-regulated LUT5, NCED3 (1), NSY (2), AAO3 (1 and 2), MoCo/ABA3 and CYP (1 and 2) (Fig. 5.6-7c). Thus, ABA biosynthesis and catabolism genes are up-regulated in leaves of both intact plants and are attenuated in girdled plants as the soil dries.

Compared to intact plants, girdling up-regulated LUT5, ZEP/ABA1, ZEP/VDE, NCED3 (1 and 2), SDR, SDR/ABA2, AAO3 (1 and 2), MoCo/ABA3 and CYP (2) genes in the roots after one day (Fig. 5.6-7d). Two days after girdling, the roots up-regulated LUT5, ZEP/ABA1, ZEP/VDE, SDR, AAO3 (1 and 2), MoCo/ABA3 and CYP (2) genes in well-watered plants, and up-regulated ZEP/ABA1, ZEP/VDE, NCED3 (1 and 2), NSY (1), SDR, AAO3 (1 and 2) and CYP (2) genes in droughted plants (Fig. 5.6-7e). Three days after girdling, all analysed genes were up-regulated, except for NSY (1), which was down-regulated in well-watered plants and not changed in droughted plants (Fig. 5.6-7f). Thus girdling upregulates most of the genes in the ABA biosynthesis (and metabolism) pathways.

Furthermore, girdling up-regulated SDR, MoCo/ABA3 and CYP (1) genes in the leaves after one day (Fig. 5.6-7a). Two days after girdling, the leaves down-regulated NCED3s (1 and 2) genes in well-watered plants, while these genes were up-regulated and NSYs (1 and 2) and CYP (2) were down-regulated in droughted plants (Fig. 5.6-7b). Three days after girdling, all analysed genes were upregulated, except for CrtZ, ZEP/ABA1, NSY (1) and SDR genes in well-watered plants, and for LUT5, NCED3 (1), NSY (1), SDR and MoCo/ABA3 genes which were downregulated in droughted plants (Fig.5.6-7c). Thus girdling tended to have opposing effects on gene expression in the ABA biosynthesis and metabolism pathways in well-watered and droughted plants.

Expression of root and leaf ABA signalling genes did not change after one day, independently of soil moisture (Fig. 5.8a, d). In roots of girdled plants, soil drying just up-regulated the PYL2 gene on Day 2 (Fig. 5.8e) and the PYL2 and PP2C genes

on Day 3, while in roots of intact plants, soil drying down-regulated the PYL2 and PP2C genes on Day 3 (Fig. 5.8f). Two days of soil drying up-regulated PYL2, PP2C 3 and ABF/ABRE genes in leaves of intact plants, while down-regulating the PP2C 3, SnRK2 and ABF/ABI5 genes in leaves of girdled plants (Fig. 5.8b). Three days of soil drying up-regulated all analysed ABA signalling genes in leaves of intact plants, except for PP2C that was down-regulated. In contrast, all analysed signalling genes, except for PP2C and ABF/ABI5 that did not change, were down-regulated in leaves of girdled plants (Fig. 5.8c). Thus soil drying had greater effects on the expression of ABA signalling genes in leaves than roots of intact plants, while droughted girdled plants up-regulated ABA signalling genes in the roots after 3 days.

One day after girdling was applied, the roots up-regulated all analysed ABA signalling genes, except for PP2C that was down-regulated (Fig. 5.8d). All these genes remained up-regulated in droughted girdled plants on Day 2, while there was no change in PYL2 and ABF/ABI5 gene expression in well-watered girdled plants (Fig. 5.8e). The same pattern was observed three days after girdling was applied, with all analysed genes up-regulated in roots of droughted plants, while in well-watered plants just PP2C 3 remain unchanged (Fig. 5.8f). In leaves, girdling up-regulated the ABF/ABI5 gene on Day 1, while down-regulating the PP2C 3 gene (Fig.5.8a). After two days, girdling up-regulated all analysed genes, except for PP2C and SnRK2 3, in leaves of well-watered plants. After three days, girdling up-regulated all analysed genes in leaves of well-watered plants, while PP2C 3 was up-regulated and ABF/ABRE down-regulated in leaves of droughted plants (Fig.5.8c). Thus, girdling up-regulated ABA signalling genes in the roots throughout the

experiment irrespective of soil moisture status, while in the leaves ABA signalling genes were more expressed in well-watered plants.

Thus, girdling clearly increased gene expression in the ABA biosynthesis, catabolism and signalling pathways in the roots compared to the leaves, before any physiological or molecular effects of soil drying were detected. Between Days 2 and 3, the interaction between girdling and drought stress stimulated the expression of ABA biosynthesis genes in the roots, with different patterns of gene regulation (up- and down-) between root and leaf tissues. In girdled plants, soil drying seemed to induce a faster and prolonged (ABA biosynthesis) gene expression response in the roots than in the leaves.

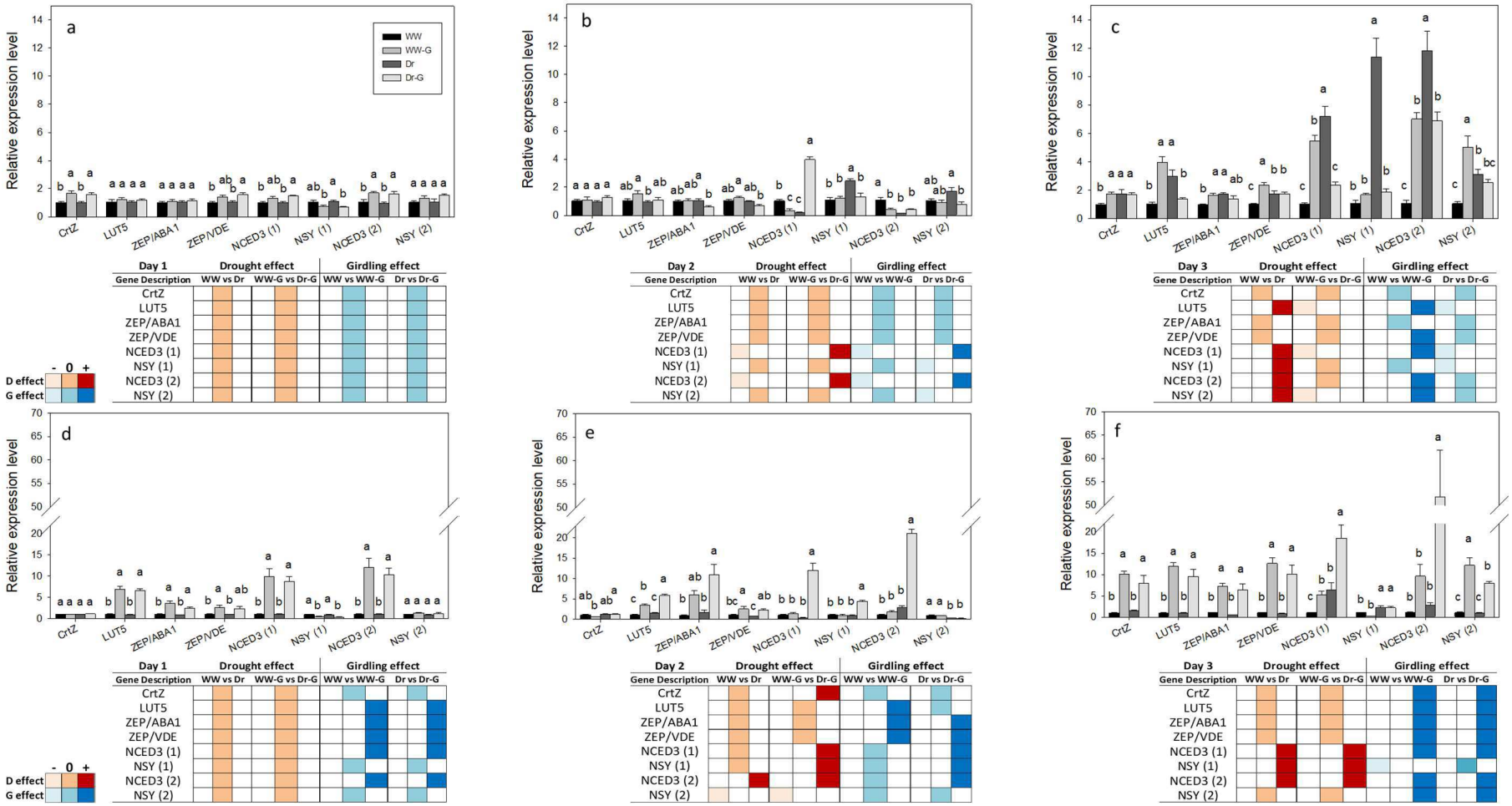


Figure 5.6. Real-time quantitative PCR expression level of ABA biosynthesis genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of ABA biosynthesis under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

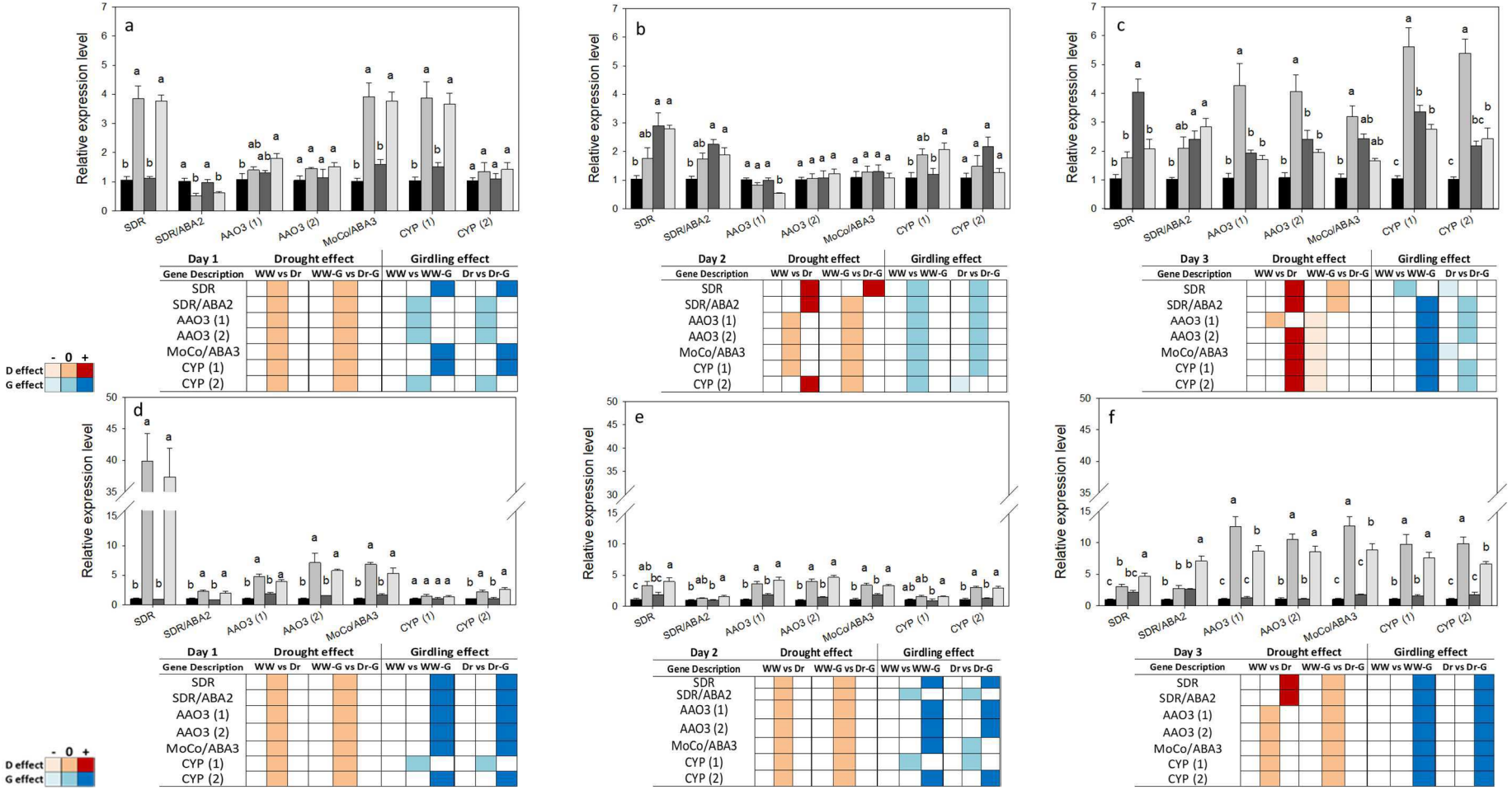


Figure 5.7. Real-time quantitative PCR expression level of ABA biosynthesis and catabolism genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of ABA biosynthesis and catabolism under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

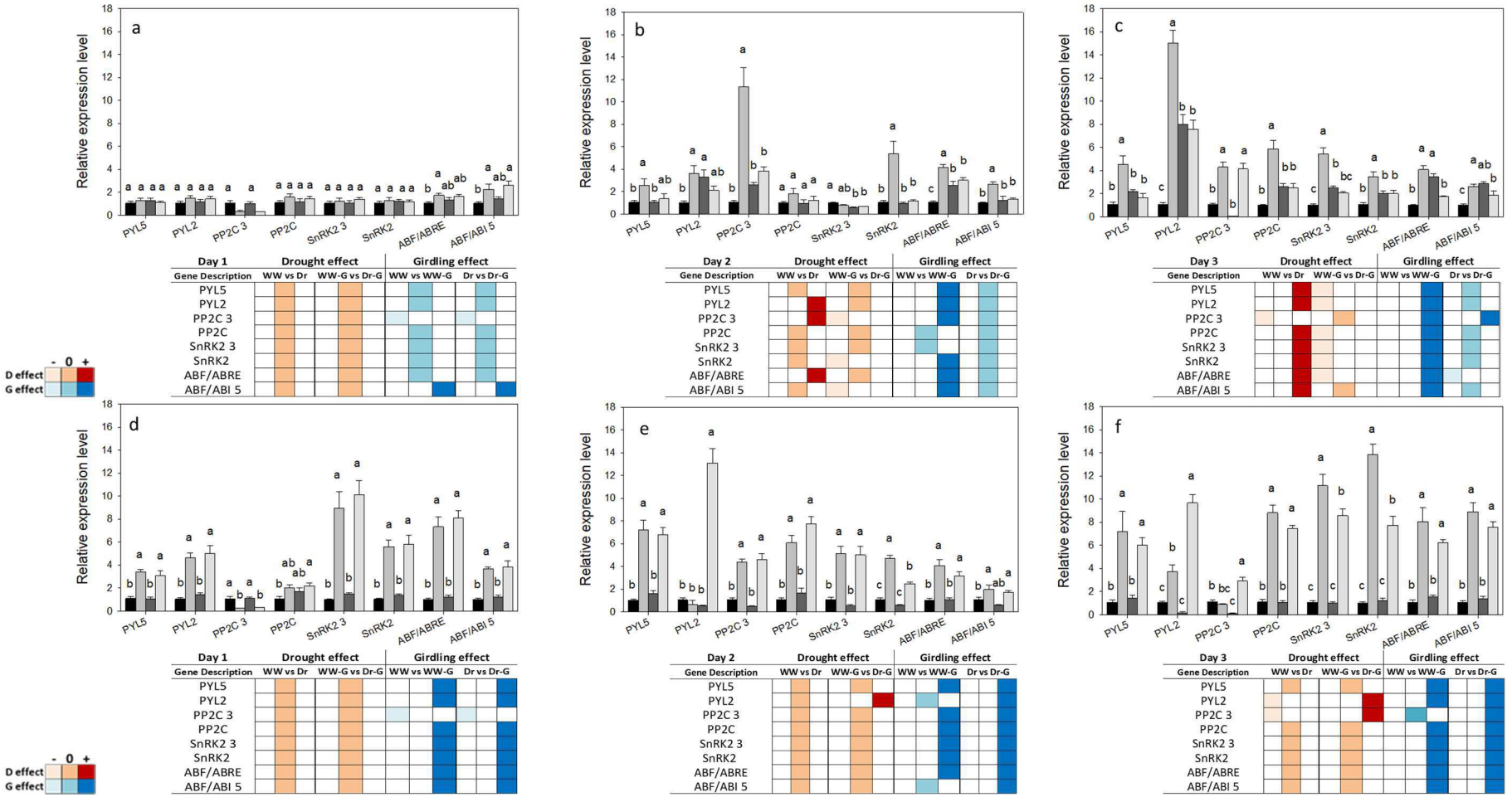


Figure 5.8. Real-time quantitative PCR expression level of ABA signalling genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of ABA signalling under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

5.3.4 Soil drying and girdling effects on JA biosynthesis, catabolism and signalling related genes

Soil drying did not affect leaf and root gene expression in the JA biosynthesis pathway of intact and girdled plants on Day 1 (Fig. 5.9-10a, d). Two days of soil drying up-regulated α-linolenic acid (2), 13(S)-HpOTrE, 12-OPDA (3), OPCL1 and Jasmonate (2) genes in the roots of intact plants, while none of these genes were affected in girdled plants (Fig. 5.9-10e). Three days of soil drying up-regulated α-linolenic acid (2), 12-OPDA (1 and 2), OPCL1 and both Jasmonate (1 and 2) genes in the roots of intact plants, while the 12,13-EOTrE and 12-OPDA (2) genes were down-regulated in the roots of girdled plants (Fig. 5.9-10f). Thus soil drying up-regulated gene expression in the JA biosynthesis pathway in roots of intact plants, while opposing effects occurred in roots of girdled plants.

Two days of soil drying up-regulated α-linolenic acid (2), 12,13-EOTrE and 12-OPDAs (2 and 3) genes in leaves of intact plants, and up-regulated α-linolenic acid (1) and 12-OPDA (1) genes in girdled plants (Fig. 5.9-10b). Three days of soil drying caused intact and girdled plants to show opposite responses in gene expression in the JA biosynthesis pathways. In intact plants, soil drying up-regulated most of the analysed genes except for α-linolenic acid (1), 13(S)-HpOTrE, 12,13-EOTrE, 3-Oxo-OPC8-CoA and Jasmonate (1) which showed no response, while in girdled plants most of the genes were down-regulated except for α-linolenic acid (2), 13(S)-HpOTrE, OPC8-CoA and 3-Oxo-OPC8-CoA genes (Fig. 5.9-10c). Thus soil drying up-regulated genes in the JA biosynthesis pathway in leaves of intact plants, while

girdled plants showed more variable foliar gene expression (up- and down-regulation on Days 1 and 2 respectively).

Compared to intact plants, girdling up-regulated α-linolenic acid (1), 13(S)-HpOTrE, 12-OPDA (4), OPC8-CoA, MFP2 and Jasmonate (2) genes in roots of well-watered plants but down-regulated the 12-OPDA (2) gene within one day (Fig. 5.9-10d). After two days, girdling up-regulated almost all analysed genes (except for the 12,13-EOTrE and 12-OPDAs (1 and 2) genes that didn´t change) in roots of well-watered plants and down-regulated the 3-Oxo-OPC8-CoA gene. Girdling up-regulated α-linolenic acid (1 and 2), 12-OPDA (4), OPC8-CoA, MFP2 and Jasmonate (1) genes in roots of droughted plants and down-regulated the 12,13-EOTrE, 12-OPDA (1) and 3-Oxo-OPC8-CoA genes (Fig. 5.9-10e). Three days after girdling, all genes except the α-linolenic acid (1) gene (that did not change) remained up-regulated in roots of well-watered plants, while girdling didn't change α-linolenic acid (1 and 2), 13(S)-HpOTrE, 12,13-EOTrE and 12-OPDAs (1 and 2) gene expression in droughted plants (Fig. 5.7-8f). Throughout the experiment, girdling affected more of the analysed JA-biosynthesis genes in roots of well-watered plants than those exposed to drying soil.

Within one day, girdling up-regulated almost all analysed genes in the leaves, except the α-linolenic acid (1), 13(S)-HpOTrE, OPC8-CoA and MFP2 genes that did not change (Fig. 5.9-10a). Two days after girdling, only the 12-OPDA (3) gene was up-regulated and the α-linolenic acid (1), 12,13-EOTrE and 12-OPDA (1) genes were down-regulated in leaves of well-watered plants, while only the α-linolenic acid (2) and 12,13-EOTrE genes were down-regulated in leaves of droughted plants (Fig.

5.9-10b). Three days after girdling, all analysed genes were up-regulated in leaves of well-watered plants except the α-linolenic acid (1) gene that did not change, while only the 12-OPDA (4) gene was downregulated in leaves of droughted plants (Fig. 5.9-10c). Thus girdling increased foliar gene expression in the JA biosynthesis pathway to a greater extent in well-watered plants than those exposed to drying soil.

Soil drying had no effect on root and leaf JA signalling genes on Day 1 (Fig. 5.11a, d). In the roots, two days of soil drying up-regulated MYC2 (2) in intact plants, and up-regulated JAZ and down-regulated MYC2 (2) in girdled plants (Fig. 5.11e). Three days of soil drying up-regulated MYC2 (2) in roots of intact plants, but had no significant effect in roots of girdled plants (Fig. 5.11f). In the leaves, soil drying up-regulated JAR (1) and MYC2 (2) genes in intact plants after two days, while there was no change in girdled plants (Fig. 5.11b). After three days, soil drying up-regulated JAR (1 and 2), COI1 and MYC2 (2) in intact plants, while JAZ was up-regulated and COI1 down-regulated in girdled plants (Fig. 5.11c). Thus, continued soil drying up-regulated some genes in the JA signalling pathway in roots and leaves of both intact and girdled plants.

Compared to intact plants, girdling up-regulated JAR (1 and 2), COI1 and MYC2 (2) genes in the roots within one day (Fig. 5.11-d). After two days, girdling up-regulated all analysed genes in the roots of well-watered plants except JAZ that did not change, while JAR (2) and COI1 were up-regulated and MYC2 (2) down-regulated in roots of droughted plants (Fig. 5.11e). After three days, girdling caused all analysed genes to be highly expressed in the roots, independently of soil moisture (Fig.

5.11f). In the leaves, girdling up-regulated JAR1 (2) and MYC2 (2) after one day (Fig. 5.11a). After two days, girdling had no effect in well-watered plants while JAR1 (1) and MYC2 (2) were down-regulated in droughted plants (Fig. 5.11b). After three days, girdling up-regulated JAR1 (2) and MYC2 (2) in leaves of well-watered plants, while JAZ and down-regulated COI1 were up-regulated in leaves of droughted plants (Fig. 5.11c). Independently of soil moisture, girdling highly up-regulated JA signalling genes in the roots, but attenuated the expression of JA signalling genes in the leaves.

Taken together, girdling clearly increased the expression of JA biosynthesis and signalling genes in roots and leaves within one day. Three days after girdling, expression of these genes was attenuated in the leaves, but still promoted in the roots. By the end of the experiment, soil drying induced a greater response in intact plants than girdled plants in both tissues Combining both girdling and soil drying treatments may lead to more changes in the analysed genes in roots than in leaves, promoting root-to-shoot co-ordination.

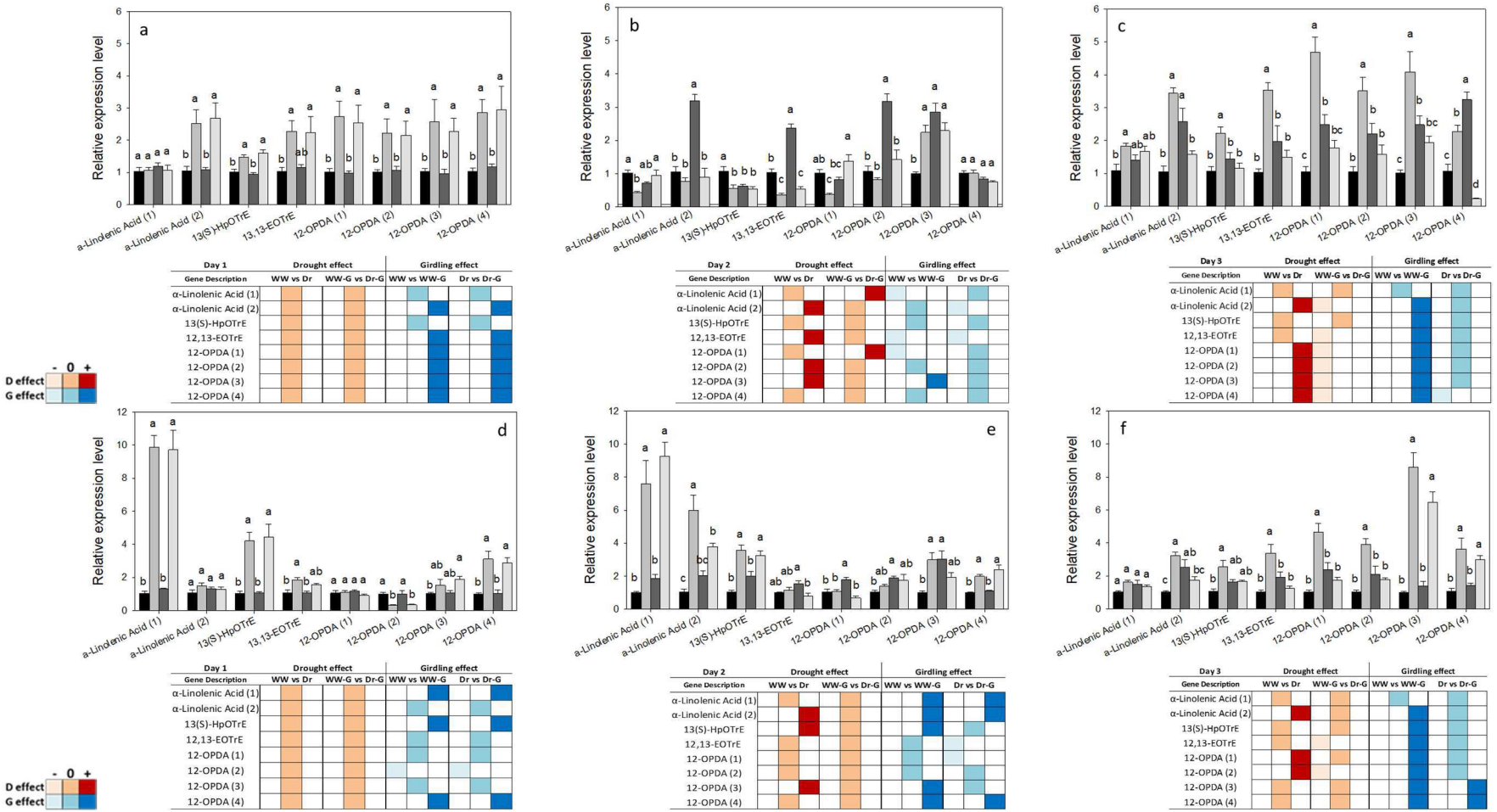


Figure 5.9. Real-time quantitative PCR expression level of JA biosynthesis genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of JA biosynthesis under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

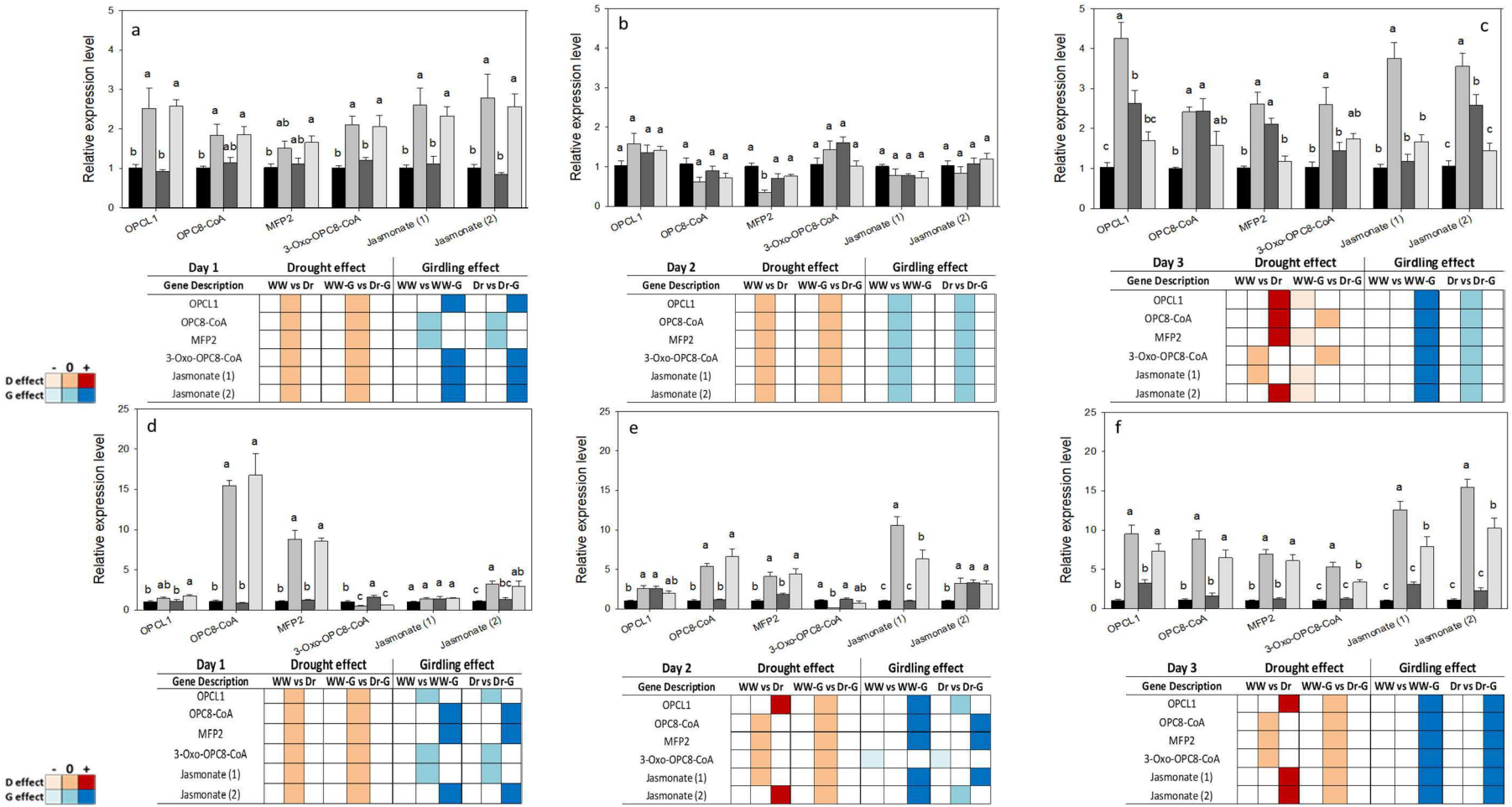


Figure 5.10. Real-time quantitative PCR expression level of JA biosynthesis genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of JA biosynthesis under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

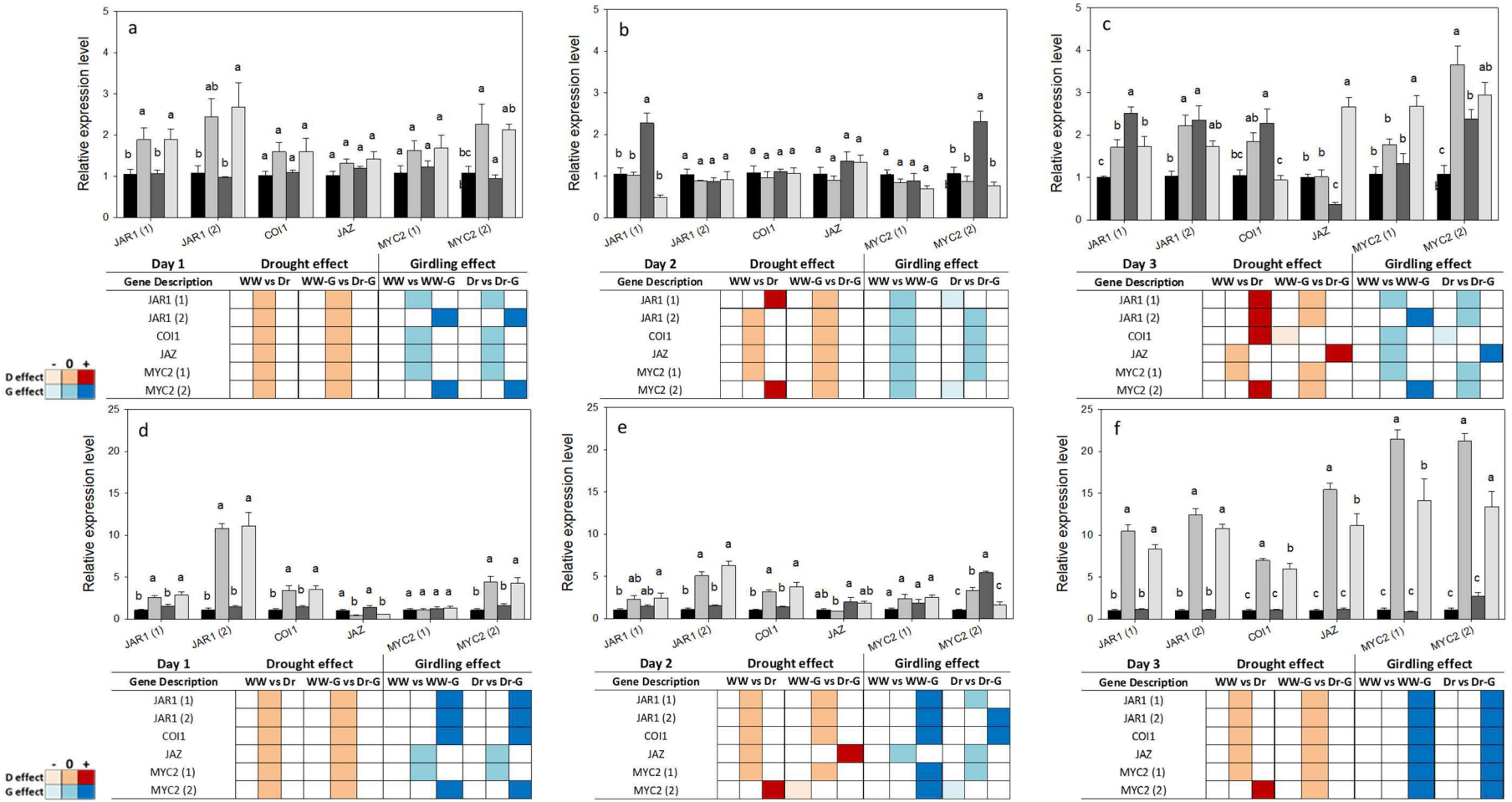


Figure 5.11. Real-time quantitative PCR expression level of JA catabolism and signalling genes in leaf (a-c) and root (d-f) tissue of well-watered intact plants (WW), well-watered girdled plants (WW-G), droughted intact plants (Dr) and droughted girdled plants (Dr-G) from Days 1 to 3 after treatments. Vertical bars indicate relative expression level mean ± s.e. (n=6) compared to well-watered intact plants (WW). Different letters indicate significant differences ($P < 0.05$) according to the Tukey´s test on each gene. Relative expression for the analysed genes of JA signalling under drought (red) and girdling (blue) effects. Gradient colours indicate log2 fold-change by down-regulated (-), no-change in (0) and up-regulated (+) gene expression of each comparison.

5.4 Discussion

Soil drying decreased g_s independently of girdling, while girdling caused partial stomatal closure of well-watered plants without affecting leaf water potential and soil water content (Fig. 5.3). Girdling increased the DEGs (up- and down-regulated) in roots of well-watered and droughted plants 3 days after the treatments were imposed (Fig. 5.4), which could reflect the importance of root gene expression in stimulating root-to-shoot signalling.

Foliar ABA and JA accumulation of girdled plants, perhaps in response to enhanced expression of ABA and JA biosynthesis genes, caused partial stomatal closure within 26 hours, and further closure (Fig. 4.2f-g, m-n) as the soil dries (Murata *et al.*, 2015; de Ollas *et al.*, 2018). Alternatively, upregulation of ABA and JA biosynthesis genes in the roots, and subsequent root-to-shoot hormone transport could explain foliar hormone accumulation and hence stomatal closure.

ABA genes response to girdling

Girdling upregulated more genes in the ABA biosynthesis pathway in roots than in leaves within 26 hours, prior to any stomatal closure or leaf water potential decreases caused by soil drying (Fig. 5.3; Fig. 5.6-7a, d). At this time, the rate-limiting NCEDs and CYP genes in the biosynthesis and catabolism pathway respectively were expressed (Zheng *et al.*, 2012; Ji *et al.*, 2014; Sussmilch *et al.*, 2017). Two days after girdling, more ABA-related genes were upregulated in the roots than in the leaves (Fig. 5.6-8b, e), but the NCED genes were only upregulated in roots of droughted girdled plants. This suggests that up-regulating the first steps

(NCED genes) of ABA biosynthesis is critical in determining ABA status, since at the end of the experiment both roots and leaves up-regulated the majority of the genes selected (Okamoto *et al.*, 2009; Boursiac *et al.*, 2013). By the end of the experiment, roots of both well-watered and droughted girdled plants had the highest expression level in the ABA biosynthesis and catabolism pathways. In contrast, well-watered girdled plants had increased foliar expression level of these genes only on Day 3, suggesting that root ABA synthesis (and transport to the shoot), in addition to the disruption of basipetal shoot-to-root ABA signalling caused by girdling, can allow foliar ABA accumulation prior to ABA biosynthesis.

In addition, root and leaf expression of ABA biosynthesis genes in well-watered girdled plants was almost completely up-regulated compared to well-watered intact plants, while under drought conditions only roots showed higher expression in girdled plants. Thus girdling induced a higher and prolonged gene expression of the ABA signalling pathway in roots compared to leaves.

Biological activity of any hormone accumulation is suggested by measuring expression of signalling-related genes, where most of these genes were up-regulated in roots of girdled plants throughout the entire experiment (Fig. 5.8; Zheng *et al.*, 2012; Ji *et al.*, 2014). Expression of these signalling genes also increased in the leaves of both intact and girdled well-watered plants (Windsor and Zeevaart, 1997; Priest *et al.*, 2006). Thus girdling stimulated ABA synthesis and perception-related genes in roots, suggesting that shoot-to-root signalling ordinarily suppresses ABA biosynthesis and response in roots of intact plants.

ABA genes response to soil drying

As the soil dries, expression of ABA biosynthesis genes in roots of intact plants was attenuated after an initial increase, whereas leaves up-regulated more genes in the last steps of the synthesis (SDR-related genes) and catabolism (CYP (2)) pathways. While NCED genes were more promoted in both roots and leaves of droughted girdled plants than in intact plants, SDR genes were more expressed in leaves of intact plants than in girdled plants exposed to drying soil (Windsor and Zeevaart, 1997; Priest et al., 2006; Ma *et al.*, 2018). Thus, by the end of the experiment, based on relative changes in gene expression, leaves may be the main organ to synthesise and degrade ABA compared to roots (Manzi *et al.*, 2015; Castro *et al.*, 2019). In contrast, withholding water to girdled plants down-regulated ABA synthesis and catabolism genes in leaves, which may explain decreased foliar ABA accumulation compared to intact plants (Fig. 4.2e). In the roots, soil drying up-regulated NCED genes (Shinozaki and Yamaguchi-Shinozaki, 1997; Jia *et al.,* 2002) in girdled plants, stimulating root tissue ABA accumulation between Day 2 and 3, consistent with the root ABA accumulation seen in Chapter 3 (Castro *et al.,* 2019) and Chapter 4 (Fig. 4.2l).

For intact plants, leaf gene expression in the ABA signalling pathway was higher than root gene expression three days after withholding water (Fig. 5.8). However, in roots of droughted girdled plants, PYL2 and PP2C 3 genes were up-regulated, suggesting that the relationship between PYR/PYL receptors and class A PP2C enhanced ABA perception, which could promote root growth in regions with low

water potential as in the *Arabidopsis* ABA-hypersensitive pp2c quadruple mutant (Antoni *et al.,* 2013; Merilo *et al.,* 2013; Yin *et al.,* 2016).

JA genes response to girdling

Girdling increased the expression of JA biosynthesis and catabolism genes similarly in roots and leaves within 26 hours of treatments. Moreover, girdling induced higher expression of 12-OPDAs and Jasmonate (1 and 2) genes in leaves compared to roots (Fig. 5.9-11a, d; Koo *et al.,* 2009; Wasternack and Song, 2017), consistent with leaf and root JA accumulation seen in Chapter 4 (Fig. 4.2f, m). Two days after girdling, all genes selected in the JA biosynthesis pathway were up-regulated in the roots, while leaves did not show any difference in expression level compared to intact plants (Fig. 5.9-10b, e). At this time, the lower leaf [JA] of girdled plants (Fig. 4.2f) could suggest that stimulation of root JA biosynthesis was not accompanied by increased root-to-shoot JA signalling. At the end of the experiment, girdling still promoted JA biosynthesis and catabolism genes in roots and leaves of well-watered plants, and in roots of plants exposed to drying soil (Fig. 5.9-10d, f). Furthermore, roots of girdled plants up-regulated both JAR1 genes selected (which promote synthesis of the active form JA-Ile) during the entire experiment, while leaves just up-regulated JAR1 (2) on Day 1 and 3 (Fig. 5.11). This highlights a similar paradigm of systemic JA signalling (eg. JA production in wounded tissues is transported to, and sensed by, distal leaves -Schilmiller and Howe, 2005) at least within 26 hours of girdling, whereas girdled roots were unable to decrease gene expression due to the lack of communication with the shoot, and a possible subsequent cross-talk with ABA receptors (PYR/PYL) (Aleman *et al.,* 2016).

Roots of girdled plants up-regulated JA signalling genes throughout the experiment compared to intact plants, and in comparison to leaves of droughted girdled plants (Fig. 5.11). Leaves up-regulated MYC2 genes on Days 1 and 3 which realised JA signalling, while from Day 2 both MYC2 genes were up-regulated in roots of girdled plants. In addition, soil drying down- and up-regulated the COI1 and JAZ genes respectively in leaves on Day 3, suggesting an increase in transcription factor (JAZ) response would enhance the JA response (Fonseca *et al.,* 2009; Pauwels and Goossens, 2011; Roberts *et al.*, 2016). Thus in well-watered girdled plants, roots were still promoting JA hormone and signalling genes, while leaves of droughted plants had down-regulated expression of JA synthesis genes, but were still responding to JA perception (up-regulation of signalling genes), which may cause a consistent stomatal closure response independently of girdling.

JA genes response to soil drying

After withholding water for two days, roots of intact plants had up-regulated the first steps of JA biosynthesis, such as 12-OPDAs genes, consistent with root JA accumulation at this time (Fig. 4.2m). Gene expression in roots may have been systemically regulated, since soil drying induced a greater response in roots of intact plants than girdled plants on Days 2 and 3 respectively (Fig. 5.9-10d, f; Dave and Graham, 2012). Since roots of girdled plants did not up-regulate JA biosynthesis and catabolism genes, this would explain their limited root JA accumulation in response to drying soil.

Similarly, as the soil dries, intact plants systemically up-regulated JA biosynthesis and catabolism genes in the leaves throughout the experiment, consistent with leaf

JA accumulation (Fig. 4.2f). In contrast, leaves of girdled plants down-regulated the majority of the genes selected in the JA biosynthesis pathway (Fig. 5.9-10a, c), explaining their limited leaf JA accumulation by the end of the experiment. Thus leaves of intact plants accumulated 1.5-fold higher JA levels than well-watered plants in response to soil drying (Fig. 4.2f) which is qualitatively similar to previous soil drying experiments in tomato (de Ollas et al., 2018), while leaves of girdled plants did not accumulate JA.

Leaves and roots showed similar trends in JA biosynthesis, catabolism and signalling genes in intact plants, with increased expression as the soil dries (Fig. 5.11). While roots of girdled plants up-regulated the transcription factor (JAZ) on Day 2, leaves up.regulated it on Day 3. The majority of JA signalling genes were not changed in girdled plants as the soil dries, as with similar up-regulated gene expression of both well-watered and droughted plants (Pauwels and Goossens, 2011; Roberts *et al.*, 2016).

In conclusion, ABA and JA accumulation were related to the expression of biosynthesis, catabolism and signalling genes of both hormones. Combining both girdling and soil drying treatments caused quicker changes in root (up-regulation) than leaf (down-regulation) expression of JA-related genes (Day 1). Higher foliar up-regulation of JA biosynthesis genes increased leaf JA accumulation in distal leaves concurrent with stomatal closure within 24 hours. Meanwhile, increased expression of ABA biosynthesis genes, throughout the experiment, may regulate ABA accumulation in both tissues as the soil dries independently of girdling. Increased expression of ABA biosynthesis genes in girdled plants (relative to intact

plants) was inconsistent with their lower drought-induced ABA accumulation, suggesting important roles for root-to-shoot transport and/or ABA catabolism in fine regulation of tissue ABA concentrations. Thus local hormone biosynthesis (as indicated by changes in tissue gene expressions - Yin *et al.*, 2016) or/and long-distance hormone transport may decrease and/or increase JA and ABA concentrations in different tissues.

Chapter 6 – General Discussion

6.1 Stomatal conductance was better explained by xylem sap ABA concentration than leaf water status as the soil dries

Stomatal conductance is one of the most important physiological parameters to consider for breeding drought-tolerant genotypes, since stomatal closure could restrict photosynthesis thereby limiting soybean yields (Bunce, 1977). Alternatively, prompt stomatal closure following exposure to drying soil early in vegetative development could conserve water, making more water available to the plant during the critical grain filling stages (Tardieu, 2013). Therefore it is necessary to understand the regulation of stomatal conductance. Different signals could cause stomatal closure, even though there are considerable interactions between signalling systems (eg. Tardieu and Davies 1992; Pantin *et al.,* 2013). Whereas loss of leaf turgor (a hydraulic signal) can stimulate a biochemical (ABA accumulation) signal (Pierce and Raschke, 1980; Buckley, 2005; Christmann *et al.*, 2007), some studies have shown that a putatively root-sourced biochemical signal can mediate stomatal closure prior to any change in leaf water relations (Davies and Zhang, 1991; Liu *et al.,* 2003a; 2005c). Whether there is genotypic variation in such stomatal regulation within a crop species, and soybean is particular, has attracted little attention.

In Chapter 2, several soybean (Williams 82, C08, C12, LH1 and LH2) genotypes were allowed to dry the soil. The rate of soil drying differed between genotypes (Fig. 2.1),

with Williams 82 drying the soil slower due to its lower leaf area (Table 2.3). Thus Williams 82 maintained higher stomatal conductance and leaf water potential values than the other genotypes as it was exposed to higher soil water availability (Fig. 2.2 and 2.3). Nevertheless, all genotypes presented the same relationship between stomatal conductance or leaf water potential and soil moisture (Fig. 2.5 and 2.6; Hossain *et al.,* 2014), suggesting limited variation in plant water relations responses to drying soil. However, genotypic variation was observed in the relationship between stomatal conductance and leaf water potential of the second trifoliate leaf (Fig. 2.10b, d; Gilbert *et al.,* 2011), where C08, LH1 and LH2 were more sensitive to Ψ_{leaf} than W82 and C12. In contrast, in the first trifoliate leaf, there was a common relationship across all genotypes between stomatal conductance and leaf water potential (Fig. 2.10a, c). Thus leaf age may affect stomatal responses to leaf water status (Oosterhuis *et al.,* 1987). Overall, there were no genotypic differences in stomatal responses to soil water availability, and limited genotypic differences in stomatal responses to leaf water potential.

However, there was pronounced genotypic variation in ABA relations only in Experiment 1, with C12 showing lower [ABA] as the soil dries (Fig. 2.7). Also, the relationship between stomatal conductance and leaf xylem sap [ABA] differed between genotypes, with C12 more sensitive than W82 and C08 (Fig. 2.8). In addition, the relationship between leaf xylem sap [ABA] and leaf water potential differed between genotypes, with C12 less sensitive to leaf water potential changes (Fig. 2.9). To summarise, the C12 genotype had lower xylem sap [ABA] as the soil dried (Fig. 2.7a) or Ψ_{leaf} declined (Fig. 2.9a), but its stomata were more sensitive to

ABA (Fig. 2.8a). Thus genotypes may differ in regulation of stomatal closure, being sensitive to very small (soil drying-induced) increases in ABA concentration or controlling their stomata by redistributing existing ABA, as with two different cultivars (Cacahuate-72 and Michoacan-12A3) of *Phaseolus vulgaris* L. This study suggested that genotypic differences in the timing of stomatal closure may be caused by different `root-sourced signal strength promoting differences in xylem ABA and bulk leaf ABA concentration during soil drying (Trejo and Davies, 1991).

In contrast, all genotypes studied showed variation in the relationship between leaf tissue [ABA] and soil water content, with C08 and LH2 accumulating higher leaf [ABA] as the soil dries (Fig. 2.7). In addition, the relationship between leaf tissue [ABA] and stomatal conductance differed between genotypes, with C12 and LH1 more sensitive than W82 and LH2 (Fig. 2.8). Furthermore, all genotypes presented genotypic variation in the relationship between leaf tissue [ABA] and leaf water potential, where C08 and LH2 were more sensitive than W82, C12 and LH1 (Fig. 2.9). Also, genotypic differences in leaf tissue [ABA] were observed a day earlier than the differences in xylem sap [ABA] under progressive soil drying conditions (Fig. 2.4). This suggests there are greater genotypic differences in leaf tissue [ABA] than genotypic variation in Ψ_{leaf} at this time (Fig. 2.3-2.4). Thus ABA accumulation slightly better explained (higher r^2 values) variation in stomatal conductance than Ψ_{leaf} in the same leaf as the soil dries (Fig. 2.8, 2.10).

Furthermore, in all genotypes, $[X\text{-}ABA]_{leaf}$ was better correlated with g_s (Fig. 2.8a, c) than was $[ABA]_{leaf}$ (Fig. 2.8b, d), even if the experimental design required measurements in leaves of different ages. This suggests that the stomata could

discriminate between root-sourced and leaf-sourced ABA, accentuating the need for an effective mechanism of metabolizing or compartmentalizing the ABA that arrives via the xylem (Zhang and Davies, 1989a; Davies and Zhang, 1991).

Two paradigms arise for describing how ABA is moved throughout the plant. One idea is that the leaf synthesises ABA (leaf-sourced paradigm) in response to decreased cellular turgor to regulate stomatal conductance, and that some of this ABA is transported to the roots (Bauer *et al.,* 2013; McAdam and Brodribb, 2016; Sack *et al.,* 2018). Alternatively, roots are the first organs to lose turgor during soil drying, generating a root-to-shoot signal to the leaves to initiate stomatal closure (root-sourced paradigm). Since ABA is a key regulator of stomatal movement, different studies have aimed to determine where it is produced and transported within the plant. To discriminate these potential sources of ABA appearing in the xylem, girdling (which disrupts shoot-to-root ABA transport) were conducted.

6.2 Shoot to root ABA transport has a predominant role in regulating stomatal responses to girdling and soil drying

To better understand the physiological importance of the different long-distance ABA signalling paradigms, girdling was applied to eliminate shoot-to-root ABA transport, altering plant physiology such as stomatal conductance, root tissue [ABA], and leaf, shoot and root xylem sap [ABA] (Fig. 3.2-3.5). There was a unifying relationship between stomatal conductance and leaf xylem [ABA] independently of whether the plants were girdled (Fig. 3.6b), while stomatal conductance and leaf water potential were only correlated in intact plants (Fig. 3.6a). Thus it is difficult to sustain the argument that leaf water potential is the primary regulator of stomatal

responses, and that leaf water status requires a "second messenger" (ABA) to exert a physiological effect. Moreover, there are examples in the literature where higher stomatal conductance is associated with lower leaf water potential, presumably because high transpiration rates decrease leaf water status (Jones 1983; Kudoyarova *et al.,* 2007)

In addition, leaf, shoot and root xylem sap ABA concentration was better correlated with soil water content than tissue water status (Table 3.2), suggesting that ABA accumulation promotes stomatal closure prior to any change in Ψ_{leaf} as the soil dries (Fig. 3.6c; Liu *et al.*, 2005c), and such ABA accumulation acts to maintain Ψ_{leaf} (Fig. 3.3a; Mitchell *et al.,* 2016). It is important to determine whether shoot xylem ABA concentration regulates foliar ABA accumulation, since stomatal closure in some species required a xylem ABA concentration 100 times higher than that occurring in droughted plants (Munns and King, 1988).

Girdling attenuated root ABA accumulation throughout the experiment independently of soil moisture (Fig. 3.5; Manzi *et al.,* 2015), although the roots of droughted girdled plants still accumulated higher ABA concentrations than the roots of well-watered girdled plants on Day 3, suggesting that both local ABA synthesis and shoot-to-root signalling regulated root [ABA] (Davies and Zhang, 1991). However it is not clear whether the reduced ability of roots for *de novo* ABA synthesis in response to soil drying is due to a limited supply of an ABA precursor from the shoot, since there was no direct correlation between carotenoid abundance and root ABA accumulation in some studies (Ren *et al.*, 2007; Manzi *et al.,* 2016).

Girdling attenuated root tissue and shoot xylem ABA concentration as the soil dries, while both these variables increased continuously in intact plants (Fig. 3.4). Furthermore, girdling attenuated shoot hormone export to the root, thus enhancing ABA accumulation in the aerial parts as the soil dries (Fig. 3.7). This suggests that ABA recycles between shoot and root via the phloem, thus the shoots make a variable contribution to root-to-shoot ABA signalling (Wolf *et al.*, 1990; Slovik *et al.*, 1995).

Girdling decreased stomatal conductance while increasing leaf xylem [ABA] independent of soil moisture (cf. Fig. 3.2, Fig. 3.4a). Thus ABA transported from the shoot to the roots via the phloem will not only determine root ABA accumulation but also leaf xylem [ABA], since leaves had much higher [ABA] than roots (Liu *et al.*, 2005c; Manzi *et al.*, 2015; McAdam *et al.*, 2016b). Thus export of shoot-sourced ABA attenuates foliar [ABA] accumulation and was necessary to maximise root accumulation in response to soil drying. In addition, the consistent response between leaf xylem [ABA] and stomatal conductance in both well-watered and droughted girdled plants (Fig. 3.6b) suggests that girdling had no long-term influence on ABA-induced stomatal closure. Nevertheless, girdling may induce a short-term wounding response and the phytohormonal impacts of this were investigated next (Chapter 4).

6.3 Multiple plant hormones are involved in stomatal responses to soil drying and girdling

Stomatal closure in response to girdling on Day 1 (Chapter 3) was again observed within 26 hours in Chapter 4. This closure was independent of shoot and root water

status and soil water content (these were equivalent in girdled and intact plants - Fig. 4.1a, c, d), and may represent an early wounding response caused by changes in leaf hormone concentrations such as iP, tZ, GA3, ABA, JA and SA (Fig. 4.2a-g). Girdling increased leaf and shoot xylem [ABA] levels of well-watered plants in Chapter 3 by 2.5-fold (Fig. 3.4) and leaf tissue [ABA] by 2-fold in Chapter 4 (Fig. 4.2e), as well as in citrus leaves after water stress treatment was imposed (Manzi *et al.,* 2015).

Furthermore, girdling drastically increased leaf JA concentrations by 5.5- and 6-fold, after 4 and 26 hours respectively, in Chapter 4 (Fig. 4.2f). Mechanically wounding the stem caused stomatal closure of unwounded leaves, just as in other studies where stomatal closure occurred in mechanically wounded leaves and also in unwounded distal leaves in *Arabidopsis* within 2h (Foster *et al.,* 2019).

Both ABA and JA accumulation were significantly inversely correlated with stomatal conductance (Table 4.2), suggesting both hormones could act as antitranspirants (Arbona and Gómez-Cadenas 2008; Savchenko *et al.*, 2014; de Ollas *et al.,* 2018). However, girdling did not alter stomatal sensitivity to these hormones (Fig. 4.5). Nevertheless, the sustained decrease in g_s of well-watered girdled plants after Day 3 coincides with increased leaf xylem ABA concentration observed in Chapter 3 (Fig. 3.2, 3.4a). Moreover, girdling had no long-term influence on ABA-mediated stomatal closure since the relationship between leaf xylem ABA concentration and stomatal conductance was consistent independent of girdling (Fig. 3.6b). Thus stomatal closure in the first 26 hours could be a response to rapid leaf JA accumulation caused by wounding, which could stimulate ABA synthesis for further

sustained stomatal closure (Pinheiro *et at.*, 2011; de Ollas and Dodd, 2016; Per *et at.,* 2018).

Girdling induced some changes in root ACC hormone concentrations within 26 h (Fig. 4.2h), with substantial (10-fold) root [ACC] accumulation possibly affecting root-to-shoot ACC signalling (Jackson, 2002; Acharya and Assman, 2009), which may antagonize ABA-mediated stomatal closure (Wilkinson *et al.,* 2012). However, since ACC was not detected in leaf samples, it is not certain whether the precursor of ethylene (ACC) was involved in the root-to-shoot signalling. Further measurements of leaf ethylene evolution may also prove instructive.

Hormone relations during the first 26 hours of the experiment differed from those occurring during the entire experiment. Overall, leaf tZ, GA4 and ABA concentrations were correlated with both stomatal conductance and soil water content (Table 4.4), but as the soil dries only tZ and ABA accumulation were correlated with stomatal closure (Fig. 4.2b, e). Thus in the absence of any treatment effect (soil drying or girdling) on leaf GA accumulation, the relative magnitude of ABA/cytokinin levels may play a role in stomatal movement since tZ antagonises ABA-induced stomatal closure (Wilkinson and Davies, 2002; Hansen and Dörffling, 2003). Furthermore, girdling seems to enhance [tZ] and decrease [ABA] compared to intact plants, since changes in leaf tZ and ABA concentrations were observed as the soil dried (Fig. 4.2c, e). Thus leaf tZ and ABA accumulation may be regulated by shoot-to-root transport, as discussed in Chapter 3.

On the other hand, soil drying increased root GA3 and JA concentrations (Fig. 4.6b, d), making it important to distinguish the contributions of local hormone synthesis

versus shoot-to-root transport as the soil dries. At the same time, girdling affected the correlation between root and leaf concentrations of tZ, ABA and JA during the entire experiment (Table 4.5; Fig. 4.9). Well-watered girdled plants had higher leaf ABA and JA concentrations (Fig. 4.9b, c). Both girdled and non-girdled plants increased leaf and root ABA concentrations, suggesting that co-ordination of root and shoot ABA concentrations was not perturbed by girdling. However, leaf JA concentrations decreased drastically as root JA concentrations increased in girdled plants (Fig. 4.9b), suggesting local regulation of JA status in the absence of shoot-to-root signalling.

Roots of girdled plants were still capable of accumulating ABA as the soil dried by the end of the experiment (Fig. 4l), suggesting that localised root ABA biosynthesis occurred (Speirs *et al.,* 2013). This could enhance root ABA export via xylem to the shoot contributing to stomatal closure (Davies and Zhang, 1990) or re-distribute ABA within the roots to locally promote root growth (Sharp *et al.,* 2004). Therefore of all the different plant hormones studied in this thesis, leaf and root tissue ABA concentrations seem to act as a key regulator of plant water balance.

While the experiments of Chapter 4 go some way to exploring the phytohormonal complexity of plant responses to girdling and soil drying, they do not provide direct evidence of hormone biosynthesis, which was sought in Chapter 5 by measuring gene expression. While multiple genes involved in hormone synthesis were measured, Chapter 5 focused on ABA and JA because of their putative physiological significance at different times during the experiment (Hildmann *et al.,* 1992), and

their contrasting co-ordination of root and leaf hormone concentrations (Fig. 4.9 b, c).

6.4 Girdling upregulated ABA and JA gene expression pathways within one day and ABA gene expression is maintained in roots and leaves as the soil dries

Most selected genes in the ABA biosynthesis, catabolism and signalling pathways were up-regulated in roots of girdled plants before any changes in soil water content were detected (Fig. 5.4-6a, d). At the same time, only two genes in the last part of the ABA biosynthesis (SDR, MoCo/ABA3), one catabolism gene (CYP(1)) and one transcription factor (ABF/ABI 5) were upregulated in the leaves. Compared to the hormone concentrations (Fig. 4.2e, l), root [ABA] decreased and leaf [ABA] increased in girdled plants within 24 hours. In addition, two stimuli could occur in leaves to promote ABA concentration at the same time. By up-regulating the last steps of the ABA biosynthesis and catabolism pathway (Fig. 5.6), these girdled plants could increase foliar ABA concentration. A secondary response is a long-distance effect where roots contribute to foliar ABA accumulation by enhanced root-to-shoot ABA signalling (Davies and Zhang, 1991; Zhang *et al.,* 2012).

By the end of the experiment, those root-expressed genes remained up-regulated in both well-watered and droughted girdled plants (Fig. 5.4-6f), suggesting enhanced root-to-shoot signalling as the soil dries (Fig. 3.7). At this point, almost all genes of the ABA biosynthesis pathway were up-regulated in leaves of well-watered girdled plants (Fig. 5.4-6c), suggesting that girdling stimulated foliar ABA accumulation (Fig. 4.2e) even though basipetal phloem transport was impeded (Zheng *et al.*, 2012; Ji *et al.*, 2014). At this time, both roots and leaves of intact

droughted plants upregulated some key genes in ABA biosynthesis such as NCED (1), NSY (1), SDR and MoCo/ABA3 compared with girdled plants under drought (Shinozaki and Yamaguchi-Shinozaki, 1997; Jia *et al.,* 2002). This girdling-mediated difference in gene expression may explain why girdled plants had lower root and leaf ABA accumulation at the end of the experiment (Fig. 4.2e, l).

With prolonged soil drying, intact plants up-regulated ABA biosynthesis and catabolism genes similarly in leaves and roots at the end of the experiment, although signalling genes were highly expressed in leaves, likely triggering further stomatal closure. This suggests that roots and leaves both play an important role in ABA homeostasis, making it difficult to distinguish directions of root-shoot communication as both tissues seem important (Okamoto *et al.*, 2009; Boursiac *et al.*, 2013). Girdled plants had high root expression of the key biosynthesis (NCEDs) and signalling (PP2C) genes, while foliar gene expression was attenuated in droughted plants compared with well-watered plants. Again, this suggests that roots of girdled plants perceive a soil drying stimulus (Shinozaki and Yamaguchi-Shinozaki, 1997; Jia *et al.,* 2002) that may be important in ABA homeostasis *in vivo*.

On the first day after girdling, JA genes throughout the whole pathway were up-regulated in both leaves and roots, highlighting the up-regulation of 12-OPDAs genes in leaves and JAR1 expression in roots (promoting synthesis of the JA-Ile molecule), either of which could induce stomatal closure (Fonseca *et al.,* 2009; Dave and Graham, 2012; de Ollas *et al.*, 2018). Increased leaf JA concentrations while root JA concentrations did not change on the first day of the experiment (Fig 4.2f, m) belied that both tissues detected a wound response based on gene expression

changes (in response to long-distance signals as the sampled roots and leaves were distal to the girdling position) and that the roots exported jasmonates to the leaves. Thereafter, leaf JA biosynthesis genes of girdled plants, independently of soil moisture, were down-regulated on Day 2, while only well-watered girdled plants genes were up-regulated on Day 3. Thus JA biosynthesis was upregulated in both intact and girdled droughted plants. In contrast, roots of girdled plants (both well-watered and droughted) progressively increased the number of upregulated genes day by day. Thus fluctuations in gene expression closely matched fluctuations in hormone accumulation.

As the soil dries, intact plants up-regulated more JA-related genes in the leaves than the roots, while there was not much difference in gene expression level between roots of girdled plants at different soil moisture status. It seems that leaves down-regulated JA synthesis but were still responding to JA, as the JA signalling genes COI1 and JAZ were down- and up-regulated respectively in girdled plants. Girdling-induced foliar JA accumulation within 26 hours, but this was not sustained by 48 hours (Fig 4.2f), suggesting that initial JA production could be followed by leaf JA homeostasis, where JA production in wounded tissues is transported to, and sensed by, distal leaves (Schilmiller and Howe, 2005). In addition, although both tissues up-regulated JA-related genes throughout the whole biosynthesis pathway, changes in signalling pathway genes suggest that only the leaves respond to hormone accumulation changes, while roots do not perceive feedback signalling from the upper part of the plant, as they could not stop up-regulating genes due to a lack of communication between both organs.

Within one day, girdling clearly increased root and leaf gene expression of the JA biosynthesis pathway. Thereafter, as the soil dries, expression of JA biosynthesis genes in the leaves declined (Fig. 5.7-9a, b, c) while there was moderate (1.8-fold) foliar JA accumulation compared with the 6-fold accumulation observed at 26h (Fig. 4.2f). Thus suggests that even though phloem communication was interrupted (thus preventing shoot-to-root JA transport), root expression of JA biosynthesis genes ultimately lead to increased root-to-shoot JA signalling and some foliar JA accumulation.

In intact plants, foliar expression of ABA biosynthesis genes increased through the experiment (Figure 5.4-6a, b, c), coincident with exaggerated (45-fold) foliar ABA accumulation on Day 3 (Fig. 4.2e), appearing to leave little role for root-to-shoot ABA signalling in modulating foliar ABA homeostasis. In contrast, ABA biosynthesis genes were down-regulated in leaves of droughted girdled plants in comparison with droughted intact plants, consistent with their lower ABA concentration. Thus the relative importance of local hormone biosynthesis *versus* root-to-shoot signalling in regulating leaf hormone levels varies between hormones.

Soil drying up-regulated ABA biosynthesis genes in the roots during the entire experiment, highlighting the first biosynthesis steps in the plastid (e.g. NCEDs) (Fig. 5.4-6d, e, f) coincident with increased root ABA concentrations as the soil dries (Fig. 4.2l). Interestingly, girdled droughted plants had similar gene expression patterns as intact plants, since both had no fold-change difference in the expression level in response to soil drying. At the same time, leaf JA biosynthesis genes expression increased within 24 hours of girdling (Fig. 5.7-9), coinciding with higher foliar JA

concentrations (Fig. 4.2f). Thereafter expression of JA biosynthesis genes decreased in the leaves coincident with lower leaf JA concentrations by the end of the experiment, while roots increased both genes expression and hormone accumulation over the same time. Thus gene expression and hormone concentrations of each tissue followed a similar tendency both analysis throughout the experiment where it could be possible to describe that ABA acts as a long-distance signalling hormone and JA acts as a tissue-dependent hormone.

6.5 Closing remarks

This thesis evaluated the role of leaves and roots in mediating ABA transport and signalling to elicit soil-drying induced stomatal closure in one of the major cultivated crops: soybean. Understanding the relationships between physiological (water potential and stomatal conductance), biochemical (phytohormones) and gene expression changes can inform genotype selection and offers opportunities to enhance water-limited yields.

ABA was a good predictor of water availability, as its leaf xylem sap concentration was highly correlated with stomatal conductance (more so than leaf water status) across different genotypes. Two contrasting paradigms of root-shoot communication provided a theoretical basis to test the relative importance of long-distance transport in regulating hormone concentrations in each tissue (Davies and Zhang, 1991; McAdam *et al.,* 2016a). Several girdling experiments that impeded shoot-to-root hormone transport revealed decreased root ABA accumulation in girdled plants, suggesting an important role of shoot-to-root ABA transport in soybean. As the soil dried, the roots of girdled plants showed increased expression

of ABA biosynthesis genes and ABA accumulation, although less than in intact plants. Thus root ABA concentrations are determined by both local and long-distance processes.

Furthermore, since girdling wounded the stem tissue and disrupted basipetal phloem transport, other hormones such as JA accumulated in the leaves, while others (iP and tZ, GA_3) decreased. These changes may also be involved in regulating stomatal closure within one day of girdling. Such hormone perturbations were transient (except for iP which persisted throughout the experiment) and disappeared 48 h after girdling, by which time their antitranspirant effect was likely replaced by ABA accumulation. These observations suggest considerable hormonal cross-talk in regulating each others accumulation and activity. For example, JA accumulation could induce ABA accumulation in roots (de Ollas *et al.,* 2013), thereby affecting stomatal closure assuming that some of this ABA is exported from the roots. The physiological significance of such hormone interactions can best be tested by evaluating the stomatal responses of double mutants (eg. deficient in both ABA and JA production) to soil drying and/or girdling.

The same pattern was found in the genes expression, where roots and leaves increased and decreased respectively ABA-related genes, but this time, both ABA and JA hormone biosynthesis was stimulated in roots although the phloem communication was interrupted. Thus the plant regulates gene expression in both leaf and root tissues to fine-tune hormone movement throughout the plant to elicit physiological responses.

Since roots upregulated ABA-related genes coincident with root tissue ABA accumulation, while leaves down-regulated ABA biosynthesis genes in comparison with well-watered girdled plants, the root-to-shoot paradigm seems more plausible. Nevertheless, decreased root ABA accumulation of girdled plants is consistent with a shoot-sourced model. The disparity between gene expression and hormone levels makes it difficult to favour a specific signalling paradigm.

Further work is needed to establish whether precursors and derivatives of both hormones, such as ABA-GE, OPDAs and JA-Ile may directly affect stomatal behaviour by measuring their concentrations in roots, xylem sap and leaves, to observe the export and/or import from different tissues. Manipulating phytohormone signalling by using mutants and/or transgenics could be combined with transcriptomic analysis to determine whether hormone accumulation and/or perception are affected. Finally, possible cross-talk of ABA with other hormones as cytokinins or gibberellins could improve our understanding of physiological responses such as stomatal behaviour.

References

Acharya, B. and Assmann, S., 2009. Hormone interactions in stomatal function. *Plant Molecular Biology*. **69**(4), 451-462.

Aftab, T., Khan, M. M. A., Idrees, M., Naeem, M., 2010. Salicylic acid acts as potent enhancer of growth, photosynthesis and artemisinin production in *Artemisia annua* L. *Journal of Crop Science and Biotechnology*. **3**, 183-188.

Alam, M. M., Nahar, K., Hasanuzzaman, M., Fujita M., 2014. Exogenous jasmonic acid modulates the physiology, antioxidant defense and glyoxalase systems in imparting drought stress tolerance in different *Brassica* species. *Plant Biotechnology Reports*. **8**, 279–293.

Albacete, A., Ghanem, M. E., Martinez-Andujar, C., Acosta, M., Sanchez-Bravo, J., Martinez, V., Lutts, S., Dodd, I. C., Perez-Alfocea, F., 2008. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. *Journal of Experimental Botany.* **59**(15), 4119-4131.

Alderfasi, A. A. and Nielsen, D. C., 2001. Use of crop water stress index for monitoring water status and scheduling irrigation in wheat. *Agricultural Water Management*. **47**(1), 69-75.

Aloni, R., Langhans, M., Aloni, E., Dreieicher, E., Ullrich, C. I., 2005. Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream. *Journal of Experimental Botany*, **56**(416), 1535-1544.

Alvarez, S., Marsh, E. L., Schroeder, S. G., Schachtman, D. P., 2008. Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant Cell & Environment*. **31**(3), 325-340.

Anderson, B. E., Ward, J. M., Schroeder, J. I., 1994. Evidence for an extracellular reception site for abscisic-acid in commelina guard-cells. *Plant Physiology*. **104**(4), 1177-1183.

Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Peirats-Llobet, M., Pizzio, G. A., Fernandez, M. A., ... & Rodriguez, P. L., 2013. PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. *Plant Physiology*. **161**(2), 931-941.

Arbona, V. and Gómez-Cadenas, A., 2008. Hormonal modulation of citrus responses to flooding. *Journal of Plant Growth Regulation*. **27**(3), 241-250.

Arbona, V., Argamasilla, R., Gómez-Cadenas, A., 2010. Common and divergent physiological, hormonal and metabolic responses of Arabidopsis thaliana and Thellungiella halophila to water and salt stress. *Journal of Plant Physiology*. **167**(16), 1342-1350.

Athar, H. R., Ashraf, M., 2009. Strategies for crop improvement against salinity and drought stress: an overview. In: Ashraf M, Ozturk M, Athar HR (eds) *Salinity and water stress: improving crop efficiency*, 3rd edn. Dodrecht, The Netherlands, pp 1–16.

Atkinson, N. J., Urwin, P. E., 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*. **63**(10), 3523–3543.

Audran, C., Liotenberg, S., Gonneau, M., North, H., Frey, A., Tap-Waksman, K., Vartanian, N., Marion-Poll, A., 2001. Localisation and expression of zeaxanthin epoxidase mRNA in *Arabidopsis* in response to drought stress and during seed development. *Australian Journal of Plant Physiology.* **28,** 1161–1173.

Balbi, V., Devoto, A., 2008. Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytologist*. **177**(2), 301–18.

Banerjee, S. and Bose, I., 2011. Transient pulse formation in jasmonate signaling pathway. *Journal of Theoretical Biology*, **273**(1), 188-196.

Battisti, R. and Sentelhas, P. C., 2017. Improvement of Soybean Resilience to Drought through Deep Root System in Brazil. *Agronomy Journal*. **109**(4), 1612-1622.

Bauer, H., Ache, P., Lautner, S., Fromm, J., Hartung, W., Al-Rasheid, K. A., Sonnewald, S., Sonnewald, U., Kneitz, S., Lachmann, N., Mendel, R. R., Bittner, F., Hetherington, A. M., Hedrich, R., 2013. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Current Biology*. **23**, 53-7.

Bernard, R. L. and Lindahl, D. A., 1972. Registration of williams soybean 1 (reg. no. 94). *Crop Science*. **12**(5), 716-716.

Blum, A., 1988. *Plant breeding for stress environments*. CRC press, 43–61.

Blum, A., 1996. Crop responses to drought and the interpretation of adaptation. *Plant Growth Regulators*. **20**, 135–148.

Blum, A., 2005. Drought resistance, water-use efficiency, and yield potential - are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research*, **56**(11), 1159-1168.

Borel, C. and Simonneau, T., 2002. Is the ABA concentration in the sap collected by pressurizing leaves relevant for analysing drought effects on stomata? Evidence from ABA-fed leaves of transgenic plants with modified capacities to synthesize ABA. *Journal of Experimental Botany*. **53**, 287-296.

Bostock, R. M., 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology*. **43**, 545-580.

Bostock, R. M., Stermer, B. A., 1989. Perspectives on wound healing in resistance to pathogens. *Annual Review of Phytopathology*. **27**, 343–71

Bouman, B. A. M. and Tuong, T. P., 2001. Field water management to save water and increase its productivity in irrigated lowland rice. *Agricultural Water Management*, **49**(1), 11-30.

Boursiac, Y., Leran, S., Corratge-Faillie, C., Gojon, A., Krouk, G., Lacombe, B., 2013. ABA transport and transporters. *Trends in Plant Science*, **18**(6), 325-333.

Boyer, J.S., 1982. Plant productivity and environment. *Science* **218**, 443–448.

Brash, A., Baertschi, S., Ingram, C., Harris, T., 1988. Isolation and characterization of natural allene oxides: unstable intermediates in the metabolism of lipid hydroperoxides. *Proceedings of the National Academy of Sciences*. **85**(10), 3382–3386.

Brodribb, T. J., Holbrook, N. M., 2003. Stomatal closure during leaf dehydration, correlation with other leaf physiological traits. *Plant Physiology.* **132,** 2166–2173.

Brossa, R., López-Carbonell, M., Jubany-Marí, T., Alegre, L., 2011. Interplay between abscisic acid and jasmonic acid and its role in water-oxidative stress in wild-type, ABA-deficient, JA-deficient, and ascorbate-deficient Arabidopsis plants. *Journal of Plant Growth Regulation*. **30**(3), 322-333.

Bruce, W. B., Edmeades, G. O., Barker, T. C., 2002. Molecular and physiological approaches to maize improvement for drought tolerance. *Journal of Experimental Botany*. **53**(366), 13-25.

Buckley, T. N., 2005. The control of stomata by water balance. *New Phytologist*. **168**(2), 275-292.

Bunce, J. A., 1977. Leaf Elongation in Relation to Leaf Water Potential in Soybean. *Journal of Experimental Botany*. **28**, 156-161.

Castro, P., Puertolas, J., Dodd, I. C., 2019. Stem girdling uncouples soybean stomatal conductance from leaf water potential by enhancing leaf xylem ABA concentration. *Environmental and Experimental Botany*. **159**, 149-156.

Catinot, J., Buchala, A., Abou-Mansour, E., Métraux, J. P., 2008. Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Letters*. **582**, 473–478.

Cattivelli, L., Rizza, F., Badeck, F. W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., ... & Stanca, A. M., 2008. Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research*, **105**(1-2), 1-14.

Çevik, V., Kidd, B. N., Zhang, P., Hill, C., Kiddle, S., Denby, K. J., ... & Beynon, J., 2012. MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in Arabidopsis. *Plant physiology*, **160**(1), 541-555.

Chaves, M. M., Flexas, J., Pinheiro, C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany*. **103**, 551–60.

Chaves, M. M., Maroco, J. P., Pereira, J. S., 2003. Understanding plant responses to drought—from genes to the whole plant. *Functional Plant Biology*. **30**, 239–264.

Chen, R., Jiang, H., Li, L., Zhai, Q., Qi, L., Zhou, W., ... & Li, C., 2012. The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors. *The Plant Cell*, **24**(7), 2898-2916.

Cheng, W. H., Endo, A., Zhou, L., Penney, J., Chen, H. C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., Sheen, J., 2002. A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell.* **5,** 2723–2743.

Cheng, Z., Sun, L., Qi, T., Zhang, B., Peng, W., Liu, Y., Xie, D., 2011. The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in Arabidopsis. *Molecular Plant*. **4**, 279–288.

Cheong, Y. H., Chang, H. S., Gupta, R., Wang, X., Zhu, T., Luan, S., 2002. Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. *Plant physiology*. ***129***(2), 661-677.

Christensen, S. A., Huffaker, A., Kaplan, F., Sims, J., Ziemann, S., Doehlemann, G., ... & Mori, N., 2015. Maize death acids, 9-lipoxygenase–derived cyclopente (a) nones, display activity as cytotoxic phytoalexins and transcriptional mediators. *Proceedings of the National Academy of Sciences*, **112**(36), 11407-11412.

Christmann, A., Hoffmann, T., Teplova, I., Grill, E., Muller, A., 2005. Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed Arabidopsis. *Plant Physiology*. **137**, 209-219.

Christmann, A., Weiler, E. W., Steudle, E., Grill, E., 2007. A hydraulic signal in root-to-shoot signalling of water shortage. *Plant Journal*. **52**(1), 167-174.

Coelho Filho, M. A., Colebrook, E. H., Lloyd, D. P., Webster, C. P., Mooney, S. J., Phillips, A. L., ... & Whalley, W. R., 2013. The involvement of gibberellin signalling in the effect of soil resistance to root penetration on leaf elongation and tiller number in wheat. *Plant and Soil*. **371**(1-2), 81-94.

Colebrook, E. H., Thomas, S. G., Phillips, A. L., Hedden, P., 2014. The role of gibberellin signalling in plant responses to abiotic stress. *Journal of Experimental Botany*. **217**(Pt 1), 67-75.

Comstock, J. P., 2002. Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. *Journal of Experimental Botany*. **53**(367), 195-200.

Cornish, K. and Zeevaart, J. A. D., 1985. Abscisic acid accumulation by roots of *Xanthium strumarium* L. and *Lycopersicon esculentum* Mill. in relation to water stress. *Plant Physiology*. **79**, 653-658.

Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., Shinozaki, K., 2011. Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biology*. **11**(1), 163–77.

Craufurd, P. Q., Peacock, J. M., 1993. Effect of heat and drought stress on sorghum. *Experimental Agriculture*. **B**, 77–86.

da Silva, E., Goncalves, A. O., Pereira, R. A., Fattori, I. M., Sobenko, L. R., Marin, F. R., 2019. Soybean irrigation requirements and canopy-atmosphere coupling in Southern Brazil. *Agricultural Water Management*. **218**, 1-7.

Dai, J. L. and Dong, H. Z., 2011. Stem girdling influences concentrations of endogenous cytokinins and abscisic acid in relation to leaf senescence in cotton. *Acta Physiologiae Plantarum*. **33**(5), 1697-1705.

Dave, A. and Graham, I. A., 2012. Oxylipin signaling: a distinct role for the jasmonic acid precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA). *Frontiers in Plant Science*, **3**, 42.

Davies, W. J. and Zhang, J., 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Biology*. **42**(1), 55-76.

Davies, W. J., Mansfield, T. A., Hetherington, A. M., 1990. Sensing of soil-water status and the regulation of plant-growth and development. *Plant Cell and Environment*. **13**(7), 709-719.

Davies, W. J., Tardieu, F., Trejo, C. L., 1994. How do chemical signals work in plants that grow in drying soil?. *Plant Physiology*. **104**(2), 309-314.

de Ollas, C. and Dodd, I. C., 2016. Physiological impacts of ABA-JA interactions under water-limitation. *Plant Molecular Biology*. **91**(6), 641-650.

de Ollas, C., Arbona, V., Gomez-Cadenas, A., Dodd, I. C., 2018. Attenuated accumulation of jasmonates modifies stomatal responses to water deficit. *Journal of Experimental Botany*. **69**(8), 2103-2116.

de Ollas, C., Hernando, B., Arbona, V., Gómez-Cadenas, A., 2013. Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. *Physiologia plantarum*. **147**(3), 296-306.

Doares, S. H., Narváez-Vásquez, J., Conconi, A., Ryan, C. A., 1995. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant physiology*. **108**(4), 1741-1746.

Dodd, I. C., 2003. Hormonal interactions and stomatal responses. *Journal of Plant Growth Regulation.* **22**(1), 32-46.

Dodd, I. C., 2005. Root-to-shoot signalling: assessing the roles of 'up' in the up and down world of long-distance signalling in planta. *Plant and Soil*. **274**(1-2), 251-270.

Dodd, I. C., 2007. Soil moisture heterogeneity during deficit irrigation alters root-to-shoot signalling of abscisic acid. *Functional Plant Biology*. **34**(5), 439-448.

Dodd, I. C., 2013. Abscisic acid and stomatal closure: a hydraulic conductance conundrum?. *New Phytologist*. **197**, 6-8.

Doherty, H. M., Selvendran, R. R., Bowles, D. J., 1988. The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids. *Physiological and Molecular Plant Pathology*, **33**(3), 377-384.

Doss, B. D., Pearson, R. W., Rogers, H. T., 1974. Effect of soil water stress at various growth stages on soybean yield. *Agronomy Journal*. **66**, 297-299.

Earl, H. J., 2003. A precise gravimetric method for simulating drought stress in pot experiments. *Crop Science*. **43**, 1868–1873.

Eck, H. V., Mathers, A. C., Musick, J. T., 1987. Plant water stress at various growth stages and growth and yield of soybeans. *Field Crops Research*. **17**, 1-16.

Else, M. A. and Jackson, M. B., 1998, Transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in the transpiration stream of tomato (*Lycopersicon esculentum*) in relation to foliar ethylene production and petiole epinasty. *Australian journal of Plant Physiology*. **25**, 453–458

Emery, R. N. and Atkins, C. A., 2002. *Roots and cytokinins*. In Plant Roots, 656-679. CRC Press.

Evans, N. H., 2003. Modulation of guard cell plasma membrane potassium currents by methyl jasmonate. *Plant Physiologist*. **131**(1), 8–11.

Faiss, M., Zalubìlová, J., Strnad, M., Schmülling, T., 1997. Conditional transgenic expression of the ipt gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *The Plant Journal*. **12**(2), 401-415.

FAO (Food and Agriculture Organization), 2003. http://apps.fao.org.

FAO (Food and Agriculture Organization), 2017; 2018. FAOSTAT. http://faostat.fao.org.

Feussner, I. and Wasternack, C., 2002. The lipoxygenase pathway. *Annual Review of Plant Biology*. **53**, 275–297.

Finkelstein, R., 2013. Abscisic acid synthesis and response. *The Arabidopsis book/American Society of Plant Biologists*, **11**.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., . . .& Solano, R., 2009. (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature Chemical Biology*. **5**(5), 344-350.

Forster, S., Schmidt, L. K., Kopic, E., Anschutz, U., Huang, S., Schlucking, K., . . . & Becker, D., 2019. Wounding-induced stomatal closure requires jasmonate-mediated activation of GORK K(+) channels by a Ca(2+) Sensor-Kinase CBL1-CIPK5 Complex. *Developmental Cell*. **48**(1), 87-99.e6.

Franks, P. J., 2013. Passive and active stomatal control: either or both? *New Phytologist*, **198**(2), 325-327.

Franks, P. J. and Farquhar, G. D., 1999. A relationship between humidity response, growth form and photosynthetic operating point in C3 plants. *Plant, Cell & Environment*. **22**, 1337–1349.

Fried, H. G., Narayanan, S., Fallen, B., 2019. Evaluation of soybean [*Glycine max* (L.) Merr.] genotypes for yield, water use efficiency, and root traits. *PloS One*, **14**(2), e0212700.

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., ... & Kobayashi, M, 2009. Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant and Cell Physiology*. **50**(12), 2123-2132.

Gilbert, M. E., Zwieniecki, M. A., Holbrook, N. M., 2011. Independent variation in photosynthetic capacity and stomatal conductance leads to differences in intrinsic water use efficiency in 11 soybean genotypes before and during mild drought. *Journal of Experimental Botany*. **62**(8), 2875-2887.

Gil-Quintana, E., Larrainzar, E., Seminario, A., Díaz-Leal, J. L., Alamillo, J. M., Pineda, M., Arrese-Igor, C., Wienkoop, S., González, E. M., 2013. Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean. *Journal of Experimental Botany*. **64,** 2171–2182.

Glauser, G., Grata, E., Dubugnon, L., Rudaz, S., Farmer, E. E., Wolfender, J. L., 2008. Spatial and temporal dynamics of jasmonate synthesis and accumulation in Arabidopsis in response to wounding. *Journal of Biological Chemistry*. **283**(24), 16400-16407.

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review Phytopathology*. **43**, 205-227.

Goldsmith, P. D., (2008). Economics of soybean production, marketing, and utilization. In *Soybeans*. AOCS Press. 117-150.

Gollan, T., Richards, R. A., Rawson, H. M., Passioura, J. B., Johnson, D. A., Munns, R., 1986. Soil water status affects the stomata1. *Functional Plant Biology*. **13**(4), 459-464.

Gómez-Cadenas, A., Tadeo, F. R., Talon, M., Primo-Millo, E., 1996. Leaf abscission induced by ethylene in water-stressed intact seedlings of Cleopatra mandarin requires previous abscisic acid accumulation in roots. Plant Physiology. 112(1), 401-408.

González-Guzmán, M., Apostolova, N., Bellés, J. M., Barrero, J. M., Piqueras, P., Ponce, M. R., Micol, J. L., Serrano, R., Rodríguez, P. L., 2002. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell*. **14**(8), 1833–1846

Gowing, D. J. G., Jones, H. G., Davies, W. J., 1993. Xylem-transported abscisic-acid - the relative importance of its mass and its concentration in the control of stomatal aperture. *Plant Cell and Environment*. **16**(4), 453-459.

Grebner, W., Stingl, N. E., Oenel, A., Mueller, M. J., Berger, S., 2013. Lipoxygenase6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of Arabidopsis. *Plant Physiology*. **161**, 2159–2170.

Gunderson, C. A. and Taylor, G. E., 1991. Ethylene directly inhibits foliar gas-exchange in *Glycine max*. *Plant Physiology.* **95**(1), 337-339.

Hadiarto, T. and Tran, L. S. P., 2011. Progress studies of drought-responsive genes in rice. *Plant Cell Reports*. **30**(3), 297-310.

Hansen, H. and Dorffling, K., 2003. Root-derived trans-zeatin riboside and abscisic acid in drought-stressed and rewatered sunflower plants: interaction in the control of leaf diffusive resistance?. *Functional Plant Biology*. **30**(4), 365-375.

Harb, A., Krishnan, A., Ambavaram, M. M., Pereira, A., 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant Physiology*. **154**(3), 1254-1271.

Hartung, W., Radin, J. W., Hendrix, D. L., 1988. Abscisic Acid movement into the apoplastic solution of water-stressed cotton leaves. *Plant Physiology*. **86**, 908-913.

HavlovA, M., Dobrev, P. I., Motyka, V., ŠTORCHOVÁ, H., LIBUS, J., DobrA, J., ... & VankovA, R., 2008. The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin O-glucosyltransferase gene under 35S or SAG12 promoters. *Plant, Cell & Environment*. **31**(3), 341-353.

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A., 2010. Effect of exogenous salicylic acid under changing environment: a review. *Environmental and Experimental Botany*. **68**(1), 14-25.

Hayat, S., Hasan, S. A., Fariduddin, Q., Ahmad, A., 2008. Growth of tomato (*Lycopersicon esculentum*) in response to salicylic acid under water stress. *Journal of Plant Interactions*. **3**(4), 297-304.

He, J., Du, Y. L., Wang, T., Turner, N. C., Xi, Y., Li, F. M., 2016. Old and New Cultivars of Soya Bean (*Glycine max* L.) Subjected to Soil Drying Differ in Abscisic Acid Accumulation, Water Relations Characteristics and Yield. *Journal of Agronomy and Crop Science*. **202**(5), 372-383.

He, Q., Zhao, S., Ma, Q., Zhang, Y., Huang, L., Li, G., Hao, L., 2014. Endogenous salicylic acid levels and signaling positively regulate Arabidopsis response to polyethylene glycol-simulated drought stress. *Journal of Plant Growth Regulation*, **33**(4), 871-880.

Heitz, T., Smirnova, E., Widemann, E., Aubert, Y., Pinot, F., Ménard, R., 2016. The rise and fall of jasmonate biological activities. In *Lipids in plant and algae development*. Springer, Cham. 405-426.

Heitz, T., Widemann, E., Lugan, R., Miesch, L., Ullmann, P., Désaubry, L., ... & Werck-Reichhart, D., 2012. Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. *Journal of Biological Chemistry*. **287**(9), 6296-6306.

Hetherington, A. M. and Woodward, F. I., 2003. The role of stomata in sensing and driving environmental change. *Nature*. **424**(6951), 901-908.

Hildmann, T., Ebneth, M., Peiia-Cortth, H., Sánchez-Serrano, J. J., Willmitzer, L., Prat, S., 1992. General roles of Abscisic and Jasmonic Acids in gene activation as a result of mechanical wounding. *Plant Cell*. **4**, 1157-1170.

Hirt, H. and Shinozaki, K. (2003). *Plant responses to abiotic stress* (Vol. 4). Springer Science and Business Media.

Horváth, E., Brunner, S., Bela, K., Papdi, C., Szabados, L., Tari, I., Csiszár, J., 2015. Exogenous salicylic acid-triggered changes in the glutathione transferases and peroxidases are key factors in the successful salt stress acclimation of Arabidopsis thaliana. *Functional Plant Biology*, **42**(12), 1129-1140.

Hossain, M. A., Munemasa, S., Uraji, M., Nakamura, Y., Mori, I. C., Murata, Y., 2011. Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. *Plant Physiology*, **156**(1), 430-438.

Hossain, M. M., Lam, H.-M., Zhang, J., 2015. Responses in gas exchange and water status between drought-tolerant and -susceptible soybean genotypes with ABA application. *The Crop Journal*. **3**(6), 500-506.

Hossain, M. M., Liu, X., Qi, X., Lam, H.-M., Zhang, J., 2014. Differences between soybean genotypes in physiological response to sequential soil drying and rewetting. *The Crop Journal*. **2**(6), 366-380.

Huang, D., Wu, W., Abrams, S. R., Cutler, A. J., 2008. The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *Journal of Experimental* Botany. **59**(11), 2991-3007.

Hufstetler, E. V., Boerma, H. R., Carter, T. E., Earl, H. J., 2007. Genotypic variation for three physiological traits affecting drought tolerance in soybean. *Crop Science*. **47**(1), 25-35.

Ikegami, K., Okamoto, M., Seo, M., Koshiba, T., 2009. Activation of abscisic acid biosynthesis in the leaves of *Arabidopsis thaliana* in response to water deficit. *Journal of Plant Research.* **122**(2), 235.

Ioio, R. D., Linhares, F. S., Sabatini, S., 2008. Emerging role of cytokinin as a regulator of cellular differentiation. *Current opinion in plant biology*, **11**(1), 23-27.

Iqbal, N., Nazar, R., Syeed, S., Masood, A., Khan, N. A., 2011. Exogenously-sourced ethylene increases stomatal conductance, photosynthesis, and growth under optimal and deficient nitrogen fertilization in mustard. *Journal of Experimental Botany*. **62**(14), 4955-4963.

Ismail, M. R., Davies, W. J., Awad, M. H., 2002. Leaf growth and stomatal sensitivity to ABA in droughted pepper plants. *Scientia Horticulturae*. **96**(1-4), 313-327.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., . . .& Shinozaki, K., 2001. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant Journal*, **27**(4), 325-333.

Jackson, M. B., 2002. Long-distance signalling from roots to shoots assessed: the flooding story. *Journal of Experimental Botany*. **53**(367), 175-181.

Janda, T., Horváth, E., Szalai, G., Paldi, E., 2007. Role of salicylic acid in the induction of abiotic stress tolerance. In *Salicylic acid: A plant hormone.* 91-150. Springer, Dordrecht.

Ji, K., Kai, W., Zhao, B., Sun, Y., Yuan, B., Dai, S., . . .& Leng, P., 2014. SlNCED1 and SlCYP707A2: key genes involved in ABA metabolism during tomato fruit ripening. *Journal of Experimental Botany*. **65**(18), 5243-5255.

Jia, W., Xing, Y., Zhao, J., Zhang, J., Zhang, S.Q., 2002. Cellular stress signaling from water deficit-perception to ABA accumulation. *Advanced Plant Sciences*. **4**, 205–212.

Jongdee, B., Fukai, S., Cooper, M., 2002. Leaf water potential and osmotic adjustment as physiological traits to improve drought tolerance in rice. *Field Crops Research*. **76**, 153–163.

Kaiser, H., Legner, N., 2007. Localization of mechanisms involved in hydropassive and hydroactive stomatal responses of *Sambucus nigra* to dry air. *Plant Physiologist*. **143**(2), 1068–77.

Kazan, K. and Manners, J. M., 2013. MYC2: the master in action. *Molecular Plant*, **6**(3), 686-703.

Kazan, K., 2015. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. *Trends in Plant Science.* **20**(4), 219-229.

Kende, H., 1993. Ethylene biosynthesis. *Annual Review of Plant Biology*. **44**(1), 283-307.

Khan, W., Prithviraj, B., Smith, D.L., 2003. Photosynthetic responses of corn and soybean to foliar application of salicylates. *Journal of Plant Physiology*. **160**, 485–492.

King, C. A., Purcell, L. C., Brye, K. R., 2009. Differential wilting among soybean genotypes in response to water deficit. *Crop Science*. **49**(1), 290-298.

Kong, L. S., von Aderkas, P., Owen, S. J., Jaquish, B., Woods, J., Abrams, S. R., 2012. Effects of stem girdling on cone yield and endogenous phytohormones and metabolites in developing long shoots of Douglas-fir (*Pseudotsuga menziesii*). *New Forests*. **43**(4), 491-503.

Koo, A. J., Cooke, T. F., Howe, G. A., 2011. Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proceedings of the National Academy of Sciences*. **108**(22), 9298-9303.

Kramer, P. J., 1988. Changing concepts regarding plant water relations. *Plant, Cell & Environment*. **11**, 565-568.

Kudoyarova, G. R., Vysotskaya, L. B., Cherkozyanova, A., Dodd, I. C., 2007. Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (*Lycopersicon esculentum* Mill.) xylem sap and leaves. *Journal of Experimental Botany*. **58**, 161-168.

Kuromori, T., Miyaji, T., Yabuuchi, H., Shimizu, H., Sugimoto, E., Kamiya, A., ... & Shinozaki, K., 2010. ABC transporter AtABCG25 is involved in abscisic acid transport and responses. *Proceedings of the National Academy of Sciences*. **107**(5), 2361-2366.

Kuromori, T., Mizoi, J., Umezawa, T., Yamaguchi-Shinozaki, K., Shinozaki, K., 2014a. Drought stress signaling network. *Molecular Biology*, 383-409.

Kuromori, T., Sugimoto, E., Shinozaki, K., 2014b. Intertissue signal transfer of abscisic acid from vascular cells to guard cells. *Plant Physiology*. **164**(4), 1587-1592.

Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., ... & Nambara, E.,2004. The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. *The EMBO Journal*, **23**(7), 1647-1656.

Lam, H. M., Xu, X., Liu, X., Chen, W., Yang, G., Wong, F. L., . . .& Zhang, G., 2010. Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. *Nature Genetics*. **42**(12), 1053-1059.

Larque-Saavedra, A., 1979. Stomatal closure in response to acetylsalicylic acid treatment. *Zeitschrift für Pflanzenphysiologie*. **93** (4), 371–375.

Le, D. T., Nishiyama, R. I. E., Watanabe, Y., Mochida, K., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L. S. P., 2011. Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. *DNA Research*. **18**(4), 263-276.

Le, D. T., Nishiyama, R., Watanabe, Y., Tanaka, M., Seki, M., Yamaguchi-Shinozaki, K., ... & Tran, L. S. P., 2012. Differential gene expression in soybean leaf tissues at late developmental stages under drought stress revealed by genome-wide transcriptome analysis. *PloS One*. **7**(11), e49522.

Lee, K. H., Piao, H. L., Kim, H. Y., Choi, S. M., Jiang, F., Hartung, W., ... & Hwang, I., 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell*. **126**(6), 1109-1120.

Leff, B., Ramankutty, N., Foley, J. A., 2004. Geographic distribution of major crops across the world. *Global Biogeochemical Cycles*. **18**, 1–27.

Lei, W., Tong, Z., Shengyan, D., 2006a. Effect of drought and rewatering on photosynthetic physioecological characteristics of soybean. *Acta Ecologica Sinica*. **26**, 2073–2078.

Lei, Y., Yin, C., Li, C., 2006b. Differences in some morphological, physiological, and biochemical responses to drought stress in two contrasting populations of *Populus przewalskii*. *Physiologia Plantarum*. **127**(2), 182-191.

Levitt, J., 1980. *Responses of Plants to Environmental Stress, Volume 1: Chilling, Freezing, and High Temperature Stresses*. Academic Press.

Li, J., Li, C., Smith, S. M., 2017. *Hormone metabolism and signaling in plants*. Academic Press.

Li, Y., Zhang, J., Zhang, J., Hao, L., Hua, J., Duan, L., Zhang, M., Li, Z., 2013. Expression of an Arabidopsis molybdenum cofactor sulphurase gene in soybean enhances drought tolerance and increases yield under field conditions. *Plant Biotechnology Journal*. **11**, 747-58.

Liang, J., Zhang, J., Wong, M. H., 1997. How do roots control xylem sap ABA concentration in response to soil drying?. *Plant and Cell Physiology*. **38**, 10-16.

Liu, A., Xiao, Z., Li, M. W., Wong, F. L., Yung, W. S., Ku, Y. S., . . . & Lam, H. M., 2019. Transcriptomic reprogramming in soybean seedlings under salt stress. *Plant, Cell & Environment*. **42**(1), 98-114

Liu, F. L., Andersen, M. N., Jacobsen, S. E., Jensen, C. R., 2005c. Stomatal control and water use efficiency of soybean (*Glycine max* (L). Merr.) during progressive soil drying. *Environmental and Experimental Botany*. **54**, 33–40.

Liu, F. L., Andersen, M. N., Jensen, C. R., 2003b. Loss of pod set caused by drought stress is associated with water status and ABA content of reproductive structures in soybean. *Functional Plant Biology*. **30**, 271-280.

Liu, F. L., Jensen, C. R., Andersen, M. N., 2003a. Hydraulic and chemical signals in the control of leaf expansion and stomatal conductance in soybean exposed to drought stress. *Functional Plant Biology*. **30**, 65-73.

Liu, F., Andersen, M. N., Jensen, C. R., 2004. Root signal controls pod growth in drought-stressed soybean during the critical, abortion-sensitive phase of pod development. *Field Crops Research*. **85**(2-3), 159-166.

Liu, F., Jensen, C. R., Andersen, M. N., 2005b. A review of drought adaptation in crop plants: changes in vegetative and reproductive physiology induced by ABA-based chemical signals. *Australian Journal of Agricultural Research*. **56**(11), 1245-1252.

Liu, F., Jensen, C. R., Shahanzari, A., Andersen, M. N., Jacobsen, S. E., 2005a. ABA regulated stomatal control and photosynthetic water use efficiency of potato (*Solanum tuberosum* L.) during progressive soil drying. *Plant Science*. **168**(3), 831-836.

Liu, S., Lv, Y., Wan, X. R., Li, L. M., Hu, B., Li, L., 2014. Cloning and expression analysis of cDNAs encoding ABA 8'-hydroxylase in peanut plants in response to osmotic stress. *PLoS One*. **9**(5), e97025.

Liu, Z. R., Chen, S. G., Zhou, Y., Xie, C. H., Zhu, B. F., Zhu, H. M., . . . & Ji, Y. H., 2015. Deciphering the venomic transcriptome of killer-wasp *Vespa velutina*. *Scientific Reports*. **5**, 9454.

Lopez, R., Brossa, R., Gill, L., Pita, P., 2015. Stem girdling evidences a trade-off between cambial activity and sprouting and dramatically reduces plant transpiration due to feedback inhibition of photosynthesis and hormone signaling. *Frontiers in Plant Science*, **6**, 285.

López-López, A., Rosenblueth, M., Martínez, J., Martínez-Romero, E., 2010. Rhizobial symbioses in tropical legumes and non-legumes. *Soil Biology and Agriculture in the Tropics*. Springer, Berlin, Heidelberg, 163–184.

Luo, X., Chen, Z., Gao, J., Gong, Z., 2014. Abscisic acid inhibits root growth in Arabidopsis through ethylene biosynthesis. *The Plant Journal*. **79**(1), 44-55.

Ma, Q. Q., Wang, W., Li, Y. H., Li, D. Q., Zou, Q., 2006. Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum*) by foliar-applied glycinebetaine. *Journal of Plant Physiology*. **163**(2), 165-175.

Ma, Y., Cao, J., He, J., Chen, Q., Li, X., Yang, Y., 2018. Molecular mechanism for the regulation of ABA homeostasis during plant development and stress responses. *International Journal of Molecular Sciences*. **19**(11), 3643.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., Grill, E., 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science*, **324**(5930), 1064-1068.

Mahajan, S. and Tuteja, N., 2005. Cold, salinity and drought stresses: an overview. *Archives of Biochemistry and Biophysics*. **444**(2), 139-158.

Mahouachi, J., Arbona, V., Gómez-Cadenas, A., 2007. Hormonal changes in papaya seedlings subjected to progressive water stress and re-watering. *Plant Growth Regulation.* **53**(1), 43-51.

Manavalan, L. P., Guttikonda, S. K., Tran, L. S. P., Nguyen, H. T., 2009. Physiological and molecular approaches to improve drought resistance in soybean. *Plant and Cell Physiology*. **50**(7), 1260-1276.

Manzi, M., Lado, J., Rodrigo, M. J., Arbona, V., Gomez-Cadenas, A., 2016. ABA accumulation in water-stressed Citrus roots does not rely on carotenoid content in this organ. *Plant Science*. **252**, 151-161.

Manzi, M., Lado, J., Rodrigo, M. J., Zacarias, L., Arbona, V., Gomez-Cadenas, A., 2015. Root ABA accumulation in long-term water-stressed plants is sustained by hormone transport from aerial organs. *Plant Cell Physiology*. **56**, 2457-2466.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., ... & Marion-Poll, A., 1996. Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *The EMBO Journal*. **15**(10), 2331-2342.

Masia, A., Pitacco, A., Braggio, L., Giulivo, C., 1994. Hormonal responses to partial drying of the root-system of *Helianthus-annuus*. *Journal of Experimental Botany*. **45**(270), 69-76.

Masuda, T. and Goldsmith, P. D., 2009. World soybean production: area harvested, yield, and long-term projections. *International food and agribusiness management review*. **12**(1030-2016-82753), 1-20.

McAdam, S. A. M. and Brodribb, T. J., 2016. Linking turgor with ABA biosynthesis: implications for stomatal responses to vapour pressure deficit across land plants. *Plant Physiology*. **171**, 2008-2016.

McAdam, S. A. M. and Brodribb, T. J., 2018. Mesophyll cells are the main site of abscisic acid biosynthesis in water-stressed leaves. *Plant Physiology*. **177**, 911-917.

McAdam, S. A. M., Brodribb, T. J., Ross, J. J., 2016b. Shoot-derived abscisic acid promotes root growth. *Plant, Cell & Environment*. **39**, 652-659.

McAdam, S. A. M., Manzi, M., Ross, J. J., Brodribb, T. J., Gomez-Cadenas, A., 2016a. Uprooting an abscisic acid paradigm: Shoots are the primary source. *Plant Signal and Behavior*. **11**(6), e1169359.

McKenzie, M. J., Mett, V., Reynolds, P. H. S., Jameson, P. E., 1998. Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. *Plant Physiology*. **116**(3), 969-977.

Melkonian, J. J., Wolfe, J., Steponkus, P. L., 1982. Determination of the Volumetric Modulus of Elasticity of Wheat Leaves by Pressure-Volume Relations and the Effect of Drought Conditioning 1. *Crop Science*. **22**(1), 116-123.

Merilo, E., Jalakas, P., Laanemets, K., Mohammadi, O., Horak, H., Kollist, H., Brosche, M., 2015. Abscisic acid transport and homeostasis in the context of stomatal regulation. *Molecular Plant*, **8**(9), 1321-1333.

Merilo, E., Laanemets, K., Hu, H., Xue, S., Jakobson, L., Tulva, I., ... & Kollist, H., 2013. PYR/RCAR receptors contribute to ozone-, reduced air humidity-, darkness-, and CO2-induced stomatal regulation. *Plant Physiology*. **162**(3), 1652-1668.

Meuwly, P., Molders, W., Buchala, A., Metraux, J. P., 1995. Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiology*. **109**(3), 1107-1114.

Mitchell, P. J., McAdam, S. A. M., Pinkard, E. A., Brodribb, T. J., 2016. Significant contribution from foliage-derived ABA in regulating gas exchange in *Pinus radiata*. *Tree Physiology*. **37**, 236-245.

Miura, K., Lee, J., Jin, J. B., Yoo, C. Y., Miura, T., Hasegawa, P. M., 2009. Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. *Proceedings of the National Academy of Sciences*. **106**(13), 5418-5423.

Miyakawa, T., Fujita, Y., Yamaguchi-Shinozaki, K., Tanokura, M., 2013. Structure and function of abscisic acid receptors. *Trends in Plant Science*, **18**(5), 259-266.

Miyawaki, K., Matsumoto-Kitano, M., Kakimoto, T., 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. *The Plant Journal*. **37**(1), 128-138.

Mochida, K., Yoshida, T., Sakurai, T., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.-S.P., 2010. Genome-wide analysis of two-component systems and prediction of stress-responsive two-component system members in soybean. *DNA Research*. **17**, 303–324.

Morgan, J. M., 1984. Osmoregulation and water stress in higher plants. *Annual review of plant physiology*. **35**(1), 299–319.

Morgan, J. M., King, R. W., 1984. Association between loss of leaf turgor, abscisic acid levels and seed set in two wheat cultivars. *Australian Journal of Plant Physiology*. **11**, 143-50.

Munemasa, S., Oda, K., Watanabe-Sugimoto, M., Nakamura, Y., Shimoishi, Y., Murata, Y., 2007. The coronatine-insensitive 1 mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. *Plant Physiology*. **143**(3), 1398-1407.

Munns, R., 2002. Comparative physiology of salt and water stress. *Plant Cell and Environment*. **25**(2), 239-250.

Munns, R., James, R. A., Sirault, X. R., Furbank, R. T., Jones, H. G., 2010. New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. *Journal of Experimental Botany*. **61**(13), 3499-3507.

Munns, R., King, R. W., 1988. Abscisic Acid is not the only stomatal inhibitor in the transpiration stream of wheat plants. *Plant Physiology*. **88**, 703-708.

Muñoz-Espinoza, V. A., López-Climent, M. F., Casaretto, J. A., Gómez-Cadenas, A., 2015. Water stress responses of tomato mutants impaired in hormone biosynthesis reveal abscisic acid, jasmonic acid and salicylic acid interactions. *Frontiers in plant science*. ***6***, 997.

Murata, Y., Mori, I. C., Munemasa, S., 2015. Diverse stomatal signaling and the signal integration mechanism. *Annual Review of Plant Biology*. **66**, 369-392.

Murata, Y., Mori, I.C., 2014. Stomatal regulation of plant water status. In: Jenks MA, Hasegawa PM, editors. *Plant abiotic stress*. 2nd ed. Ames, IO: John Wiley and Sons. 47–67.

Nakashima, K., Tran, L. S. P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., ... & Yamaguchi-Shinozaki, K., 2007. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *The Plant Journal*, **51**(4), 617-630.

Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Frontiers in plant science*. **5**, 170.

Neves-Borges, A. C., Guimaraes-Dias, F., Cruz, F., Mesquita, R. O., Nepomuceno, A. L., Romano, E., . . .& Alves-Ferreira, M., 2012. Expression pattern of drought stress marker genes in soybean roots under two water deficit systems. *Genetics and Molecular Biology*, **35**(1), 212-U237.

Newell, C. A. and Hymowitz, T., 1983. Hybridization in the genus Glycine subgenus Glycine willd (Leguminosae, Papilionoideae). *American Journal of Botany*. **70**(3), 334-348.

Nir, I. D. O., Moshelion, M., Weiss, D., 2014. The A rabidopsis GIBBERELLIN METHYL TRANSFERASE 1 suppresses gibberellin activity, reduces whole-plant transpiration and promotes drought tolerance in transgenic tomato. *Plant, Cell & Environment*. **37**(1), 113-123.

Nishimura, N., Sarkeshik, A., Nito, K., Park, S. Y., Wang, A., Carvalho, P. C., ... & Yates, J. R., 2010. PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. *The Plant Journal*. **61**(2), 290-299.

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D. T., Kojima, M., Werner, T., ... & Sakakibara, H., 2011. Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *The Plant Cell*. **23**(6), 2169-2183.

Niu, Y., Figueroa, P., Browse, J., 2011. Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *Journal of Experimental Botany*. **62**(6), 2143-2154.

Okamoto, M., Tanaka, Y., Abrams, S. R., Kamiya, Y., Seki, M., Nambara, E., 2009. High humidity induces abscisic acid 8'-hydroxylase in stomata and vasculature to regulate local and systemic abscisic acid responses in Arabidopsis. *Plant physiology*. **149**(2), 825-834.

Oosterhuis, D. M. and Wullschleger, S. D., 1987. Osmotic adjustment in cotton (Gossypium hirsutum L.) leaves and roots in response to water stress. *Plant Physiology*. **84**(4), 1154-1157.

Pandey, D. M., Goswami, C. L., Kumar, B., 2004. Physiological effects of plant hormones in cotton under drought. *Biologia Plantarum*. **47**(4), pp.535–540.

Pantin, F., Monnet, F., Jannaud, D., Costa, J. M., Renaud, J., Muller, B., Simonneau, T., Genty, B., 2013. The dual effect of abscisic acid on stomata. *New Phytologist*. **197**, 65-72.

Pardo, E. M., Vellicce, G. R., Aguirrezabal, L., Pereyra, I. G., Rocha, C. M. L., García, M. G., Prieto, A. S., Welin, B., Sanchez, J., Ledesma, F., Castagnaro, A. P., 2015. Drought tolerance screening under controlled conditions predicts ranking of water-limited yield of field-grown soybean genotypes. *Journal of Agronomy and Crop Science*. **201**, 95-104.

Park, S. Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., ... & Alfred, S. E., 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science*. **324**(5930), 1068-1071.

Passioura, J. B., 1988. Response to Dr. P. J. Kramer's article, "Changing concepts regarding plant water relations". *Plant, Cell & Environment*. **11**, 569-571.

Pathan, M. S., Lee, J. D., Shannon, J. G., Nguyen, H. T., 2007. Recent advances in breeding for drought and salt stress tolerance in soybean. In: Jenks, M.A., Hasegawa, P.M., Jain, S.M. (Eds.), *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops*. Springer, Netherlands.

Pathan, S. M., Lee, J. D., Sleper, D. A., Fritschi, F. B., Sharp, R. E., Carter, T. E., Shannon, J. G., 2014. Two soybean plant introductions display slow leaf wilting and reduced yield loss under drought. *Journal of Agronomy and Crop Science*. **200**(3), 231-236.

Pauwels, L. and Goossens, A., 2011. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. *The Plant Cell*. **23**(9), 3089-3100.

Peleg, Z. and Blumwald, E., 2011. Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology*. **14**(3), 290-295.

Per, T. S., Khan, M. I. R., Anjum, N. A., Masood, A., Hussain, S. J., Khan, N. A., 2018. Jasmonates in plants under abiotic stresses: Crosstalk with other phytohormones matters. *Environmental and Experimental Botany*. **145**, 104-120.

Pierce, M. and Raschke, K., 1980. Correlation between loss of turgor and accumulation of Abscisic Acid in detached leaves. *Planta*. **148**, 174-182.

Pinheiro, C., Antonio, C., Ortuno, M. F., Dobrev, P. I., Hartung, W., Thomas-Oates, J., Ricardo, C. P., Vanková. R., Chaves, M. M., Wilson, J. C., 2011. Initial water deficit effects on Lupinus albus photosynthetic performance, carbon metabolism, and hormonal balance: metabolic reorganization prior to early stress responses. *Journal of Experimental Botany*. **62**(14), 4965-4974.

Pospisilova, J., 2003. Participation of phytohormones in the stomatal regulation of gas exchange during water stress. *Biologia Plantarum*. **46**(4), 491-506.

Price, A. H., Cairns, J. E., Horton, P., Jones, H. G., Griffiths, H., 2002. Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. *Journal of Experimental Botany*. **53**(371), 989-1004.

Priest, D. M., Ambrose, S. J., Vaistij, F. E., Elias, L., Higgins, G. S., Ross, A. R. S., . . .& Bowles, D. J., 2006. Use of the glucosyltransferase UGT71B6 to disturb abscisic acid homeostasis in Arabidopsis thaliana. *Plant Journal*, **46**(3), 492-502.

Puertolas, J., Alcobendas, R., Alarcon, J. J., Dodd, I. C., 2013. Long-distance abscisic acid signalling under different vertical soil moisture gradients depends on bulk root water potential and average soil water content in the root zone. *Plant, Cell & Environment*. **36**, 1465-1475.

Qin, X. and Zeevaart, J. A., 2002. Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiology*. **128**(2), 544-551.

Qiu, L. J., Chang, R. Z., 2010. The origin and history of soybean 2010. In: Singh, G. (Ed.), *The Soybean: botany, production, uses*. CAB International, Wallingford, UK, 1–23.

Quarrie, S. A., Whitford, P. N., Appleford, N. E. J., Wang, T. L., Cook, S. K., Henson, I. E., Loveys, B. R., 1988. A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cerealand lupin leaves. *Planta*. **173**, 330-339.

Raghavendra, A. S., Gonugunta, V. K., Christmann, A., Grill, E., 2010. ABA perception and signalling. *Trends in Plant Science*, **15**(7), 395-401.

Raskin, I., 1992. Role of salicylic acid in plants. *Annual review of plant biology*. **43**(1), 439-463.

Reddy, A. R., Chaitanya, K. V., Vivekanandan, M., 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology*. **161**(11), 1189-1202.

Ren, H., Gao, Z., Chen, L., Wei, K., Liu, J., Fan, Y., Davies, W. J., Jia, W., Zhang, J., 2007. Dynamic analysis of ABA accumulation in relation to the rate of ABA catabolism in maize tissues under water deficit. *Journal of Experimental Botany*. **58**, 211-219.

Reymond, P., Weber, H., Damond, M., Farmer, E. E., 2000. Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell*, **12**(5), 707-719.

Rivas, F., Fornes, F., Rodrigo, M. J., Zacarías, L., Agustí, M., 2011. Changes in carotenoids and ABA content in Citrus leaves in response to girdling. *Scientia Horticulturae*. **127**(4), 482-487.

Robert, J. W., 1986. *The soybean solution: Meeting world food needs*. NIT-College of Agriculture, University of Illinois at Urbana, Champaign, USA.

Roberts, M. R., 2016. Jasmonic Acid Signalling. In: eLS. John Wiley & Sons, Ltd: Chichester.

Robert-Seilaniantz, A., Grant, M., Jones, J. D., 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology*. **49**, 317-343.

Rodríguez-Navarro, D. N., Oliver, I. M., Contreras, M. A., Ruiz-Sainz, J. E., 2011. Soybean interactions with soil microbes, agronomical and molecular aspects. *Agronomy for Sustainable Development*. **31**(1), 173-190.

Rossato, L., MacDuff, J. H., Laine, P., Le Deunff, E., Ourry, A., 2002. Nitrogen storage and remobilization in Brassica napus L. during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation. *Journal of Experimental Botany*. **53**(371), 1131-1141.

Rothwell, S. A., Elphinstone, E. D., Dodd, I. C., 2015. Liming can decrease legume crop yield and leaf gas exchange by enhancing root to shoot ABA signalling. *Journal of Experimental Botany*. **66**, 2335-2345.

Sack, L., John, G. P., Buckley, T. N., 2018. ABA accumulation in dehydrating leaves is associated with decline in cell volume, not turgor pressure. *Plant Physiology*. **176**, 489-493.

Saibo, N. J. M., Vriezen, W. H., Beemster, G. T. S., Van Der Straeten, D., 2003. Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. *Plant Journal*. **33**(6), 989-1000.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., Sakata, K., Mizutani, M., 2004. Arabidopsis CYP707As encode (+)-abscisic acid 8'-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. *Plant Physiology*. **134**(4), 1439-1449.

Sakakibara, H., Suzuki, M., Takei, K., Deji, A., Taniguchi, M., Sugiyama, T., 1998. A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. *The Plant Journal*. **14**(3), 337-344.

Salvagiotti, F., Cassman, K. G., Specht, J. E., Walters, D. T., Weiss, A., Dobermann, A., 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. *Field Crops Research*. **108**, 1–13.

Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S. Y., Jamin, M., Cutler, S. R., Rodriguez, P. L., Márquez, J. A., 2009. The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature*. **462**, 665–668.

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V., Flors, V., 2013. Jasmonate signaling in plant development and defense response to multiple (a) biotic stresses. *Plant Cell Reports*. **32**(7), 1085-1098.

Savchenko, T., Kolla, V. A., Wang, C. Q., Nasafi, Z., Hicks, D. R., Phadungchob, B., ... & Dehesh, K., 2014. Functional convergence of oxylipin and abscisic acid pathways controls stomatal closure in response to drought. *Plant Physiology*. **164**(3), 1151-1160.

Schachtman, D. P. and Goodger, J. Q., 2008. Chemical root to shoot signaling under drought. *Trends in Plant Science*. **13**(6), 281-287.

Schilmiller, A. L. and Howe, G. A., 2005. Systemic signaling in the wound response. *Current Opinion in Plant Biology*, **8**(4), 369-377.

Schmittgen, T. D. and Livak, K. J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, **3**(6), 1101-1108.

Schmülling, T., 2002. New insights into the functions of cytokinins in plant development. *Journal of plant growth regulation*. **21**(1).

Schurr, U. and Schulze, E. D., 1996. Effects of drought on nutrient and ABA transport in *Ricinus communis*. *Plant, Cell & Environment*. **19**, 665-674.

Schwartz, S. H., Qin, X., Zeevaart, J. A., 2003. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology*. **131**(4), 1591-1601.

Seiler, C., Harshavardhan, V. T., Rajesh, K., Reddy, P. S., Strickert, M., Rolletschek, H., ... & Sreenivasulu, N., 2011. ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. *Journal of Experimental Botany*. **62**(8), 2615-2632.

Seiler, C., Harshavardhan, V. T., Reddy, P. S., Hensel, G., Kumlehn, J., Eschen-Lippold, L., ... & Selvaraj, G., 2014. Abscisic acid flux alterations result in differential abscisic acid signaling responses and impact assimilation efficiency in barley under terminal drought stress. *Plant Physiology*. **164**(4), 1677-1696.

Sellin, A., Niglas, A., Õunapuu, E., Karusion, A., 2013. Impact of phloem girdling on leaf gas exchange and hydraulic conductance in hybrid aspen. *Biologia plantarum*. **57**(3), 531–539.

Seo, M., Aoki, H., Koiwai, H., Kamiya, Y., Nambara, E., Koshiba, T., 2004. Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. *Plant and Cell Physiology*. **45**(11), 1694-1703.

Serraj, R. and Sinclair, T. R., 2002. Osmolyte accumulation: can it really help increase crop yield under drought conditions?. *Plant, Cell & Environment*. **25**(2), 333-341.

Setter, T. L., 2012. Analysis of constituents for phenotyping drought tolerance in crop improvement. *Frontiers in Physiology*. **3**, 180.

Setter, T. L., Brun, W. A., Brenner, M. L., 1980. Effect of obstructed translocation on leaf Abscisic Acid, and associated stomatal closure and photosynthesis declinel. *Plant Physiology*. **65**, 1111-1115.

Setter, T. L. and Brun, W. A., 1981. Abscisic Acid translocation and metabolism in soybeans following depodding and petiole girdling treatments. *Plant Physiology*. **67**, 774-779.

Sharp, R. E. and LeNoble, M. E., 2002. ABA, ethylene and the control of shoot and root growth under water stress. *Journal of Experimental Botany*. **53**(366), 33-37.

Sharp, R. E., 2002. Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant, Cell & Environment*. **25**(2), 211-222.

Sharp, R. E., LeNoble, M. E., Else, M. A., Thorne, E. T., Gherardi, F., 2000. Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: evidence for an interaction with ethylene. *Journal of Experimental Botany.* **51**(350), 1575-1584.

Sheard, L. B., Tan, X., Mao, H. B., Withers, J., Ben-Nissan, G., Hinds, T. R., . . .& Zheng, N., 2010. Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature*. **468**(7322), 400-U301.

Shinozaki, K. and Yamaguchi-Shinozaki, K., 1997. Gene expression and signal transduction in water-stress response. *Plant Physiology*. **115**(2), 327-334.

Shinozaki, K. and Yamaguchi-Shinozaki, K., 2007. Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany.* **58**(2), 221-227.

Shinozaki, K., 2007. Acceleration of soybean genomics using large collections of DNA markers for gene discovery. *DNA Research*. **14**(6), 235-235.

Singh, R. J., 2006. *Genetic Resources, Chromosome Engineering, and Crop Improvement: Vegetable Crops*. CRC Press, London, UK

Slovik, S., Daeter, W., Hartung, W., 1995. Compartmental redistribution and long-distance transport of abscisic acid (ABA) in plants as influenced by environmental changes in the rhizosphere - a biomathematical mode. *Journal of Experimental Botany*. **46**, 881-894..

Sobeih, W. Y., Dodd, I. C., Bacon, M. A., Grierson, D., Davies, W. J., 2004. Long-distance signals regulating stomatal conductance and leaf growth in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying. *Journal of Experimental Botany*. **55**(407), 2353-2363.

Solomon, S., Qin, D., Manning, M., Marquis, M., Averyt, K., Tignor, M., 2007. IPCC: climate change 2007: the physical science basis. In: Miller HL, Chen Z, editors. *Contribution of working groups I, II, and III to the fourth assessment report of the intergovernmental panel on climate change*. Cambridge, UK: Cambridge University Press;

Sperry J. S., 2000. Hydraulic constraints on gas exchange. *Agricultural and Forest Meteorology*. **104**, 13–23.

Spollen, W. G., LeNoble, M. E., Samuels, T. D., Bernstein, N., Sharp, R. E., 2000. Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. *Plant Physiology*. **122**(3), 967-976.

Staswick, P. E. and Tiryaki, I., 2004. The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell*. **16**(8), 2117-2127.

Stratmann, J. W., 2003. Long distance run in the wound response - jasmonic acid is pulling ahead. *Trends in Plant Science*. **8**(6), 247-250.

Sulieman, S., Ha, C. V., Nasr Esfahani, M., Watanabe, Y., Nishiyama, R., Pham, C. T. B., Nguyen, D. V., Tran, L. S. P., 2015. DT2008: A promising new genetic resource for improved drought tolerance in soybean when solely dependent on symbiotic N2 fixation. *BioMed Research International*. 1–7.

Sun, T. P. and Gubler, F., 2004. Molecular mechanism of gibberellin signaling in plants. *Annual Review of Plant Biology*. **55**, 197-223.

Sussmilch, F. C., Brodribb, T. J., McAdam, S. A. M., 2017. Up-regulation of NCED3 and ABA biosynthesis occur within minutes of a decrease in leaf turgor but AHK1 is not required. *Journal of Experimental Botany*. **68**(11), 2913-2918.

Szilagyi, L., 2003. Influence of drought on seed yield components in common bean. *Bulgarian Journal of Plant Physiology*. (Special issue), 320-330.

Taiz, L. and Zeiger, E., 2010. *Plant physiology* 5th edition. Sunderland, Massachusetts, USA: Sinauer Assosiates inc., puplishers.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., ... & Sakakibara, H., 2004. AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. *Plant and Cell Physiology*. **45**(8), 1053-1062.

Tan, B. C., Joseph, L. M., Deng, W. T., Liu, L., Li, Q. B., Cline, K., McCarty, D. R., 2003. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *The Plant Journal*, **35**(1), 44-56.

Tang, J., Riley, W. J., Niu, J., 2015. Incorporating root hydraulic redistribution in CLM 4.5: Effects on predicted site and global evapotranspiration, soil moisture, and water storage. *Journal of Advances in Modeling Earth Systems*. **7**(4), 1828-1848.

Tardieu, F. and Davies, W. J., 1992. Stomatal response to abscisic acid is a function of current plant water status. *Plant Physiology*. **98**(2), 540-545.

Tardieu, F. and Davies, W. J., 1993. Integration of hydraulic and chemical signaling in the control of stomatal conductance and water status of droughted plants. *Plant Cell and Environment*. **16**(4), 341-349.

Tardieu, F. and Tuberosa, R., 2010. Dissection and modelling of abiotic stress tolerance in plants. *Current Opinion in Plant Biology*. **13**(2), 206-212.

Tardieu, F., 2013. Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit. *Frontiers in Physiology*. **4**, 17.

Tardieu, F., Lafarge, T., Simonneau, T., 1996. Stomatal control by fed or endogenous xylem ABA in sunflower: interpretation of correlations between leaf water potential and stomatal conductance in anisohydric species. *Plant, Cell & Environment*. **19**, 75-84.

Tran, L. S. P., Quach, T. N., Guttikonda, S. K., Aldrich, D. L., Kumar, R., Neelakandan, A., ... & Nguyen, H. T., 2009. Molecular characterization of stress-inducible GmNAC genes in soybean. *Molecular Genetics and Genomics*, **281**(6), 647-664.

Tran, L. S. P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., Yamaguchi-Shinozaki, K., 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proceedings of the National Academy of Sciences*. **104**(51), 20623-20628.

Trejo, C. L. and Davies, W. J., 1991. Drought-induced closure of *Phaseolus vulgaris* L. stomata precedes leaf water deficit and any increase in xylem ABA concentration. *Journal of Experimental Botany.* **42**(245), 1507-1515.

Trewavas, A. J., Jones, H. G., 1991. An assessment of the role of ABA in plant development. In: Davies WJ, Jones HG, editors. *Abscisic acid: physiology and biochemistry*. Oxford: Bio Scientific Publishers. 169–188.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., ... & Shinozaki, K., 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proceedings of the National Academy of Sciences*. **106**(41), 17588-17593.

USDA Statistics (United States Department of Agriculture - National Agricultural Statistics Service), 2018. USDA-NASS. https://www.nass.usda.gov/Data_and_Statistics/index.php.

Valluru, R., Davies, W. J., Reynolds, M. P., Dodd, I. C., 2016. Foliar abscisic acid-to-ethylene accumulation and response regulate shoot growth sensitivity to mild drought in wheat. *Frontiers in Plant Science*. **7**, 461.

Vanstraelen, M. and Benková, E., 2012. Hormonal interactions in the regulation of plant development. *Annual Review of Cell and Developmental Biology*. **28**, 463-487.

Vernieri, P., Lenzi, A., Figaro, M., Tognoni, F., Pardossi, A., 2001. How the roots contribute to the ability of *Phaseolus vulgaris* L. to cope with chilling-induced water stress. *Journal of Experimental Botany*. **52**, 2199-2206.

Vick, B. A. and Zimmerman, D. C., 1983. The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. *Biochemical and Biophysical Research Communications*. **111**(2), 470-477.

Vignes, D., Djekoun, A., Planchon, C., 1986. Reponses de differents genotypes de soja au déficit hydrique. *Canadian journal of plant science*. **66**, 247–255.

Vikram, P., Swamy, B. M., Dixit, S., Ahmed, H. U., Cruz, M. T. S., Singh, A. K., Kumar, A., 2011. qDTY 1.1, a major QTL for rice grain yield under reproductive-stage drought stress with a consistent effect in multiple elite genetic backgrounds. *BMC genetics*. **12**(1), 89.

Visentin, I., Vitali, M., Ferrero, M., Zhang, Y., Ruyter-Spira, C., Novak, O., Strnad, M., Lovisolo, C., Schubert, A., Cardinale, F., 2016. Low levels of strigolactones in roots as a component of the systemic signal of drought stress in tomato. *New Phytologist*. **212**, 954-963.

Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., ... & Merlot, S., 2009. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. *The Plant Cell*, **21**(10), 3170-3184.

Vlot, A. C., Dempsey, D. M. A., Klessig, D. F., 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology*. **47**, 177-206.faru

Voisin, A. S., Reidy, B., Parent, B., Rolland, G., Redondo, E., Gerentes, D., ... & Muller, B., 2006. Are ABA, ethylene or their interaction involved in the response of leaf growth to soil water deficit? An analysis using naturally occurring variation or genetic transformation of ABA production in maize. *Plant, Cell & Environment*, **29**(9), 1829-1840.

Waadt, R., Hitomi, K., Nishimura, N., Hitomi, C., Adams, S. R., Getzoff, E. D., Schroeder, J. I., 2014. FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. *ELife*, **3**, e01739.

Wang, G. X., Zhang, J., Liao, J. X., Wang, J. L., 2001. Hydropassive evidence and effective factors in stomatal oscillations of *Glycyrrhiza inflata* under desert conditions. *Plant Science*. **160**(5), 1007-1013.

Wang, K. L. C., Li, H., Ecker, J. R., 2002. Ethylene biosynthesis and signaling networks. *The Plant Cell*. **14**(suppl 1), S131-S151.

Wang, W., Vinocur, B., Altman, A., 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*. **218**(1), 1-14.

Wang, X. J., Guo, C., Peng, J., Li, C., Wan, F. F., Zhang, S. M., . . .& Li, J. G., 2019. ABRE-BINDING FACTORS play a role in the feedback regulation of ABA signaling by mediating rapid ABA induction of ABA co-receptor genes. *New Phytologist*. **221**(1), 341-355.

Wang, Y., Ying, J., Kuzma, M., Chalifoux, M., Sample, A., McArthur, C., ... & McCourt, P., 2005. Molecular tailoring of farnesylation for plant drought tolerance and yield protection. *The Plant Journal*. ***43***(3), 413-424.

Wani, S. H., Kumar, V., Shriram, V., Sah, S. K., 2016. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal*, **4**(3), 162-176.

Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., ... & Leung, J. (2008). An update on abscisic acid signaling in plants and more…. *Molecular plant*. **1**(2), 198-217.

Wasternack, C. and Hause, B., 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of botany*. **111**(6), 1021-1058.

Wasternack, C. and Kombrink, E., 2009. Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. *ACS Chemical Biology*. **5**(1), 63-77.

Wasternack, C. and Song, S. S., 2017. Jasmonates: biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. *Journal of Experimental Botany*. **68**(6), 1303-1321.

Wasternack, C., 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of botany*, **100**(4), 681-697.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., Schmülling, T., 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant Cell*, **15**(11), 2532-2550.

Wilkinson, S. and Davies, W. J., 2002. ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant Cell & Environment*. **25**(2), 195-210.

Wilkinson, S. and Davies, W. J., 2008. Manipulation of the apoplastic pH of intact plants mimics stomatal and growth responses to water availability and microclimatic variation. *Journal of Experimental Botany*. **59**(3), 619-631.

Wilkinson, S. and Davies, W. J., 2010. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, Cell & Environment*, **33**(4), 510-525.

Wilkinson, S., Kudoyarova, G. R., Veselov, D. S., Arkhipova, T. N., Davies, W. J., 2012. Plant hormone interactions: innovative targets for crop breeding and management. *Journal of Experimental Botany.* **63**(9), 3499-3509.

Windsor, M. L. and Zeevaart, J. A. D., 1997. Induction of ABA 8'-hydroxylase by (+)-S-,(-)-R- and 8',8',8'-trifluoro-S-abscisic acid in suspension cultures of potato and Arabidopsis. *Phytochemistry*. **45**(5), 931-934.

Wingeyer, A. B., Amado, T. J., Pérez-Bidegain, M., Studdert, G. A., Varela, C. H. P., Garcia, F. O., Karlen, D. L., 2015. Soil quality impacts of current South American agricultural practices. *Sustainability*. **7**(2), 2213-2242.

Woldemariam, M. G., Baldwin, I. T., Galis, I., 2011. Transcriptional regulation of plant inducible defenses against herbivores: a mini-review. *Journal of Plant Interactions*. **6**(2-3), 113-119.

Wolf, O., Jeschke, W. D., Hartung, W., 1990. Long distance transport of Abscisic Acid in NaCl-treated intact plants of *Lupinus albus*. *Journal of Experimental Botany*. **41**, 593-600.

Wolters, H. and Jurgens, G., 2009. Survival of the flexible: hormonal growth control and adaptation in plant development. *Nature Reviews Genetics*. **10**(5), 305-317.

Yamaguchi, S., 2008. Gibberellin metabolism and its regulation. *Annual Review Plant Biology*. **59**, 225-251.

Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review Plant Biology*. **57**, 781–803.

Yang, S. H. and Zeevaart, J. A., 2006. Expression of ABA 8'-hydroxylases in relation to leaf water relations and seed development in bean. *The Plant Journal***. 47**, 675–686.

Ye, N., Zhu, G., Liu, Y., Li, Y., Zhang, J., 2011. ABA controls H2O2 accumulation through the induction of OsCATB in rice leaves under water stress. *Plant and Cell Physiology*. **52**(4), 689-698.

Yin, Y., Adachi, Y., Nakamura, Y., Munemasa, S., Mori, I. C., Murata, Y., 2016. Involvement of OST1 Protein Kinase and PYR/PYL/RCAR Receptors in Methyl Jasmonate-Induced Stomatal Closure in Arabidopsis Guard Cells. *Plant and Cell Physiology*. **57**(8), 1779-1790.

Yoshida, T., Fujita, Y., Maruyama, K., Mogami, J., Todaka, D., Shinozaki, K., Yamaguchi-Shinozaki, K., 2015. Four Arabidopsis AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress. *Plant, Cell & Environment*. **38**(1), 35-49.

Zhang, B., Chen, P., Florez-Palacios, S. L., Shi, A., Hou, A., Ishibashi, T., 2010. Seed quality attributes of food-grade soybeans from the US and Asia. *Euphytica*. **173**, 387–396.

Zhang, F. P., Sussmilch, F., Nichols, D. S., Cardoso, A. A., Brodribb, T. J., McAdam, S. A. M., 2018. Leaves, not roots or floral tissue, are the main site of rapid, external pressure-induced ABA biosynthesis in angiosperms. *Journal of Experimental Botany*. **69**(5), 1261-1267.

Zhang, J. and Davies, W. J., 1990. Changes in the concentration of ABA in xylem sap as a function of changing soil water status can account for changes in leaf conductance and growth. *Plant, Cell & Environment*. **13**(3), 277-285.

Zhang, J. and Davies, W. J., 1991. Antitranspirant activity in xylem sap of maize plants. *Journal of Experimental Botany*. **42**, 317-321.

Zhang, J. and Davies, W. J., 1989. Abscisic acid produced in dehydrating roots may enable the plant to measure the water status of the soil. *Plant, Cell & Environment*, **12**(1), 73–81.

Zhang, J. and Davies, W. J., 1989. Sequential response of whole plant water relations to prolonged soil drying and the involvement of xylem sap ABA in the regulation of stomatal behavior of sunflower plants. *New Phytologist*. **113**, 167–174.

Zhang, J., Jia, W., Yang, J., Ismail, A. M., 2006. Role of ABA in integrating plant responses to drought and salt stresses. *Field Crops Research*. **97**(1), 111-119.

Zheng, Y., Huang, Y., Xian, W., Wang, J., Liao, H., 2012. Identification and expression analysis of the Glycine max CYP707A gene family in response to drought and salt stresses. *Annals of Botany*. **110**(3), 743-756.

Zhou, R., Cutler, A. J., Ambrose, S. J., Galka, M. M., Nelson, K. M., Squires, T. M., ... & Abrams, S. R., 2004. A new abscisic acid catabolic pathway. *Plant Physiology*. **134**(1), 361-369.

Zwack, P. J. and Rashotte, A. M., 2015. Interactions between cytokinin signalling and abiotic stress responses. *Journal of Experimental Botany*. **66**(16), 4863-4871.