Plant Root Development and Hormone Signalling during Drought Stress

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Thesis submitted for the degree of Ph.D. at Lancaster University

March 2016

Declaration

Except where reference is 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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March 2016

Acknowledgements

I would like to thank my supervisors, Professor Bill Davies and Professor Brian Forde for your precious support of my PhD study. Your patience, encouragement, immense knowledge and wisdom helped me overcome various difficulties and challenges during this research and assisted me to develop as a researcher.

I am grateful to Professor Fusuo Zhang and Professor Jianbo Shen in China Agriculture University (Beijing, China) for recommending me to study in Lancaster University four years ago. I also appreciate Dr. Sally Wilkinson for her guidance in the very beginning of this research.

This thesis would not have progressed smoothly without the collaboration with my colleague and friend Dr. Lin Chen. Thank you for training me important laboratory skills and discussing some of the experiments with me. I also thank you for your companionship during my studies.

I express my gratitude to Dr. Alfonso Albacete and his friendly colleagues in CSIC (Murcia, Spain) for their assistance in the analysis of plant hormone samples. I am also grateful to Professor Ian Dodd for his help with my studies and building up the collaboration with Dr. Alfonso Albacete.

I also would like to thank the skilful technicians, scientific researchers, academic visitors and students in the Lancaster Environment Centre for their practical help and meaningful discussions: Shane Rothwell, Jaime Puértolas, Annette Ryan, Carlos José De Ollas Valverde, Debra Hurst, Maureen Harrison, Geoffrey Holroyd, Dawn Worrall, Alla Korobova, Antje Fiebig, Guofeng Hu, Lucas Gent, Richard Boyle, Sarah Donaldson, Hannah Wright, Dionysios Touliatos, Rhydian Beynon-Davies, Juan Gabriel Pérez-Pérez, Ascen Martínez Pérez, Martina Fojtů, Noorliana Mohd Zan, Phillip Nott, Wei Chen and Ying Wang .

All of my affable colleagues and friends in the Plant and Crop Science Group and in the Chinese community at Lancaster, I am very glad to meet all of you here during past four years. Thank you for your enormous help and companionship during my study here and for creating an enjoyable living and research environment.

I give my thanks for the financial support from the China Scholarship Council, the China Bridge project and the EU FP7 project EURoot.

Finally, I would like to express my gratitude to my family: my parents, parents-in-law and sister-in-law for all of their unlimited understanding and cheering. I give special thanks to my great husband, Xiaoxi Li, for always being with me during these years, taking care of me, helping with my lab work and providing constructive comments on my thesis writing.

Abstract

The plant root system is crucial for plant survival, growth and development, and it plays an important role in plant resistance to drought stress. Drought is one of the primary factors that restrict plant growth and yield, and its threat to crop yields will increase along with the growing food demand by the population of a world experiencing a changing climate. In response to drought in plants, various hormones are vital regulators, because they are able to manipulate plant development and in some cases minimise the adverse impact of drought. Therefore, understanding how the plant root system will adapt to a soil drying challenge is crucial. Of particular importance is the plant response to a non-lethal drought stress, which is often encountered in the field. Elucidation of the mechanisms underlying such responses, including hormonal regulations, may help crop scientists improve the plant performance under drought.

A six-day progressive soil drying pot experiment was designed to examine the synchronisation of physiological responses in maize (*Zea mays* L.) roots and leaves during soil drying. It was found that maize roots showed earlier responses to soil drying than leaves in changing growth rates, water potentials and hormone levels. Root growth was stimulated at soil water content of 25–32% (ca. 41% in well-watered pots), while both root growth and leaf elongation were inhibited when soil water content was below 20%. Root abscisic acid (ABA) level gradually increased when soil water content was lower than 32% during soil drying. The stimulation and inhibition of root growth during soil drying may be regulated by root ABA, depending on the degree of the concentration increase. The ethylene release rates from leaves and roots were inhibited during soil drying, which occurred later than the increase in ABA levels.

In a subsequent root phenotyping study on 14 maize genotypes, significant genetic variation was observed in root angle and size (root length, surface area and dry weight), and in the plasticity of these traits under mild and severe drought stress. Genotypes with a steeper root angle under well-watered conditions tended to display more promotion or less inhibition in root size under drought. Further analysis showed that combined traits of maize root angle, its plasticity and the root size plasticity under drought may be a better predictor for maize drought resistance than a single one of these traits. Moreover, root angle was found positively related to the leaf and root ABA levels and negatively related to the root *tZ* (a cytokinin) level under well-watered conditions.

In another study on the crosstalk of drought-related hormones using the model plant *Arabidopsis thaliana* L., the biphasic responses of root elongation to ABA were confirmed, i.e. low external ABA concentrations stimulated root growth while high ABA concentrations inhibited it. Furthermore, ethylene and auxin were found to be involved in these responses. The inhibitory effect of high ABA levels on root growth was reduced or even eliminated when Arabidopsis was chemically treated to inhibit the ethylene biosynthesis or signalling, or to block auxin influx carriers. This was confirmed using mutants with blocked ethylene or auxin signalling, or a defect in the

auxin influx carrier AUX1. On the other hand, the stimulatory effect of low ABA levels on root growth was lost when Arabidopsis seedlings were chemically treated to inhibit the auxin efflux carriers, and in mutants with blocked auxin signalling or with a defect in the PIN2/EIR1 auxin efflux carrier. These results indicate that ABA regulates root growth through two distinct pathways. The inhibitory effect that operates at high ABA concentrations is via an ethylene-dependent pathway and requires auxin signalling and auxin influx through AUX1. The stimulatory effect that operates at low ABA concentrations is via an ethylene-independent pathway and also requires auxin signalling and auxin efflux through PIN2/EIR1.

This research contributes to our understanding of the responses of plant root system to different degrees of non-lethal drought stress, and it highlights the importance of root traits that may be important to plant drought resistance. The potential involvement of hormones (ABA, ethylene, auxin and cytokinin) in these processes is clarified. The knowledge gained may be integrated in novel crop management strategies to plan irrigation and help in the development of drought resistant crop varieties.

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Abbreviations

[^{3}H]ABA	Isotope ^{3}H-labelled ABA
[ABA]	Abscisic acid concentration
ABA	Abscisic acid
ABCB	ATP-binding cassette group B
ABI	ABA insensitive
ACC	1-aminocycopopane-1-carboxylic acid
AFB	Auxin-related F-box
Ag^+	Silver ion
AREBs/ABFs	ABA response elements binding factors
ARF	Auxin response factor
AUX/IAA	Auxin/indole-3-acetic acid
AUX1	Auxin 1 (auxin influx transporter)
AVG	Aminoethoxyvinylglycine (ethylene biosynthesis inhibitor)
CE	Controlled-environment
ChlH	Magnesium cheletase
CHPAA	3-chloro-4-hydroxyphenylacetic acid (auxin influx inhibitor)
CKX	Cytokinin oxidase/dehydrogenase gene
CSIC	The Spanish National Research Council
CTR	Constitutive triple response
cZ	*cis*-zeatin
df	Degrees of freedom
DMSO	Dimethyl sulfoxide
DW	Dry weight
DZ	Dihydrozeatin
EIL	EIN3-like
EIN	Ethylene insensitive
EREBPs	Ethylene-responsive element binding proteins
ERFs	Ethylene response factor genes
ERS	Ethylene response sensor
ETR	Ethylene resistant
GA	Gibberelline acid
GC	Gas chromatography
GFP	Green fluorescent protein
GTGs	GPCR type G-proteins
H_2O_2	Hydrogen peroxide
HPLC-MS	High performance liquid chromatography-mass spectrometry

IAA	Indole-3-acetic acid
iP	Isopentenyladenine
JA	Jasmonic acid
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
LAX	Like-AUX1
MAC 252	Anti-abscisic acid antibody
MD	Mean differences
memT	Methoxy-*m*T
meoT	Methoxy-*o*T
***m*T**	*meta*-topolin
NA	Not available
NaCl	Sodium chloride
NPA	N-1-naphthylphthalamidic acid (auxin efflux inhibitor)
***o*T**	*ortho*-topolin
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PGP	Phosphogylco protein
PIN	Pin formed (auxin influx transporter)
PP2C	Clade A type 2C protein phosphatases
PYR/PYL/RCARs	Pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor
QC	Quiescent centre
QTL	Quantitative trait locus
RWC	Relative water content
SA	Salicylic acid
SnRK2	Group III sucrose non-fermenting1-related protein kinase 2
SS	Sums of squares
STS	Silver thiosulfate (ethylene signalling inhibitor)
TIBA	2,3,5-triiodobenzoic acid (auxin efflux inhibitor)
TIR	Transport inhibitor response
***t*Z**	*trans*-zeatin

Arabidopsis Lines

Wild-type

Col-8

ABA signalling mutant

snrk2.2/2.3/2.6

Auxin efflux mutants

pin2/eir1-1
pin3-4
pin3-5
pin4-3
pin7-2

Auxin influx mutants

aux1-T
aux1-7

Auxin reporter line

DR5::GFP

Auxin signalling mutants

iaa7/axr2-1
tir1-1

Cytokinin signalling mutant

ahk2 ahk3

Ethylene signalling mutants

etr1-1
ein2-1
ein3-1
eil1

Chapter 1 General Introduction

The world population is over seven billion and it is projected to reach nine billion by 2050 (Godfray *et al.*, 2010). Food, fibre and energy will be increasingly in demand (Evans, 1999; Godfray *et al.*, 2010) and it is predicted that food production will need to double by 2050 in order to feed the growing population (Tilman *et al.*, 2011). Agriculture is facing great challenges to increase the availability of food. Also, there is fast growing demand for meat and dairy products as economies grow (Godfray *et al.*, 2010). Feeding animals consumes almost one third of the global crop production (Godfray *et al.*, 2010). A challenge for agricultural science is to develop crops, which are adapted to the various abiotic environmental stresses that are currently limiting production (Araus *et al.*, 2012; Masuka *et al.*, 2012). Water deficit stress is one of the principal factors that greatly limit plant growth and yield development (Kramer and Boyer, 1995; Chaves and Oliveira, 2004). Water deficit can be caused by drought, salinity or freezing stress (Boyer, 1982). When water supplied to plants is less than the demand, water deficit occurs and prevents plants from realising the genetic yield potential even when other growing conditions are favourable (Lawlor, 2013). Additionally, the threat of drought to agriculture is predicted to increase under climate change (Easterling *et al.*, 2000, Tardieu, 2012; Porter *et al.*, 2014). The rainfall variability and the risk of high temperatures at critical crop developmental stages are projected to increase in the coming decades (Battisti and Naylor, 2009; Tardieu, 2012; Porter *et al.*, 2014) and thus significantly impact agricultural production (Tebaldi and Lobell, 2008; Tardieu, 2012).

Maize (*Zea mays* L.) is one of the world's three staple crops with rice and wheat, and maize generally has higher potential yield than the other two (Varshney *et al.*, 2012). Maize production will see a reduction in the face of the reduced availability of water in the near future (Rosegrant *et al.*, 2002). Since the 1930s, conventional breeding in maize has improved its production markedly under various environments including drought conditions (Duvick, 2005). However, it has been suggested that those traits that contribute to maize yield improvement under stressed conditions in the past decades (e.g. upright leaves, smaller tassels) have reached their potential for further selection (Duvick, 2005). Skirycz *et al.* (2011) argue that our relative lack of success in drought resistance improvement may be because lethal drought conditions are often imposed when selecting drought resistant plants. This may not help to predict the drought resistance of the same plants under moderate drought conditions, which are often encountered in the field. Additionally, the timing of drought stress occurring in plants is crucial (Boonjung and Fukai, 1996a, b; Tardieu, 2011).

Therefore, in order to address the challenges from both the growing population and the likely increase in the severity and frequency of drought stress, more research is needed to understand and improve plant drought resistance.

1.1 Drought stress

Drought occurs in all climatic regimes, including the high and low rainfall areas (Wilhite and Glantz, 1985). Compared with aridity, which is a permanent feature of climate in low rainfall regions, drought is a temporary aberration (Wilhite, 2010). In general, drought is mainly related to a decline in rainfall over a period of time

(Mishra and Singh, 2010). High temperature, high wind speeds, and low relative humidity also play important roles in inducing drought (Mishra and Singh, 2010). However, it is not possible to have a universal definition for drought due to various difficulties summarised by Wilhite and Glantz (1985). From the perspective of different disciplines, there are four categories of drought (meteorological, agricultural, hydrological and socio-economic) (Wilhite and Glantz, 1985). An 'agricultural drought' usually refers to the soil moisture declining during a period caused by below-average precipitation, less frequent rain events, or above-normal evaporation, which lead to diminished plant growth and production (Mishra and Singh, 2010; Dai, 2011). Differences in the intensity, duration and spatial coverage differentiate one drought from another (Wilhite and Glantz, 1985; Wilhite, 2010). Soil moisture content is often used to characterise agricultural drought and it is critical for the realisation of crop production potentials (Wilhite, 2010). Other indices such as precipitation and temperature have also been adopted to indicate the degree of agricultural drought (Mishra and Singh, 2010).

The soil moisture status can be indicated by several variables, e.g. soil water potential (MPa, bar) and soil water content (% of w/w or v/v) (Or and Wraith, 2002). Because of differences in soil texture, the water content at given water potential can differ from soil to soil. Important variables are saturation, field capacity, wilting point and the amount of plant available water (Figure 1.1). The degree of the drought stress challenge (i.e. drought severity) is difficult to describe accurately using only soil water content (Or and Wraith, 2002; Wilhite, 2010). The drought stress level for a plant is closely related to the amount of water it demands, which depends on the prevailing weather condition, the biological characteristics and the growth stage of

the plant, and the physical and biological properties of the soil (Mishra and Singh, 2010). Four classes are commonly used to characterize drought intensity i.e. well-watered condition, mild, moderate and severe drought. However, there is no specific definition of the range of soil or plant water potential for these four classes.

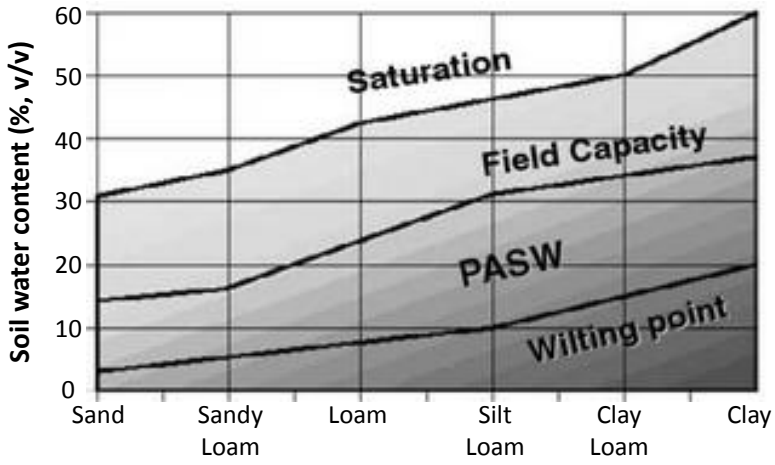


Figure 1.1: The estimated soil water content (volumetric) at the plant wilting point, field capacity and saturation, and the plant-available soil water content (PASW) in a range of soil textural classes (modified from Or and Wraith, 2002).

Water movement from soil into roots is mainly driven by the water potential gradient between the root and the soil. Water always moves from high potential to low potential in the soil-plant system (Kramer and Boyer, 1995). The highest water potential is zero and provides the resistance to water movement is not insurmountable, water will move to a location or compartment where the water potential is reduced by e.g. negative pressures, surface forces or osmotic forces (Kramer and Boyer, 1995). Soil water potential is highly heterogeneous, especially in vertical distribution. Normally, surface soil has lower water potential than deeper soil, because of gravity and evaporation from the soil surface (Sharp and Davies,

1985; Or and Wraith, 2002). For most plants, the soil water potential at the permanent wilting point is around −1.5 MPa (Kramer and Boyer, 1995).

Plant water status is often used to indicate the degree of drought stress experienced by plants under a soil drying challenge (Hsiao, 1973; Sinclair and Ludlow, 1985). For example, the maximum water content of a leaf is regarded as 100 % when it is fully saturated. By the equation [(Fresh weight − Dry weight) / (Saturated weight − Dry weight)] × 100%, the relative water content (RWC) of a leaf can be calculated (Hsiao, 1973; Lafitte, 2002). When a plant leaf exhibits a RWC of around 100%, generally the plant is well supplied with water; it is under mild drought stress when the RWC drops by 8−10%; it is under moderate drought when RWC is reduced by 10−20%, and if the RWC declines by more than 20%, it is under severe drought stress (Hsiao, 1973). These water contents will vary in plants with different dry weights and cell wall thickening, which is why comparative water potentials are generally used to compare the water status of plants (Hsiao, 1973).

Plants use different strategies to cope with different degrees of drought (avoidance and tolerance), including numerous responses to avoid water loss, continue water uptake at low soil moisture contents or tolerate a low tissue water content, and thereby minimise the reduction of crop growth and yield under drought (Verslues *et al.*, 2006; Lawlor, 2013). These avoidance and tolerance strategies are accomplished through a range of traits, such as reducing stomatal conductance and leaf area, changing root and shoot growth to enhance root to shoot ratio and maintaining turgor pressure by reducing the solute potential (osmotic adjustment) (Kramer and Boyer, 1995; Zhang *et al.*, 1999; Lawlor, 2013). Among these responses,

effects on root growth (e.g. stimulated growth) have been suggested to play important roles in crop plants under a relative mild drought since they can help maintain water uptake (Verslues *et al.*, 2006; Kano *et al.*, 2011). Interestingly, Tardieu (2012) noted that most drought relevant traits show dual effects: they may show positive effect under severe drought stress but negative effect under milder stress, or vice versa. Most of the genes that are found to be important for drought resistance in mature leaves under severe drought stress showed little effect on drought resistance under mild drought (Skirycz *et al.*, 2010). Drought resistant plants that were screened under severe drought normally show constitutive activation of mechanisms for saving water (e.g. stomatal closure), which can lead to a growth penalty (Kasuga *et al.*, 1999; Davies *et al.*, 2011; Skirycz *et al.*, 2011). Moreover, it has been suggested that apart from the severities of drought stress, the plant developmental stages will affect its response to drought (Boonjung and Fukai, 1996a, b; Tardieu, 2012). Therefore, understanding the mechanisms underlying plant growth regulation at a certain developmental stage, especially root developmental changes, in response to non-lethal drought (i.e. mild or moderate) stress is important to improve plant performance in drought resistance.

1.2 Plant root growth as soil dries

Arabidopsis and maize root systems

The root has many critical functions for a living plant, of which anchorage and acquisition of water and nutrients are the most important (López-Bucio *et al.*, 2003; Hochholdinger *et al.*, 2004). In higher plants, the root system architecture is highly diverse in terms of morphology and anatomy (Hochholdinger *et al.*, 2004; Osmont *et*

al., 2007; Hodge *et al*., 2009). *Arabidopsis thaliana* L. is one of the most widely used model plants in biological research, and the root morphology of this dicotyledonous plant is relatively simple (Malamy and Benfey, 1997a, b). Like many dicotyledonous plants, the root system of Arabidopsis consists of one embryonic primary root and branched lateral roots from the pericycle founder cells of the primary root (Osmont *et al*., 2007; Péret *et al*., 2009; Figure 1.2A). In the Arabidopsis root there is one layer of endodermal and cortical cells, and the cortical cell layer contains eight circumferential cells (Hochholdinger *et al*., 2004; Figure 1.2E, F). The typical Arabidopsis root tip is subdivided into four zones longitudinally, i.e. root cap, meristem, elongation zone and differentiation zone (Overvoorde *et al*., 2010; Figure 1.2D).

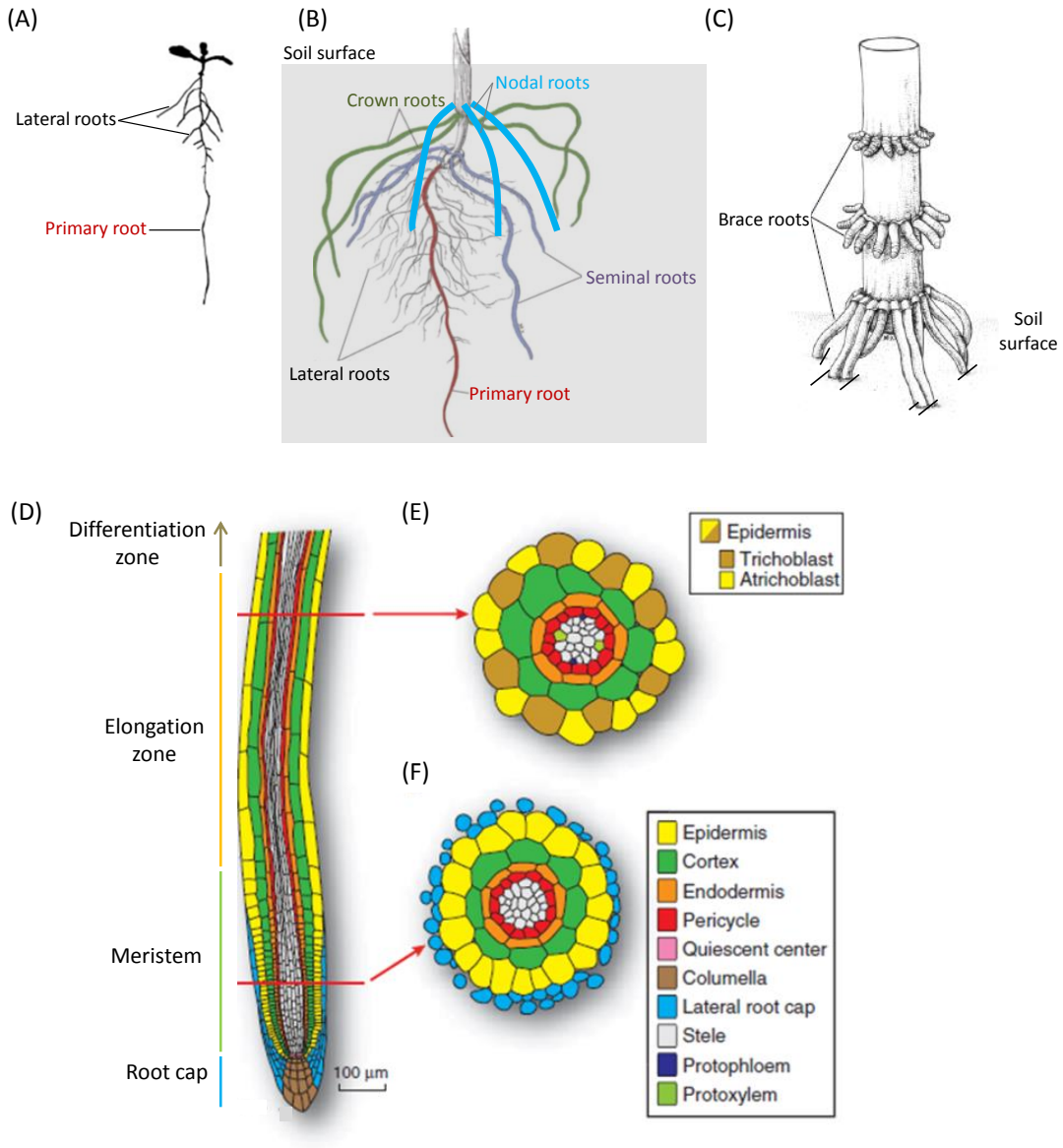


Figure 1.2: Root morphology and anatomy. (A) Arabidopsis primary and lateral roots (modified from Osmont *et al.*, 2007). (B) Maize root system (modified from Hochholdinger and Tuberosa, 2009). (C) Maize brace root (modified from Hochholdinger *et al.*, 2004). (D) The root cap, meristem, elongation zone and differentiation zone in an Arabidopsis root tip. (E) Cross section of the Arabidopsis root in the elongation zone to highlight the one endodermal cell circle and the one cortical cell circle consisted of eight cells. (F) Cross section of Arabidopsis root in the meristem zone. (D–F modified from Overvoorde *et al.*, 2010).

In contrast to Arabidopsis, maize is a monocotyledonous cereal plant. The maize root system consists of one primary and a few seminal roots that are embryonic roots, several whorls of shoot-borne roots and numerous lateral roots that are

postembryonic (Hochholdinger and Tuberosa, 2009; Figure 1.2B, C). The shoot-borne roots include the crown and nodal root, as well as the brace root if they formed above the soil surface (Hochholdinger *et al.*, 2004; Lynch, 2013). The primary root develops first after germination; then the seminal roots start; lastly, the shoot-borne roots initiate and the root development is coordinated with the shoot development (Foth, 1962; Yu *et al.*, 2014). All of these roots are able to branch and form several classes of lateral roots, which derive from pericycle and endodermal cells in order (Bell and McCully 1970; Yu *et al.*, 2014). Lateral roots are thinner than other types of maize roots but they account for the majority of the total root length and surface area of the root system (Yu *et al.*, 2014). The development of these roots is strongly associated with water and nutrient uptake (McCully, 1999; Kamoshita *et al.*, 2004). The seminal and shoot-borne roots, which are thick, determine the growing direction and distribution of a root system in the soil (Abe and Morita, 1994; Lynch, 2013; Yu *et al.*, 2014). Maize roots contain 8–15 layers of cortical cells and one endodermal cell layer, in contrast to the model example Arabidopsis root (Hochholdinger *et al.*, 2004). The number of cells in a cortical circle in maize root is not fixed (Hochholdinger *et al.*, 2004). Furthermore, the quiescent centre (QC) of the maize root is surrounded by both proximal and distal meristems and is much larger than that in Arabidopsis, which contains only four cells (Hochholdinger *et al.*, 2004).

The capacity of root to extract water and nutrients from soil is affected by both root system architecture and root function (Hammer *et al.*, 2009). Root system architecture is defined as a combination of morphological and structural traits, such as root number, length, angle, elongation, branching and the ability to penetrate hardpans (Hodge *et al.*, 2009). Under a resource-poor environment, root system

architecture is able to adapt and change markedly, which is commonly termed root plasticity (Feldman, 1984; López-Bucio *et al.*, 2003; Hodge *et al.*, 2006). Changes in root architecture can profoundly affect the ability of a plant to take up nutrient and water from the soil under unfavourable conditions. The response in root system architecture is crucial to at least partly maintain plant growth and production under drought (Manschadi *et al.*, 2006; Kano *et al.*, 2011), or nutrient deficiencies (e.g. phosphorus, nitrogen) (Mollier and Pellerin, 1999; Liao *et al.*, 2001; López-Bucio *et al.*, 2003). Therefore, it is important to gain a better understanding of the root system architectural change and its regulation under drought conditions in order to improve plant performance in drought resistance (Henry *et al.*, 2011; Moumeni *et al.*, 2011).

Root growth response to drought

When a plant is growing in a drying soil, its growth is often inhibited, with root growth usually being less inhibited than shoot growth (Munns and Camer, 1996). In some cases, under mild drought, root growth may be promoted by soil drying, which is of great importance in maintaining sufficient water supply for the plant (Sharp and Davies, 1979; Kano *et al.*, 2011). Westgate and Boyer (1985) showed that the maize nodal root could continue its elongation when the water potential in its growing region was −1.4 MPa, while the elongation of the stem, silks and leaves from the same plant was completely inhibited when the water potentials in their growing regions were −0.50, −0.75 and −1.0 MPa respectively. Similarly, the primary root elongation rates of maize, soybean, cotton and squash were reduced but maintained

when the substrate water potential was −1.6 MPa, but the shoot growth was completely inhibited at −0.8 MPa (Sharp, 2002).

Root dry weight, length and volume are widely measured to indicate the root system size (Price *et al.*, 1997; Zhang *et al.*, 2009; Kano *et al.*, 2011). The root length, especially in deep and wet soil layers, was shown to be strongly and positively correlated with water extraction (Kamoshita *et al.*, 2000; Kamoshita *et al.*, 2004). A large root dry weight under drying conditions may not mean a larger root contact area with soil, because the root structure may change (e.g. reduced root diameter) (Hodge *et al.*, 2009; Hodge, 2010). A field study in rice reported that genotypes with greater root length under mild drought accumulated higher shoot biomass (Kano *et al.*, 2011). Manschadi *et al.* (2006) found that a wheat variety (SeriM82) with a more compact root system and greater root length at depth yielded more than the standard wheat variety (Hartog) under drought. It is a widely held view that enhanced root growth and/or rooting depth under drought are important traits for a drought-resistant ideotype (Zhang *et al.*, 2009; Tardieu, 2012). For deep rooting, the distinction between relative and absolute rooting depth is also critical (Schenk and Jackson, 2002; Hodge *et al.*, 2009). However, when the soil volume is limited, or there is little water stored in deep soil layers, there may be little benefit from increased root growth or a deep root system (Tardieu, 2012; Wasson *et al.*, 2012). Under such conditions, the increased root growth can quickly deplete the small amount of extractable water that remains and then root growth will soon be significantly inhibited (Kamoshita *et al.*, 2004; Tardieu, 2012). This can be seen from the results of Sharp and Davies (1979) in a short-term gradual soil drying experiment, which showed that the total root biomass and length were stimulated when the

drought was mild but inhibited when it became severe. A similar result was found in soybean (Creelman *et al.*, 1990). Therefore, plant root growth may respond differently to different degrees of drought, and the mechanisms underlying this are still not well understood.

1.3 Traits in root phenotyping for plant drought resistance

Phenotype is defined as "the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment" (oxforddictionaries.com). Plant phenotyping is the comprehensive quantitative description of the anatomical, ontogenetical, physiological and biochemical properties of a plant (Walter *et al.*, 2015). Plant phenotyping has long been used by breeders to screen desirable genotypes for specific purposes, such as selecting drought-resistant crop plants (Walter *et al.*, 2015). The genetic differences of plant phenotypes (both shoot and root) have been extensively reported (Gregory *et al.*, 2009; Zhang *et al.*, 2009). However, most drought-resistant phenotyping studies focused on the shoot traits, such as leaf size and expansion (Den Herder *et al.*, 2010; Kuijken *et al.*, 2015). This may be due to the fact that the root is not as easily accessible as the shoot and that its structure and function can be highly affected by the growth environment, which makes it difficult to investigate and obtain consistent results (Malamy, 2005; Den Herder *et al.*, 2010). Another reason may be that root phenotyping is notoriously labour intensive and slow (Wasson *et al.*, 2012; Araus and Cairns, 2014; Kuijken *et al.*, 2015). Nevertheless, the importance of the root system in determining biomass accumulation and yield formation suggests that

root phenotyping may be a necessary and valuable way to deliver genotypes that show enhanced yield under particular conditions (Den Herder *et al.*, 2010).

So far one of the most successful and well-known traits in root phenotyping for drought resistance is a deep root system, such as in the wheat genotype SeriM82 (Manschadi *et al.*, 2006; Manschadi *et al.*, 2008; Wasson *et al.*, 2012). Previous studies in rice also suggested that a deep root system is crucial for plant drought resistance when there is no hard pan in the soil to prevent root penetration (Fukai and Cooper, 1995; Kamoshita *et al.*, 2000). Besides rooting depth, other studies suggested that the reduced root diameter, appropriate root xylem size and increased root hair number contributed to enhance water uptake, and those traits have also been proposed as drought-resistant relevant traits (Wasson *et al.*, 2012; Comas *et al.*, 2013). Apart from these traits, Zhu *et al.* (2010) found that maize genotypes with more aerenchyma formation in the root showed reduced root respiration, increased root length density in deep soil and enhanced shoot biomass under drought compared to genotypes with less aerenchyma, which makes the induction of root aerenchyma a potential trait to improve carbon economy under drought in maize (Zhu *et al.*, 2010; Comas *et al.*, 2013). Moreover, the root angle (i.e. between the root and the vertical axis) is related to root vertical distribution and it has been supposed to be another important root trait for drought resistance (Oyanagi, 1994; Manschadi *et al.*, 2008; Uga *et al.*, 2015). Abe and Morita (1994) and Kato *et al.* (2006) reported that rice plants with steeper root angles distributed more roots in deeper soil layers. Additionally, maize genotypes with steeper root angles were found to have higher grain yields under water-stressed field conditions (Ali *et al.*, 2015). Therefore, steep root angle has become a new root trait for improving plant drought resistance (Uga

et al., 2015). Although it is reported that drought-resistant wheat plants tend to show a deeper root system (Gregory, 2006; Rich and Watt, 2013), whether root angle displays a plastic response to drought in a similar way to other root traits has not been properly investigated.

1.4 Hormonal regulation of root system architecture under drought

ABA

Abscisic acid (ABA) is one of the most extensively studied drought-relevant plant hormones, and it is involved in both drought avoidance and drought tolerance (Claeys and Inzé, 2013). ABA signalling pathways have been elucidated and genetic analyses have identified three types of ABA receptors so far, (1) ChlH (magnesium cheletase) and (2) GTGs (GPCR type G-proteins), which are located in plastid and plasma membrane respectively, and (3) PYR/PYL/RCARs (pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor) that are localised in nucleus and cytosol (Cutler *et al.*, 2010; Raghavendra *et al.*, 2010; Arc *et al.*, 2013). A model for ABA action has been proposed. When ABA binds to PYR/PYL/RCAR receptors, a conformation change of receptors is induced together with the formation of a protein complex with PP2C (clade A type 2C protein phosphatases, including ABI1-ABA insensitive 1 and ABI2) (Fujii *et al.*, 2009; Soon *et al.*, 2012). Then the PP2Cs, which are the ABA signalling negative regulators, release the inhibition on SnRK2 (group III sucrose non-fermenting1-related protein kinase 2). SnRK2 positively regulates ABA signalling by phosphorylating downstream targets, e.g. the bZIP transcription factors that include AREBs/ABFs (ABA response elements binding factors) and ABI5 (Fujita *et al.*, 2005; Fujii *et al.*, 2009; Soon *et al.*, 2012).

ABA plays an important role in long-distance signalling to reduce plant stomatal aperture under drought (Davies *et al.*, 2002; Wilkinson and Davies, 2002). Many studies have shown that reduced soil water availability can enhance ABA levels in the root, xylem sap and leaf (Zhang and Davies, 1989; Davies and Zhang, 1991; Puértolas *et al.*, 2015). Sharp (2002) showed that the ABA content in maize root growth zone increased about five fold at a water potential of −1.6 MPa in the substrate. Zhang and Tardieu (1996) demonstrated that all maize root tissues could synthesise ABA under drying conditions. Furthermore, the endogenous ABA level can indicate the soil water availability (Zhang and Davies, 1989).

Generally, ABA is considered to be a plant growth inhibitor, as applied ABA inhibits plant shoot and root growth in the absence of water stress (Sharp *et al.*, 1994; Sharp and LeNoble, 2002). In other studies the high ABA levels in drought-stressed plants was thought to be responsible for the observed growth inhibition (Bensen *et al.*, 1988; Creelman *et al.*, 1990; Wilkinson and Davies, 2002). However, it has also been observed that applied ABA can have biphasic effects on plant root growth: ABA at relatively low concentrations stimulated root growth, while high concentrations can inhibit it (Watts *et al.*, 1981; Xu *et al.*, 2013). This is similar to the effect of soil drying on root growth (Sharp and Davies, 1979; Creelman *et al.*, 1990).

Furthermore, maize root growth is inhibited more severely at low water potential (−1.6 MPa) in mutants or in chemical treated plants in which high ABA accumulation was prevented (Saab e*t al.*, 1990). This result indicated that ABA accumulation is required to maintain maize root elongation under drought, an observation that changed the traditional view that substantial accumulation of ABA inhibits plant

growth (Sharp, 2002). Yamaguchi and Sharp (2010) found that maintenance of root elongation by ABA was conferred by its regulation of ion homeostasis, osmotic adjustment and cell wall extensibility. ABA was further found to act through an auxin-independent pathway to inhibit lateral root development (Casimiro *et al.*, 2003). It is suggested that ABA tended to maintain root tip growth and inhibit root branching under severe drought, which resulted in a deep but less dense root system (Tardieu *et al.*, 2010).

Ethylene

Ethylene is a gaseous plant hormone that may play important roles in plant drought responses (Schachtman and Goodger, 2008; Santner *et al.*, 2009). In Arabidopsis, five membrane-located ethylene receptors have been identified: ETR1 (ethylene resistant 1), ETR2, ERS1 (ethylene response sensor 1), ERS2 and EIN4 (ethylene insensitive 4). EIN2 works positively at the downstream of CTR1 (constitutive triple response 1), which is a serine threonine protein kinase and acts at the downstream of the receptors as a negative regulator (Bleecker and Kende, 2000). Receptors activated by ethylene can negatively regulate the CTR1 to allow EIN2 to activate transcription factors such as EIN3, EIL1 and EREBPs (ethylene-responsive element binding proteins)/ERFs (ethylene response factor genes) (Alonso *et al.*, 1999; Wang *et al.*, 2002; Yoo *et al.*, 2008). Many downstream ethylene-responsive genes are then activated by those transcription factors.

Contradictory results have been reported for plant ethylene production under drought (Morgan and Drew, 1997). Rapid desiccation of detached leaves and a rapidly developed drought stress promote ethylene production (Wright, 1977; Aharoni,

1978). In contrast, slow soil drying reduced the ethylene production rate in intact pot-grown cotton and bean plants (Morgan *et al.*, 1990). In addition, the increased ABA level under low water potentials restricted maize ethylene production (Spollen *et al.*, 2000; Sharp and LeNoble, 2002). Exogenous ABA has been shown to inhibit ethylene production in different organs of various plant species (Gertman and Fuchs, 1972; Wright, 1980). However, Arabidopsis plants exposed to a high concentration of ABA (100 μM) showed enhanced ethylene production in a recent study (Luo *et al.*, 2014).

Ethylene and its precursor ACC (1-aminocyclopropane-1-carboxylic acid) were reported to act in synergy with auxin to inhibit root growth and promote root hair initiation and elongation (Le *et al.*, 2001; Růžička *et al.*, 2007; Muday *et al.*, 2012). The increase of endogenous ethylene levels in maize has been correlated with its root elongation decrease (Alarcón *et al.*, 2009). Furthermore, ethylene or ACC treatment, or elevated ethylene production in Arabidopsis and tomato inhibited lateral root initiation, and seedlings with blocked ethylene response showed enhanced lateral root formation (Negi *et al.*, 2008; Negi *et al.*, 2010). These results suggested that ethylene may act antagonistically with auxin to inhibit lateral root formation (Muday *et al.*, 2012). In addition, ethylene inhibited ABA-induced leaf stomatal closure under drought (Tanaka *et al.*, 2005). Transgenic maize plants with reduced ethylene production improved grain yield under drought (Habben *et al.*, 2014). It is an interesting question whether ethylene itself and its interaction with other hormones (e.g. ABA and auxin) are involved in regulating plant root system architecture under drought.

Auxin

Auxin is a primary regulator in plant growth and development (Mockaitis and Estelle, 2008). Auxin binds to F-box proteins, TIR1 (transport inhibitor response 1) and AFB (auxin-related F-box) (Dharmasiri *et al.*, 2005a, b). Then auxin promotes the ubiquitination and degradation of AUX/IAA (auxin/indole-3-acetic acid) repressor proteins through the $SCF^{TIR1/AFBs}$ complex and 26S proteasomes (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005; Fukaki and Tasaka, 2009). The AUX/IAA family mediates ARF (auxin response factor) proteins negatively (Fukaki and Tasaka, 2009). The degradation of AUX/IAA will release the inhibition on ARF and allow these transcription factors to regulate downstream auxin relevant gene expression (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005).

Auxin controls root growth and gravitropic response through its activity gradients, which show a distal maximum distribution in the root tip (Friml, 2003; Swarup *et al.*, 2005; Teal *et al.*, 2006). The auxin transport system is critical to form an auxin distribution pattern and this is mainly the result of the activity of auxin influx and efflux proteins (Friml, 2003; Teal *et al.*, 2006). AUX1 (auxin 1), LAX2 (like-AUX1), and LAX3 are the major auxin influx carriers (Péret *et al.*, 2012). So far, eight PIN (pin formed, PIN1–8) protein family members are known to be major auxin efflux carriers (Petrášek *et al.*, 2006; Wiśniewska *et al.*, 2006; Kleine-Vehn and Friml, 2008), of which PIN1–4 and PIN7 are well characterized (Mravec *et al.*, 2009). In addition to the PIN family proteins, some other membrane proteins are also involved in auxin efflux, for instance members of the ABCB (ATP-binding cassette group B) auxin transporters and PGP (phosphoglycoprotein) auxin transporters (Mravec *et al.*, 2008;

Spalding, 2013). Disturbed auxin transporter system by transporter inhibitors and gene mutations can result in an altered auxin distribution pattern, which will strongly affect plant root cell expansion, elongation and gravitropism (Ottenschläger *et al.*, 2003; Swarup *et al.*, 2005; Růžička *et al.*, 2007).

Auxin was found to inhibit root elongation (Muday *et al.*, 2012). However, exogenous auxin was found to induce lateral root development, and auxin-insensitive plants showed decreased lateral root initiation (Gilbert *et al.*, 2000; Casimiro *et al.*, 2003). Xu *et al.* (2013) reported that increased ABA accumulation under moderate osmotic stress was responsible for root growth promotion which may have occurred through the regulation of auxin transport in root tips. The cross talk between ABA and auxin may play an important role in regulating root growth, which requires more investigation (Rock and Sun, 2005; Yamaguchi and Sharp, 2010).

Cytokinin

Cytokinin can regulate plant cell proliferation and differentiation, and control various plant growth and developmental processes (Sakakibara, 2006; Spíchal, 2012). Growing numbers of studies suggest that cytokinin is involved in plant drought responses (Davies *et al.*, 2005; Werner *et al.*, 2010; Nishiyama *et al.*, 2011). The natural cytokinins in plant are isoprenoid and aromatic cytokinins and the former are more frequently found and are more abundant than the latter (Sakakibara, 2006). Isopentenyladenine (iP), *trans*-zeatin (*tZ*), *cis*-zeatin (*cZ*) and dihydrozeatin (DZ) are the common natural types of isoprenoid cytokinins (Sakakibara, 2006). Among them iP- and *tZ*-type cytokinins are the major forms and they normally exhibit high bioactivities, while *cZ*-type cytokinin has low or no activity, although different

results may arise from different bioassays (Sakakibara, 2006). Substantial amounts of *c*Z-type cytokinins are found in maize and their roles in maize are still unclear (Veach *et al.*, 2003; Schäfer *et al.*, 2015). Several aromatic cytokinins such as *ortho*-topolin (*o*T), *meta*-topolin (*m*T), their methoxy-derivatives meoT and memT, and benzyladenine (BA) are only found in some plant species (Strnad, 1997; Sakakibara, 2006).

The application of BA inhibited root elongation in Arabidopsis seedlings growing in the light or the dark (Cary *et al.*, 1995). Transgenic Arabidopsis with decreased cytokinin levels (iP- and *t*Z-type) showed increased root branching, primary root growth and drought resistance (Werner *et al.*, 2001; Werner *et al.*, 2003; Werner *et al.*, 2010). Therefore cytokinins are negative regulators in root formation and elongation (Werner *et al.*, 2010). Drought stress significantly reduced the *t*Z-type cytokinins in Arabidopsis, but not the iP- and *c*Z-type cytokinins (Nishiyama *et al.*, 2011). Increased ABA levels in xylem sap and leaves of grape vines were found accompanied by decreased *t*Z-type cytokinins concentrations in root and shoot under partial root-zone drying (Stoll *et al.*, 2000). Cytokinins are postulated to antagonise the ABA effect on plant behaviour (Blackman and Davies, 1983; Pospíšilová, 2003; Nishiyama *et al.*, 2011). For example, the increase of ABA level can induce leaf stomatal closure under drought, while cytokinins can inhibit such ABA-induced stomatal closure (Blackman and Davies, 1983; Tanaka *et al.*, 2006). However, Nishiyama *et al.* (2011) suggested that cytokinin-deficient plants (containing low levels of iP-, *t*Z-, *c*Z- and DZ-type cytokinins) exhibited a strong drought resistance and this may be associated with ABA hypersensitivity rather than ABA-induced stomatal closure and stomatal density. Cytokinin and its interaction

with ABA may be important for regulating plant root system architecture under drought.

1.5 Research objectives and thesis structure

The aim of this thesis was to investigate root system architecture and its genetic variation, and the potential relation between root system architecture and key plant hormones under non-lethal drought stress using various maize and Arabidopsis genotypes. The study focused on the early seedling stages and all the trials were conducted in controlled environment conditions.

Chapter 2 intended to set up experimental system for the non-lethal drought stress treatments and examine the synchronisation of maize leaf and root physiological responses during soil drying (including changes in growth rate, water potential and hormone levels). The potential roles of ABA and ethylene in maize responses to soil drying were discussed.

Chapter 3 aimed to study the genetic variation in root angle and other root traits (length, surface area and dry weight) in 14 maize genotypes, and the root plasticity under drought. The correlations between the root angle and several other root traits and plant drought resistance (as indicated by shoot biomass change under drought) were explored. In this chapter, the possible relationship between plant hormones (ABA, ethylene and *t*Z) and root angles was investigated.

Chapter 4 was designed to investigate the biphasic responses of Arabidopsis root to applied ABA and the potential crosstalk between ABA and other hormones (ethylene and auxin) during such responses.

Finally, Chapter 5 was a general discussion of all of the work presented in this thesis.

Chapter 2 Synchronisation of Changes in Maize Hormone Levels and Leaf and Root Growth during Soil Drying

2.1 Introduction

Drought is a major factor restricting crop production in many regions of the world (Boyer, 1982; Boyer *et al.*, 2013). While maize (*Zea mays* L.) is among the top three staple crops worldwide (Varshney *et al.*, 2012), its production is likely to suffer more from drought stress in the future under a changing climate with increased risk of high temperatures and more variable precipitation (Battisti and Naylor, 2009; Tardieu, 2012). Therefore, it is important to breed drought-resistant maize varieties, which necessitates better understanding of the physiological responses of maize shoot and root growth to drought stress (Tuberosa *et al.*, 2007).

Plant shoots and roots may respond differently to the same drought stress by means of development, growth and other physiological changes (Munns and Sharp, 1993; Munns and Camer, 1996). Shoot growth is generally more inhibited by drought than root growth (Sharp and Davies, 1979). When soil turns much drier, the root can continue to grow while the shoot may be completely inhibited (Westgate and Boyer, 1985).

Phytohormones have been shown to regulate plant development and growth under drought stress (Santner *et al.*, 2009). The concentration of abscisic acid (ABA), one of the most important drought-relevant hormones, was found to increase under drought stress in many plant species (e.g. Arabidopsis, maize and potato) (Zhang and

Davies, 1989; Huang *et al.*, 2008; Puértolas *et al.*, 2015). It is also suggested that the concentration of ABA in the root could be an indicator of a local change in soil water status (Zhang and Davies, 1989). Furthermore, the high accumulation of ABA under drought stress is reported to be responsible for stomatal closure and the inhibition of shoot and root growth (Munns and Sharp, 1993; Wilkinson and Davies, 2002; Harris, 2015). Mild drought can stimulate root growth, while severe drought can inhibit it (Sharp and Davies, 1979; Creelman *et al.*, 1990). Accordingly, stimulatory and inhibitory effects on root growth were shown when ABA was applied to plants at low and high concentrations respectively (Watts *et al.*, 1981; Xu *et al.*, 2013).

Ethylene is a gaseous plant hormone, which is probably also involved in plant drought responses (Sharp and LeNoble, 2002; Schachtman and Goodger, 2008). Previous studies have indicated that drought stress may promote, restrict or not affect the ethylene production in various species (El-Beltagy and Hall, 1974; Morgan *et al.*, 1990; Sharp and LeNoble, 2002). Morgan *et al.* (1990) also reported that intact cotton and bean plants showed reduced ethylene production during slow soil drying in contrast to the responses shown by detached leaves under rapid desiccation. However, ethylene has been shown to be an inhibitor of shoot growth, root elongation and lateral root initiation (Pierik *et al.*, 2006; Muday, 2012). A series of studies have suggested that significant accumulation of ABA was necessary to prevent extra ethylene production and thus ameliorate its inhibition of maize shoot and root growth under low water potentials (Saab *et al.*, 1990; Sharp and LeNoble, 2002). Hence, it has been assumed that the interaction between ABA and ethylene plays an important role in regulating plant drought response (Sharp and LeNoble, 2002; Tanaka *et al.*, 2005). However, few studies have simultaneously investigated

the gradual changes of hormone levels and leaf and root growth in response to a gradual soil drying, let alone the timing of these changes, which is prerequisite if we are to elucidate the complex signalling pathways which are important components of the plant drought response.

By subjecting 15-d old maize plants to a 6-d soil drying episode, the responses of leaf and root growth and other physiological parameters, especially the changes of endogenous ABA and ethylene levels, were investigated synchronously.

2.2 Materials and methods

Plant growth

Commercial maize variety *Earligold* F1 (VSW041, Moles Seeds, UK) was used in this study. Two hundred and eighty seeds (0.15–0.19 g/seed) were soaked in deionized water for 48 h and then pre-germinated on wet paper towels for 72 h in a controlled-environment (CE) room in the dark (temperature: 24˚C/18˚C; photoperiod:14 h/10 h; light density: 350 μmol m^{-2} s^{-1}). Then seedlings with a root length of 4–10 cm were transplanted into 155 pots (height: 24 cm; diameter: 6.4 cm; with stainless wire mesh at the bottom) with one seedling per pot. Each pot was filled with 785 g of moist soil (ca. 628 g dry soil) to make a 22-cm tall soil column. The soil was sieved (1-cm sieve) John Innes No.2 (Foremost, UK). After transplanting, each pot was watered thoroughly by adding 200 ml water. Seedlings became visible on the next day and another 20 ml water was added to each pot. The soil column was then drained for 1 h and weighed to determine the pot capacity for water (54% of soil water content, w/w soil dry weight). All pots were weighed and watered to the pot

capacity every day until the 15th day, except on the 7th day after transplantation when 50 ml Hoagland's nutrient solution (pH = 5.8–6.0) was given to each pot. The third leaf was expanded fully (the leaf collar became visible) by the 15th day after transplantation (Figure 2.1A) (Abendroth *et al.*, 2011), which was set as the last watering day (Day 0) for the soil drying treatment.

One hundred and four plants at a similar growth stage were selected: 48 plants for the soil drying treatment and another 48 plants as the well-watered control during the following 6 d, in addition to 8 plants for sampling on Day 0 (Figure 2.1A). Control plants were watered daily to pot capacity. Eight pots of each treatment were destructively harvested every day during Day 1–6.

Soil water content and soil water potential

After removing the shoot from the soil surface, the soil column was cut into top and bottom halves from the middle (Figure 2.1B). After root tissue was removed, each part of the column was weighed ($W_{original}$), oven dried at 80˚C for about a week and weighed again for dry weight (W_{dry}). Then the soil water content (%, w/w) was calculated by $[(W_{orignial} - W_{dry})/ W_{dry}] \times 100\%$.

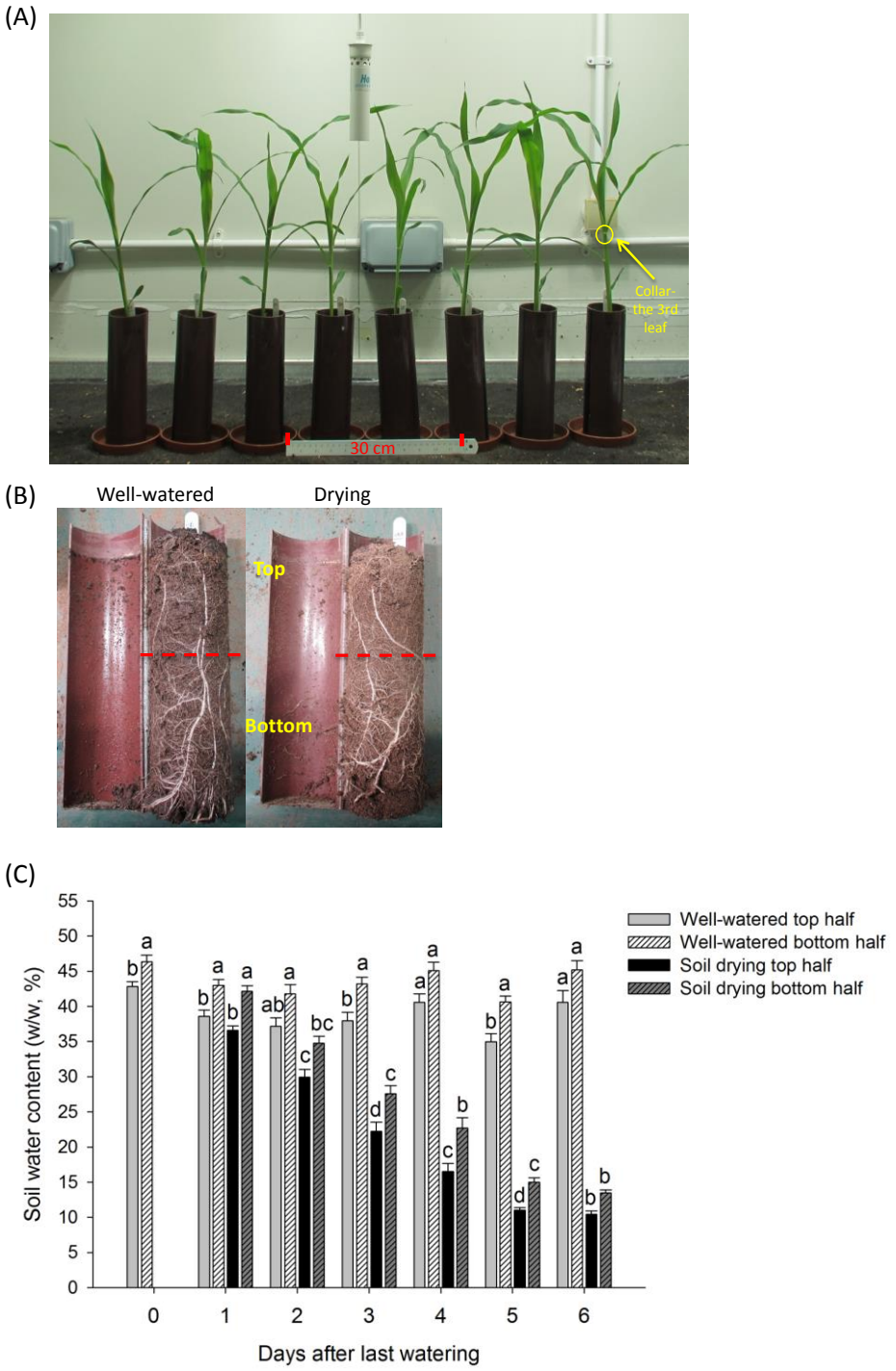


Figure 2.1: (A) Plants on the last watering day (Day 0); (B) soil columns of the well-watered and soil drying treatments on Day 6 after the last watering; (C) soil water content in top and bottom parts of well-watered and soil drying treatments (Day 0–6). Pre-germinated maize seeds (*Earligold* F1) were transplanted into pots filled with sieved soil (John Innes No.2). Seedlings germinated from the soil surface after one day. All pots were weighed and watered to the pot capacity every day until the 15th day, except on the 7th day after transplantation when 50 ml Hoagland's nutrient solution (pH = 5.8–6.0) were given to each pot. The third leaf was fully expanded on the 15th day after transplantation, and this day was set as the last watering day (Day 0). One hundred and four plants at a similar growth stage were selected and eight plants were harvested on Day 0. Forty eight plants were assigned for the soil drying

treatment and another 48 plants for the well-watered control. Control plants were watered daily to the pot capacity while watering was ceased in the soil drying treatment for 6 d. Eight pots of each treatment were destructively harvested every day during Days 1–6. Each soil column was cut into top and bottom halves from the middle to measure the soil water content in top and bottom parts. Columns and bars are means ± standard error. Different letters indicate significant difference on the same day at $P < 0.05$ (n = 4).

A soil water characteristic curve can be found in Appendix 1 Figure 1. The soil water potential was measured by thermocouple psychrometer (Wescor Inc., Utah, USA) when the soil water content was above 25% (water potential higher than –0.35 MPa) and by the WP4-T Dewpoint Potentiometer (Decagon Devices, Washington, USA) when the water content was between 5–25%.

Leaf elongation rate and root growth measurements

The length of four growing leaves (the 4th–7th leaves) was measured daily once visible. The leaf elongation rate (mm/h) was calculated. After the incubation for root ethylene (see below), the entire root system was scanned and analysed for total root length and root surface area with the WinRHIZO Pro system (Regent Instruments Inc., Quebec, Canada). In each treatment, the mean of root length or surface area in the previous day was treated as the root length or surface area for that day for calculation of the daily increase rates of these parameters (units: m/day, cm^2/day).

Leaf and root water potential and solute potential

Leaf and root water potential (Ψ_{leaf} and Ψ_{root}) were measured with thermocouple psychrometers. Leaf discs (5 mm diameter) were punched from the middle of the 3rd leaf (avoiding the midrib). The leaf disc was immediately wrapped in aluminum foil to minimise water loss and loaded into a C52 sample chamber (Wescor Inc., Utah,

USA) within minutes for 3 h. The voltage was then recorded on a HR-33T Dew Point Microvolt meter (Wescor Inc., Utah, USA). The water potential in MPa was converted from the recorded voltage based on the calibration with salt solutions of known osmotic potentials. A few roots (no root tips) were collected from the outer surface of top 2/3 soil columns after the root tips were collected for ABA assay (see below). The roots were cut into small segments (ca. 5 mm). Ten to 15 root segments were wrapped in aluminum foil and used to measure the water potential in the same way as for the leaf samples.

The same leaf and root samples were then used to measure solute potentials ($\Psi_{s\text{-}leaf}$ and $\Psi_{s\text{-}root}$) by the same psychrometer. Samples were frozen by submergence into liquid nitrogen and then stored in a –20˚C freezer, defrosting before use. The voltage was record after 30-min incubation of the plant samples and then converted to solute potential in MPa. Leaf and root turgor pressure ($\Psi_{t\text{-}leaf}$ and $\Psi_{t\text{-}root}$) were then calculated for every sample according to the equation $\Psi_t = \Psi - \Psi_s$.

Stomatal conductance

Stomatal conductance was measured daily between 7:00 and 9:00 am (photoperiod started at 6:00 am) with an AP4 porometer (Delta-T Devices, Cambridge, UK). The 3rd (fully expanded on Day 0) and the 4th (fully expanded on Day 2 or 3) leaves of each plant were measured. The measurement was on the abaxial leaf surfaces from both sides of the midrib in the middle 1/3 of each leaf. Two positions on each side of the midrib were measured and the mean value of the four readings was used to represent the stomatal conductance for an individual plant.

ABA assay for leaf and root tissues

In order to analyse the ABA concentration on enough plant material, the 3rd leaves of every two of the eight plants from the same treatment were pooled as one replicate. The leaves were cut at the collars, folded into one 15 ml centrifuge tube (Corning, New York, USA) and submerged into liquid nitrogen immediately. Around 100 root tips (ca. 3 cm) were collected from the top 2/3 of the soil column of the same two pots used for leaf sampling. The root tips were quickly washed with tap water, transferred to a 1.5 ml centrifuge tube and submerged into liquid nitrogen. All samples were stored at −20°C before being freeze-dried for 48 h. The samples were then ground, and ca. 30 mg leaf tissue and all root tips were extracted with deionised water at 1:25 mg:µl ratio in a 1.5 ml centrifuge tube and shaken at 4°C overnight. Then the competitive radioimmunoassay (Quarrie *et al.*, 1988) was used to determine ABA concentrations (ng/g DW). The extract was centrifuged at 12 000 g for 4 min and then 50 µl supernatant was pipetted into the reaction buffer. This buffer contained 200 µl of 50% 50 mM PBS buffer (pH = 6.0), 100 µl diluted antibody MAC 252, and 100 µl diluted [^{3}H] ABA. The mixture was then incubated for 45 min at 4°C. The bound radioactivity of [^{3}H] ABA was measured with a liquid scintillation counter (Packard TriCARB 1600TR liquid scintillation analyser, Canberra, CT, USA). A standard curve with 8 ABA solutions (0, 62.5, 125, 250, 500, 1000, 2000 and 2×10^{6} pg 50 μl^{-1} (+)-ABA), which was made from (±)-ABA (A1049, Sigma-Aldrich) and was measured with samples and used for calculating the ABA concentrations of samples.

Ethylene release rates from leaf and root

Four of the eight plants in each treatment were used for ethylene incubation every day. The 5th leaf and the entire root system of one plant were used to quantify the ethylene release rate respectively. The entire root system was washed out of the soil (within 30 min) after root tips were collected. Leaf and root samples were incubated in glass test tubes sealed with rubber stoppers for 1.5 h under light and dark respectively. To prevent water loss from the sample, a piece of wet filter paper was enclosed. After the incubation, 1 ml gas was taken with a syringe and injected into a gas chromatography system (GC) fitted with a FID detector (6890N, Agilent Technologies, California, USA) (Chen *et al.*, 2013a). A 20 ppm ethylene/nitrogen standard gas (BOC Limited, Surrey, UK) was used to check the ethylene peak time and also for calibration. The leaf and root samples (after root scanning, see above) were oven dried and weighed afterwards. Then leaf and root ethylene release rates (nl/ (g DW h)) were calculated.

Statistical analysis

The statistical software SPSS 21.0 (IBM, USA) was used to perform either one-way ANOVA with Tukey's *post hoc* test or *t*-test at the $P < 0.05$ level.

2.3 Results

Soil water content during soil drying

To determine the drought intensity of the soil drying treatment during the 6 d after last watering, soil water contents of top and bottom halves of each soil column were measured. The top half of the column had a lower soil water content than the bottom half of the column in both well-watered and drying treatments (Figure 2.1C). The

well-watered pots had a soil water content of 39% and 44% in the top and bottom soils on average during the 6 d, respectively (Figure 2.1C). In contrast, the water content in the drying treatment declined from 37% to 10% in the top half soil and from 42% to 13% in the bottom half soil (Figure 2.1C). Soil water contents in both top and bottom halves of the drying treatment were significantly lower than those in the well-watered pots from Day 2 (Figure 2.1C). The average water content of the soil columns in the drying treatment dropped gradually from pot capacity (54%) on Day 0 to 12% on Day 6 (Figure 2.1C), corresponding to water potentials of −0.20 and −0.75 MPa respectively (Figure 2.1C, Appendix 1 Figure 1).

Effects of soil drying on leaf and root growth

Leaf elongation rates were measured on four growing leaves, i.e. the 4th−7th leaves, when they were visible. Soil drying significantly reduced the leaf elongation rate of the 5th and 6th leaves by more than 30% and 80% during Day 4−5 and Day 5−6 respectively (Figure 2.2B, C). The elongation rates of the 4th (the oldest selected leaves) and 7th (the youngest) leaves in the soil drying treatment tended to be lower than those in the well-watered control plants after Day 4, but the differences were only significant on the final day of soil drying (Figure 2.2A, D). Therefore, the 5th leaf seemed to be the most suitable one for observing soil drying effects on leaf elongation in this study.

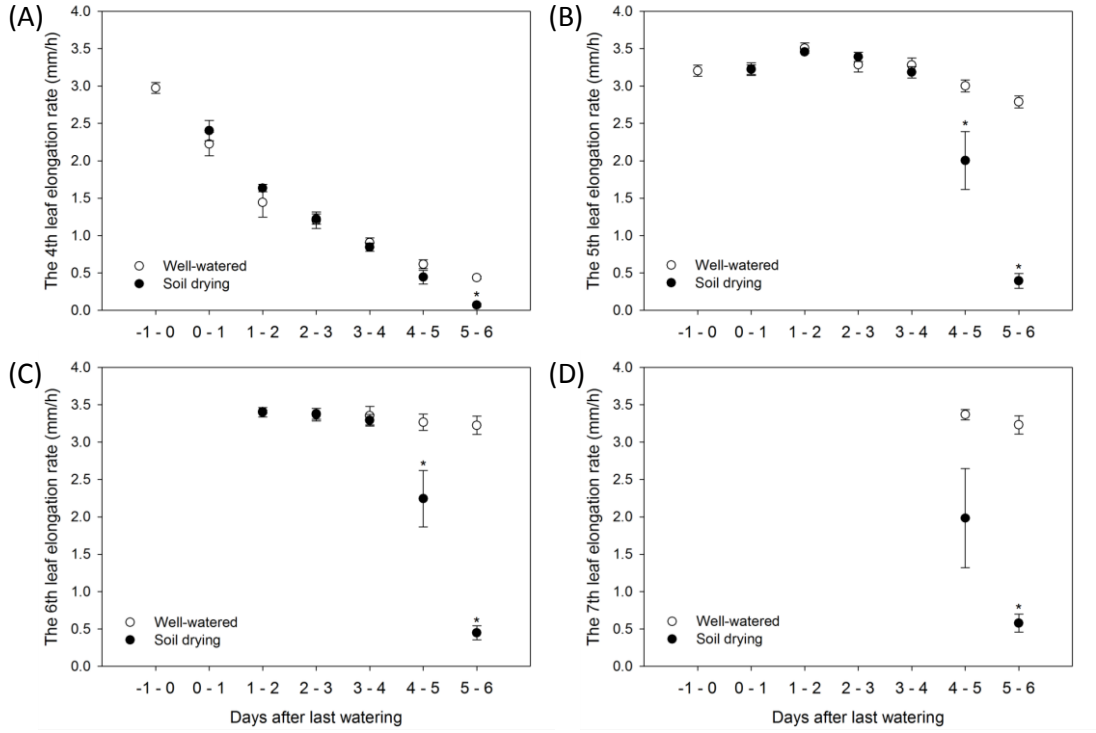


Figure 2.2: Leaf elongation rate of (A) the 4th leaf (fully expanded on Day 2 or 3), replication n = 8; (B) the 5th leaf (expanding, and was visible before the start of soil drying), replication n = 8; (C) the 6th leaf (expanding, and was visible from Day 1), replication n = 8; (D) the 7th leaf (expanding, and was visible from Day 4), replication n = 4–8. Points and bars are means ± standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$.

Earlier research has reported that maize root growth was stimulated by mild drought and inhibited when the drought became severe (Sharp and Davies, 1979; Watts *et al.*, 1981). In this experiment and several preliminary experiments (e.g. Appendix 1 Figure 2), a weak but similar trend can be seen during the 6-d soil drying, although the statistical analysis did not show significant differences in root growth between the drying treated and well-watered plants in terms of total root length, root surface area and their increase rates (Figure 2.3). Maize in the soil drying treatment tended to show greater root growth rate than the well-watered plants from Day 2–3 when drought was mild (Figure 2.3B, D), which resulted in bigger root

system on Day 3 with larger total root length and surface area (Figure 2.3A, C). However, maize in the soil drying treatment tended to have a smaller root system on Day 6 (Figure 2.3A, C), which might be due to the reduced root growth rate after Day 4 when the drought became more severe (Figure 2.3B, D).

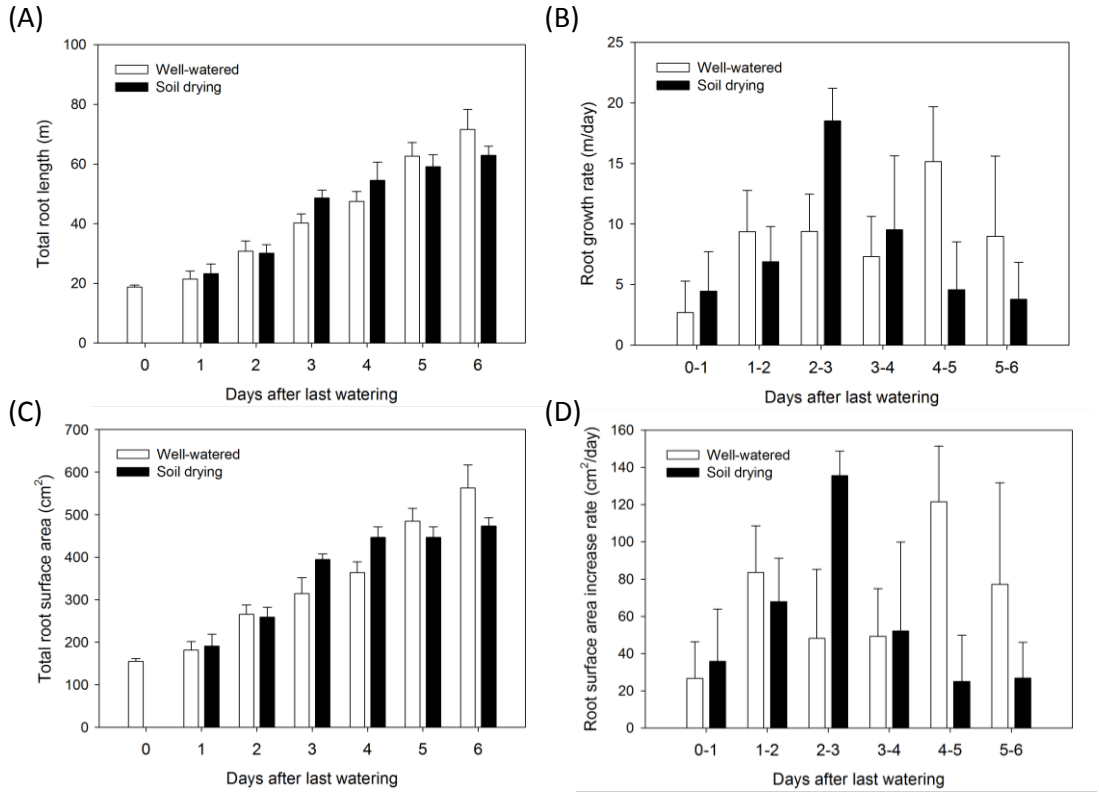


Figure 2.3: Root growth responses to soil drying. (A) Total root length during Day 0–6, replication n = 3–4. (B) Root growth rate in the 6-day soil drying treatment, replication n = 3–4. (C) Total root surface area during Day 0–6, replication n = 3–4. (D) Surface area increase rate in the 6-day soil drying treatment, replication n = 3–4. During the 6-day soil drying (see Figure 2.1), the roots that were used for ethylene incubation in each treatment were scanned and analysed for total root length and root surface area using the WinRHIZO Pro system. Columns and bars are means ± standard error.

Physiological responses to soil drying

Changes in water potential and turgor pressure of leaf and root

Leaf water potential and solute potential of the 3rd leaf was monitored as an indicator of leaf water status during soil drying. The leaf water potential in well-watered maize was between −0.25 to −0.38 MPa during the 6-d period, while in the drying treatment it dropped to −0.92 MPa on Day 5 (Figure 2.4A). On Day 6, there was no further reduction in leaf water potential in the drying treatment, which may be related to little change of soil water content during the last two days (Figure 2.1C). However, a repeat experiment did show further reduction of leaf water potential on Day 6 of soil drying, when the soil water content dropped by 3% during the last two days (Appendix 1 Figure 1, 3 and 4). The leaf turgor pressure of both well-watered and soil drying treated maize fluctuated during the 6-d period and the decrease of leaf solute potential in the drying treatment prevented leaf turgor pressure decline in the plant (Figure 2.4).

Root water status was also determined in this experiment. The root water potential was always around −0.3 MPa in the well-watered plants during the 6 d (Figure 2.5A), which was close to its average soil water potential (Figure 2.1C and Appendix 1 Figure 1). In contrast, the root water potential in soil drying treatment decreased from −0.3 to −1.1 MPa from Day 1 to 6 and was significantly lower than in the well-watered plants from Day 3 (Figure 2.5A). Root turgor pressure was maintained by reduced root solute potential during the 6 d (Figure 2.5B). It is notable that the root water potential decreased along with but remained lower than the soil water potential in the drying treatment since Day 2 (Figure 2.1C, 2.5A and Appendix 1 Figure 1). This result indicates that the root response to soil drying started as early as Day 2−3.

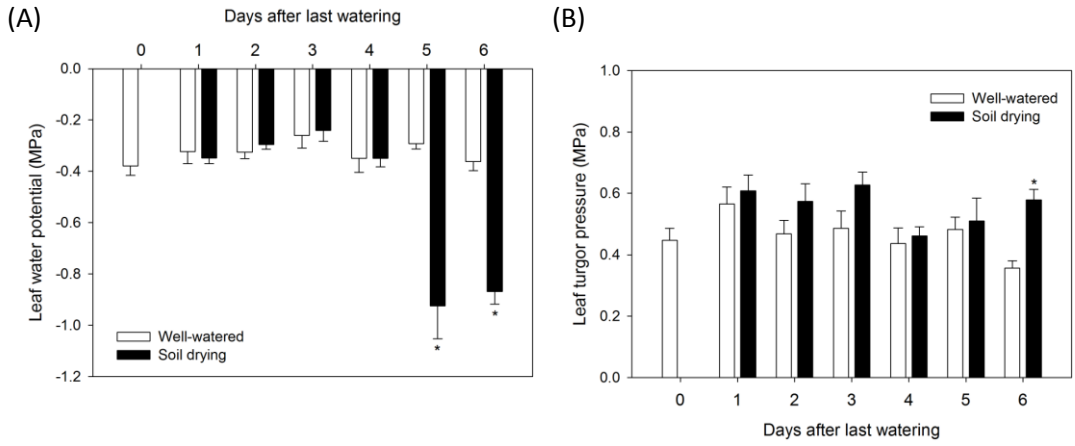


Figure 2.4: (A) The leaf water potential and (B) leaf turgor pressure of the 3rd leaf during Day 0–6. During the 6-day soil drying (see Figure 2.1), a leaf disc (5 mm diameter) from the middle of the 3rd leaf (avoiding the midrib) was incubated for 3 h in a C52 sample chamber in the thermocouple psychrometer. The voltage was then recorded on a HR-33T Dew Point Microvolt meter. The leaf samples were then frozen and defrosted before they were used to measure the solute potentials, which were also measured by the same thermocouple psychrometer used for water potential measurement. Each sample was incubated for 30 min and the voltage was recorded and converted to water potentials and solute potentials respectively. Columns and bars are means ± standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 8).

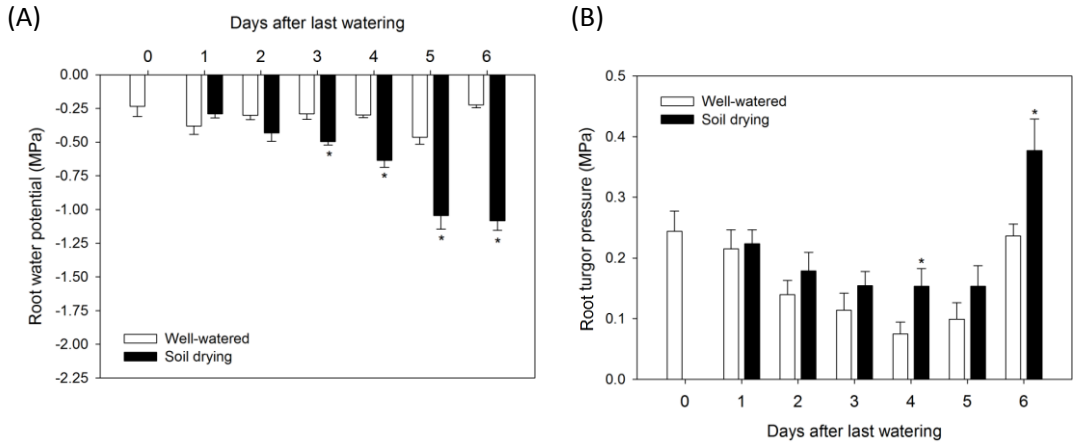


Figure 2.5: (A) The root water potential and (B) root turgor pressure during Day 0–6. During the 6-day soil drying (see Figure 2.1), 10–15 root segments (no root tips) from the top 2/3 soil columns were incubated for 3 h in a C52 sample chamber in the thermocouple psychrometer. The following procedure was the same for the leaf samples (Figure 2.4). Columns and bars are means ± standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 8).

Changes in leaf stomatal conductance

The stomata response to soil drying was monitored on a mature leaf (the 3rd) and a younger one (the 4th). The stomata conductance of the 3rd leaf decreased along with soil drying from Day 5 and decreased by 43% and 75% compared with the well-watered maize on Day 5 and 6 respectively (Figure 2.6A). However, the 4th leaf showed a higher stomatal conductance than the 3rd leaf, by around 30% on average over the 6 d (Figure 2.6). In addition, an earlier response of stomata to soil drying was seen in this younger leaf; a significant stomatal closure by 12% on Day 3 compared to the well-watered plant (Figure 2.6B). On the last two days of soil drying, the stomatal conductance in the 4th leaf decreased further (by 39% and 62% respectively) (Figure 2.6B).

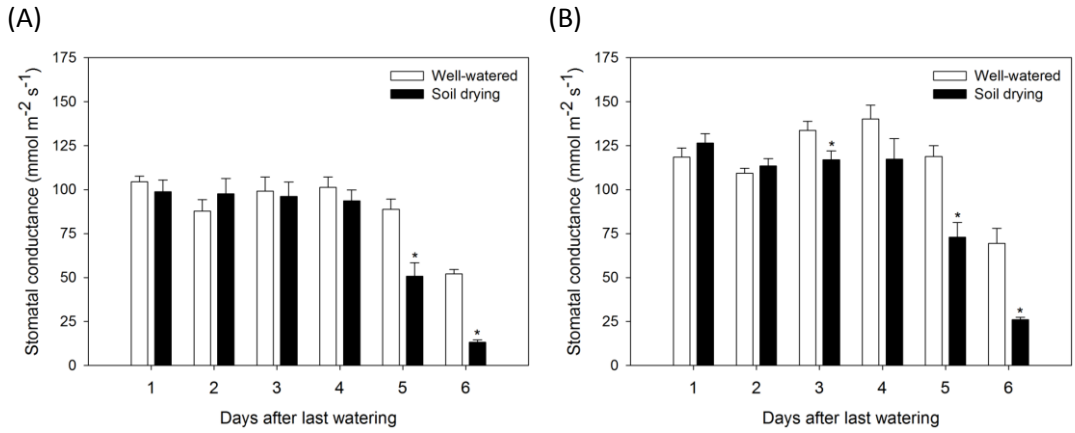


Figure 2.6: Leaf stomatal conductance of (A) the 3rd leaf (fully expanded before soil drying), (B) the 4th leaf (fully expanded on Day 2 or 3) in response to soil drying. Replication n = 8. During the 6-day soil drying (see Figure 2.1), the 3rd and 4th leaves of each plant were measured for stomatal conductance using an AP4 porometer. The measurement was on the abaxial leaf surface from both sides of the midrib in the middle 1/3 of each leaf. Two positions on each side of the midrib were measured and the mean value of the four readings represented the stomatal conductance of the respective leaf. Columns and bars are means ± standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$.

Changes of ABA concentrations and ethylene release rates in leaf and root

During the 6 d, ABA concentrations in the 3rd leaf of well-watered plants ranged between 61–141 ng/g DW (Figure 2.7A), while in the soil drying treatment the concentrations increased to more than two-fold from Day 4 (Figure 2.7A). There was no statistically significant difference in the leaf ABA concentration between the drying treated and well-watered plants on Day 4, mainly because the ABA concentration in one of the four drying replicates was similar to the watered treatment. However, from Day 5, a 29 times higher leaf ABA concentration were seen in the soil drying treatment than that in the well-watered treatment (Figure 2.7A). By contrast, soil drying only reduced the ethylene release rate of the 5th leaf on the last day by 43% (Figure 2.7B).

The ABA concentration in the root tips of well-watered maize ranged between 84–139 ng/g DW, which was similar to concentrations in the 3rd leaf (Figure 2.8A). In response to soil drying, the ABA concentration in root tips increased by 56% on Day 3, earlier than that in the 3rd leaf of the same plant, which increased only from Day 4 (Figure 2.7A). In root tips, soil drying continued to increase the ABA concentration on Day 4, 5 and 6, when the concentration was 2, 9 and 13 times of that in well-watered plants, respectively (Figure 2.8A). It has to be noted that the root tips were sampled for ABA assay while the entire root system was used for ethylene analysis. From Day 5, the root ethylene release rate in the drying treatment was significantly lower than that of the watered treatment (Figure 2.8B). In the well-watered treatment the root ethylene release rate increased by 11–60% on Day 4–6 compared with Day 1 (Figure 2.8B).

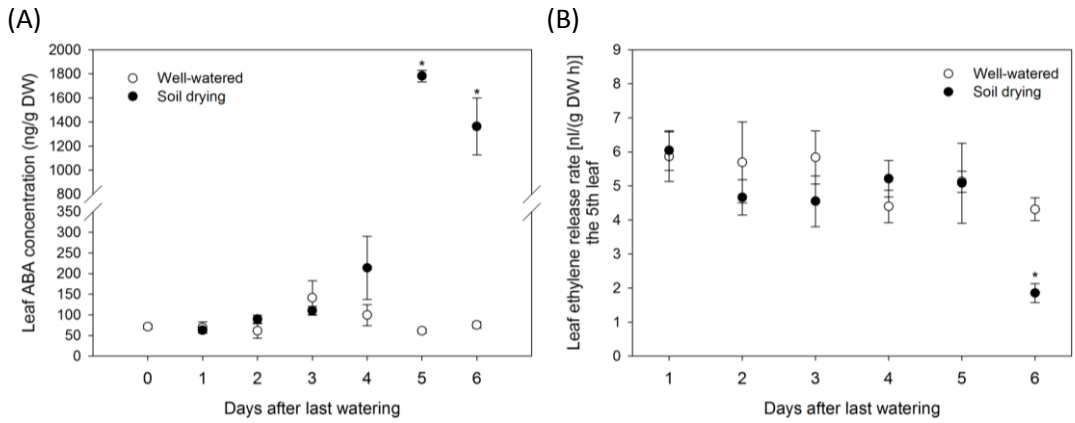


Figure 2.7: Leaf hormone responses to soil drying. (A) Leaf ABA concentration in the 3rd leaf (fully expanded before soil drying) during Day 0–6, replication n = 4. (B) Leaf ethylene release rate of the 5th leaf (expanding) during Day 1–6, replication n = 4. During the 6-day soil drying (see Figure 2.1), the 3rd leaves of every two of the eight plants from the same treatment were cut at the collars and mixed as one replicate. Leaf samples were submerged into liquid nitrogen immediately and then stored at −20˚C before being freeze-dried for 48 h. Dry samples were then ground and extracted with water. The extract was then used to determine the ABA concentration by the radioimmunoassay. Four of the eight plants in each treatment were used for ethylene incubation every day. The 5th leaf was cut from the soil surface and then incubated for 1.5 h (under light in the CE room) with a piece of wet filter paper in a sealed glass tube. Then 1 ml gas was taken with a syringe and measured with a GC system fitted with a FID detector. The 5th leaf was then oven dried and the ethylene release rate was calculated. Points and bars are means ± standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$.

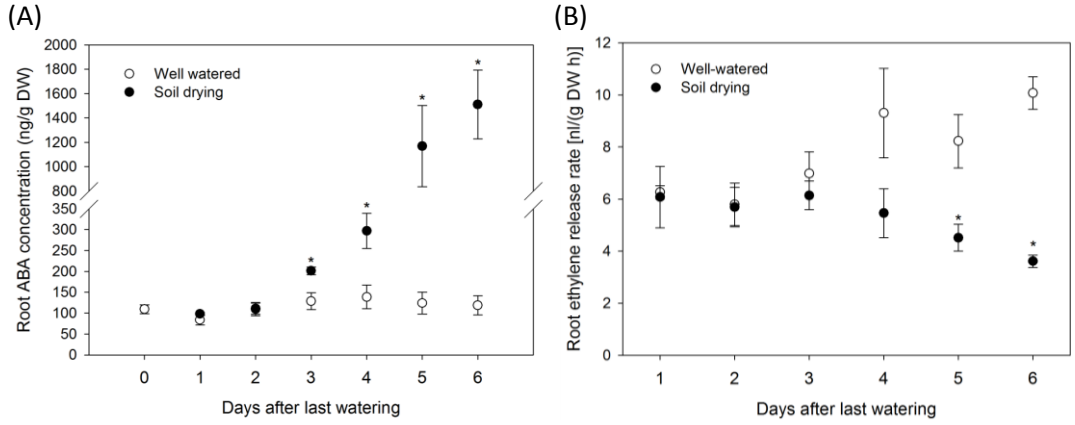


Figure 2.8: Root hormone responses to soil drying. (A) ABA concentrations in root tips during Day 0–6, replication n = 4. (B) Ethylene release rate from the entire root system during Day 1–6, replication n = 4. During the 6-day soil drying (see Figure 2.1), around 100 root tips (ca. 3 cm each) were collected from the top 2/3 of the soil column in the two pots used for leaf ABA sampling. The following procedure was the same for the leaf samples (Figure 2.7), except the root ethylene sample was incubated under dark. Points and bars are means ±

standard error. Stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$.

2.4 Discussion

Different responses of maize leaf and root growth to soil drying

Previous studies have reported that maize shoot and root growth responded differently to soil drying (Sharp and Davies, 1979; Watts *et al.*, 1981). Shoot growth (e.g. leaf area and shoot dry weight) can be impeded by soil drying (Sharp and Davies, 1979, 1985; Westgate and Boyer, 1985), while root growth (e.g. root length and root dry weight) can be stimulated under mild drought and inhibited when the drought becomes severe (Sharp and Davies, 1979; Watts *et al.*, 1981; Creelman *et al.*, 1990). Consistent results in the present study showed that growth rate of the maize shoot (leaf elongation rate) and root (root length and surface area increase rates) responded differently to the short-term soil drying (Figure 2.2, 2.3 and 2.9A). Although a statistical difference was absent (which may be due to the large variation in the root length of soil grown plants), the maize under soil drying tended to show higher root growth rate (root length and surface area increase rates) under mild drought (Day 2–3, Figure 2.3, 2.9A), but lower growth rate when drought became more severe (after Day 4) (Figure 2.3, 2.9A). In contrast, leaf elongation was inhibited by soil drying, which started from Day 4 and was later than the promotion of root growth (Figure 2.9A).

Earlier drought responses (water potential decrease) in the root than in the shoot have been reported in maize plants (Sharp and Davies, 1979; Westgate and Boyer,

1985; Saab and Sharp, 1989). In this study, the root water potential started to decrease on Day 2–3 (average soil water content: 25–32%) under soil drying, while the leaf water potential was not reduced until Day 4–5 (average soil water content: 13–20%) (Figure 2.9B). The later response in the leaf than in the root may be attributable to the stimulated root growth under mild drought, so that the root was able to take up sufficient water to maintain unstressed leaves and also leaf elongation for a while. These results are consistent with previous studies that suggested the plant leaf water potential is an indicator of plant water status, but not an ideal parameter to represent the true soil water status or the root water status (reviewed in Davies and Zhang, 1991). This is because the leaf water potential may not change synchronously as the soil water potential drops, and other physiological responses may have already been activated in roots and perhaps in leaves too (e.g. reduced stomatal conductance and leaf elongation) (Sharp and Davies, 1979; Bates and Hall, 1981; Henson *et al.*, 1989).

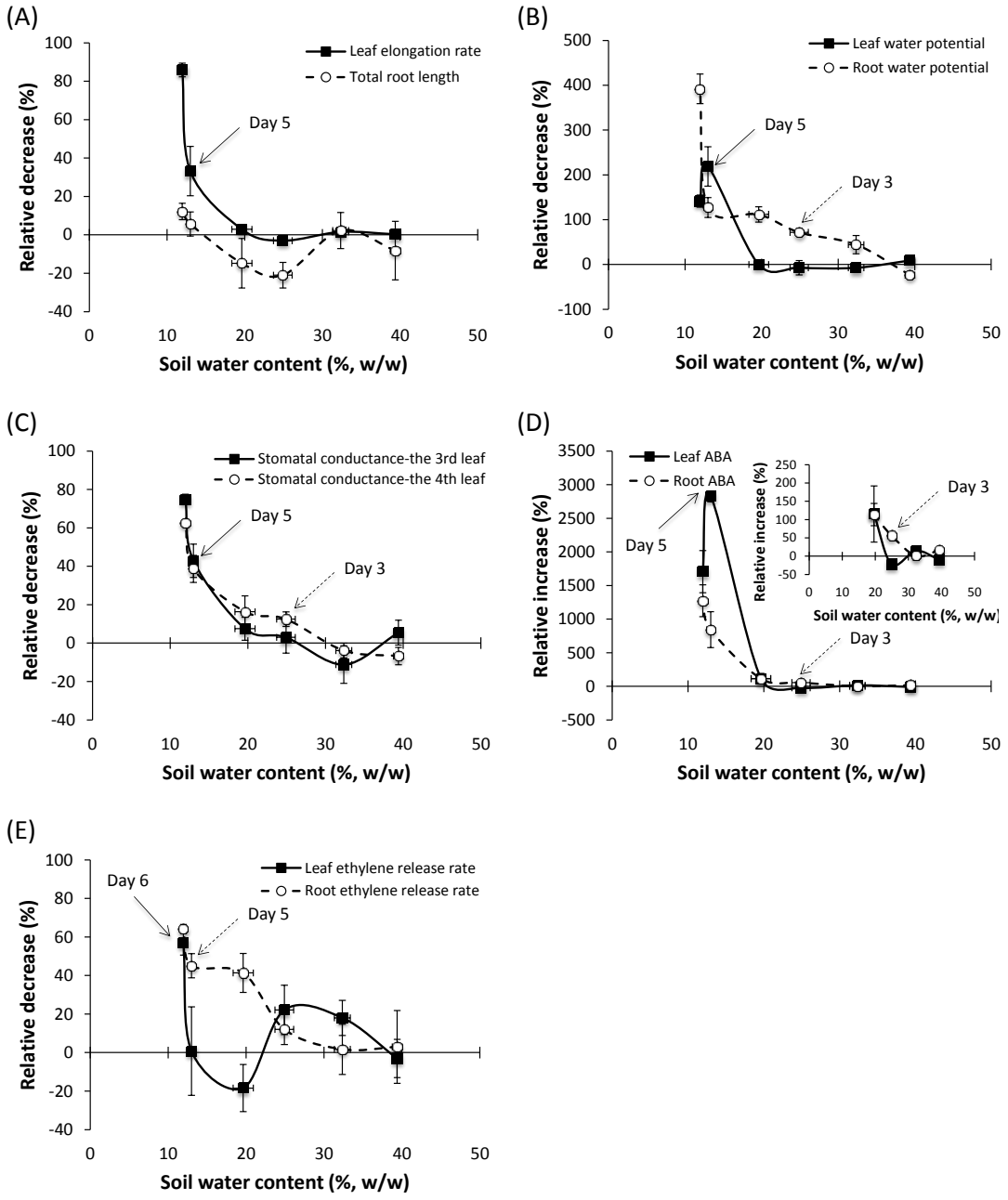


Figure 2.9: Relative changes in plant growth and physiological parameters against the soil water content during the 6-d soil drying. The relative decrease of (A) leaf and root growth; (B) leaf and root water potentials; (C) stomatal conductance of the 3rd and 4th leaves. (D) The relative increase of leaf and root ABA concentrations. (E) The relative decrease of ethylene release rate from the leaf and root. Points and bars are means ± standard error. Arrows and Day indicate the time when the difference between the two treatments became significantly.

Leaf stomatal conductance in the 3rd leaf was reduced by soil drying from Day 5, when the leaf water potential dropped (Figure 2.6, 2.9B, C). This is different from

previous reports that the closure of stomata could happen before leaf water potential is reduced by soil drying (Bates and Hall, 1981; Davies and Zhang, 1991; Tardieu *et al.*, 2010). This is particularly true with isohydric plants such as maize, which can maintain leaf water status unaffected in daytime under water deficit with stomatal closure occurring at a threshold leaf water potential (Tardieu *et al.*, 1996). Reduced stomatal aperture under drought stress is also a typical drought avoidance strategy in many plant species because it can prevent more water loss from evaporation and then postpone or minimise potential damage by more severe drought, which will probably occur when leaf water potentials decrease (Lawlor, 2013). The younger leaf (the 4th) showed lower stomatal conductance on Day 3 when root water potential was just significantly reduced by soil drying (Figure 2.5, 2.6, 2.9B, C). It is suggested that stomata of younger leaves were more sensitive to soil drying than those of the older leaves. It also indicates that the stomata of the growing leaf responded more quickly to soil drying than did its elongation rate. Leaf water potential in the 4th leaf was not measured; therefore it is not clear whether soil drying reduced both the water potential and stomata aperture in the 4th leaf at the same time or not. Nevertheless, stomata in older leaves have been found to be less sensitive to ABA-induced closure than those in younger leaves (Atkinson *et al.*, 1989), and it has been suggested that older leaves can provide ABA to sustain higher ABA concentrations in younger leaves (Zeevaart and Boyer, 1984).

The relationship between the ABA concentration, ethylene release rate and the leaf and root growth under soil drying

It was found that ABA concentrations in both root tips and leaf tissues of maize increased under soil drying (Figure 2.7A, 2.8A), which is in accordance with previous studies (Davies and Zhang, 1991; Bauerle *et al.*, 2006). In addition, the root ABA increase triggered by soil drying was accompanied by promoted root growth during the same day (Day 2–3), when the average soil water content decreased from 32% to 25% (soil water potential was from −0.33 to −0.37 MPa) (Figure 2.9A, D). The ABA was further accumulated in roots after Day 3 when the soil was drying further, with root growth inhibited from Day 4. Exogenous ABA has been found to both stimulate and inhibit root growth in maize, rice and also in Arabidopsis, depending on its concentration (Watts *et al.*, 1981; Xu *et al.*, 2013; Chapter 4). Therefore, it is implied that increased ABA levels of different magnitudes in roots under a mild or a more severe drought may have both stimulated or inhibited root growth. In contrast to the root, the ABA concentration in the leaf increased later, from Day 3 to Day 4, but only significantly from Day 5 (Figure 2.9D). The leaf elongation rate was inhibited during Day 4–5 (Figure 2.9A). This indicates that a small increase of leaf ABA (around one fold increase) was not related to leaf elongation change, while a large increase in leaf ABA level coincided with the inhibition of leaf elongation, which is consistent with previous reports that ABA is a shoot growth inhibitor (Trewavas and Jones, 1991; Munns and Sharp, 1993; Sharp and LeNoble, 2002).

In this study, root tips were sampled only from the top 2/3 of the pot to analyse the ABA concentration, because the root sampling method can be important if we want to argue that the root ABA increase occurred together with the decrease of the root water potential. Soil water was distributed heterogeneously in the pot (Figure 2.1C), so that when the top part of the soil column is dry enough to trigger an

increase of ABA concentration in the root, the lower part may still be too wet to see any enhanced root ABA level. Thus, root tips collected from the entire soil column may make it difficult to see an early increase of ABA concentration in the root even when the average soil water content had dropped to 22% in a preliminary experiment (data not shown). The leaves may not sense soil drying as early as the roots, but they may receive signals from the roots via xylem transport and also show similar ABA response as roots (Jackson, 1997; Munns and Cramer, 1996). However, our preliminary experiment showed root tips sampled from entire soil column made it difficult to determine whether the ABA response in the root occurs before it occurs in the leaf.

The present study showed that soil drying inhibited ethylene release from both maize leaf and root (Figure 2.7B, 2.8B), which is in accordance with the finding that maize ethylene emission was inhibited under low water potentials when the ABA level was increased (Sharp and LeNoble, 2002). However, the inhibitory effects of soil drying on leaf and root ethylene occurred at a later stage of the soil drying on Day 6 and 5 respectively (Figure 2.9E). Thus, the ABA concentrations in leaf and root were more susceptible to soil drying than ethylene release rates. Furthermore, both the leaf and root growth responses had happened prior to the detected changes of ethylene level during the soil drying. These non-synchronous effects suggest that changes in ethylene level do not play an important role in the regulation of leaf elongation and root growth under drought (at least before Day 4 in the current experiment). Similarly, Voisin *et al.* (2006) found that leaf elongation rate was not affected in moderately drought-stressed ABA-deficient maize plants that showed high ethylene levels. However, the ethylene levels may have been affected by the soil

drying in earlier days, but the GC equipment is not sensitive enough to detect such small change in emission (Cristescu *et al.*, 2013).

A possible explanation for the increase in root ethylene of well-watered plants from Day 4 is that the container has constrained the growing volume of root system and caused stress (Figure 2.8B). Ethylene has been reported to be a stress-induced hormone, and mechanical impedance can enhance the ethylene production without changing ABA level, while phosphorus deficit can also promote ethylene emission (Moss *et al.*, 1988; Li *et al.*, 2009).

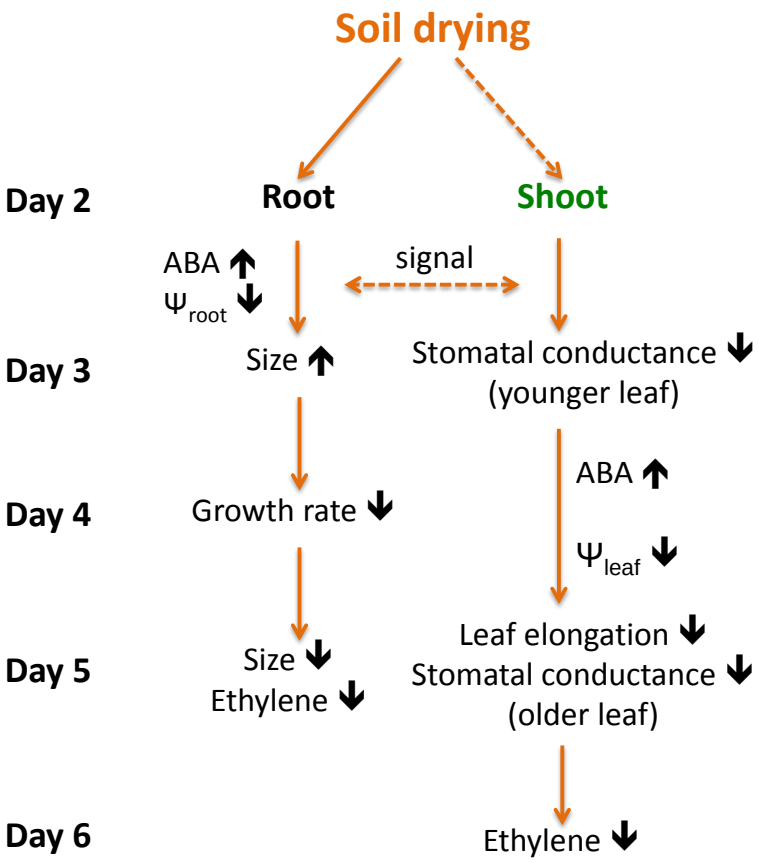


Figure 2.10: A process diagram summarised the physiological responses of maize root and shoot to the 6-day soil drying. Day indicates the number of days after the last watering. Root size indicates total root length and surface area. Upward or downward arrow in black indicates increase or decrease, respectively.

2.5 Conclusion

This study shows asynchronous physiological responses of maize leaf and root to a short-term soil drying, including changes in the growth rates, ABA concentrations and rates of ethylene release. The increase of ABA concentrations in root tissues was synchronous with the changes in root water potential and root growth rate during soil drying. The inhibition of leaf elongation occurred when the leaf ABA concentration increased to more than double that of well-watered plants. However, the decrease of ethylene release rates in leaf or root tissues happened later than the changes in ABA levels and they were not synchronous with the growth change of leaf or root during the 6-d soil drying. Those processes are summarised in Figure 2.10.

Chapter 3 Genetic Variation in Maize Root Traits and Hormone Levels under Drought

3.1 Introduction

The plant root system is crucial for plant survival and it can be important to determine plant resistance to environmental stresses, such as drought and poor nutrient availability (López-Bucio *et al.*, 2003; Zhu *et al.*, 2005; Uga *et al.*, 2013). Under drought conditions, plants with an efficient root system architecture may be able to maintain a favourable water balance to maintain their growth (Henry *et al.*, 2011). Root traits that contribute to such efficient root system architectures are the targets for plant breeding (Uga *et al.*, 2015). Although a number of root traits and their association with plant drought resistance have been studied (Wasson *et al.* 2012; Comas *et al.*, 2013), the complexity of root physiological traits makes it difficult to identify and characterise specific root traits that are related to drought resistance (Burton *et al.*, 2013).

Previous studies suggested that the xylem size, diameter and length of root and its distribution in deep soil are key traits that determine plant drought resistance (Kato *et al.*, 2006; Wasson *et al.*, 2012; Comas *et al.*, 2013). For instance, wheat and rice genotypes with deeper root biomass distribution in the field showed better yield performance under drought than those with shallower root distribution (Reynolds *et al.*, 2007; Henry *et al.*, 2011; Uga *et al.*, 2013). Closely related to the pattern of root distribution, root angle has recently become one of the target traits thought to be

influential in plant drought resistance since it determines the direction for root elongation, and thereby probably controls the efficiency of resource capture from the soil (Abe and Morita, 1994; Kato *et al.*, 2006; Uga *et al.*, 2015). Additionally, a recent study suggested that a steeper root angle under well-watered condition was correlated with higher grain yields in maize under drought in the field (Ali *et al.*, 2015). However, we still need more understanding of the importance of root angle in comparison with other root traits in determining plant drought resistance and its relation with other traits.

Plant root system architecture is controlled by both genetic and environmental factors (McCully, 1995, 1999; Rich and Watt, 2013). A plant root can display considerable plasticity in response to heterogeneous distribution of water and nutrient in the soil (Hodge, 2004; Malamy, 2005; Gruber *et al.*, 2013). For instance, maize root length and biomass may be promoted when drought is mild and inhibited when drought became severe (Sharp and Davies, 1979; Creelman *et al.*, 1990). Furthermore, under drought stress conditions, growth of fine lateral roots of perennial ryegrass plants was stimulated, and the endodermis of root was heavily suberised, which may help to protect the root stele from desiccation (Jupp and Newman, 1987). Nevertheless, how the root angles will change (plasticity) under drought is less clear.

Many plant hormones are involved in plant response to drought stress (Santner *et al.*, 2009). Abscisic acid (ABA) is well known to play a pivotal role in regulating a series of molecular and physiological processes in response to drought (Davies and Zhang, 1991; Sharp *et al.*, 2004; Boyer and Westgate, 2004). Giuliani *et al.* (2005)

identified a quantitative trait locus (QTL) *RootABA1* in maize and it exerted effects on the leaf ABA titre and root architecture. High concentrations of ABA are reported to be necessary for restricting ethylene production in maize at low water potentials, thus maintaining plant root growth (Saab *et al.*, 1990; Sharp and LeNoble, 2002). Cytokinin is considered as a negative regulator for root elongation and branching (Cary *et al.*, 1995; Werner *et al.*, 2010). Werner *et al.* (2010) reported that transgenic tobacco and Arabidopsis plants with root-specific overexpression of a cytokinin oxidase/dehydrogenase gene (*CKX*), which regulates cytokinin-breakdown, had less root cytokinin, a larger root system and better resistance to severe drought. However, the relationship between these hormones (i.e. ABA, ethylene and cytokinin) and maize root angle has not yet been explored.

This study aimed to investigate the genetic variation in maize crown and nodal root angles and the plasticity of these traits under drought by subjecting 14 maize genotypes to three soil water levels. It also intended to examine whether root angle and its plasticity correlate with other root traits, endogenous hormone levels and reduction in shoot biomass under drought.

3.2 Materials and methods

Plant growth

Fourteen genotypes of maize (*Zea mays* L.) were tested in this study. There were two inbred lines B73, UH007 which were kindly provided by Prof. Dr. Frank Hochholdinger, University of Bonn, Germany, and Dr. Wolfgang Schipprack, University of Hohenheim, Germany respectively; and another twelve F1 hybrids that

were derived from twelve inbred lines crossed with the same parental line UH007 by the EURoot Project partners.

For each experiment (one genotype), there were three water treatments (well-watered, mild drought and severe drought) and five pots for each treatment. For practical reasons, the 14 genotypes were sown and harvested one after another under the same treatments in a controlled environment (CE) room (24˚C /18˚C, 14 h/10 h, and a light density of 350 µmol m^{-2} s^{-1}). The experiment was repeated once for all 14 genotypes, totalling 28 batches of experiment, which were conducted consecutively during July–November 2015. For seeds germination in each batch of experiments, forty-five seeds of one genotype were selected by weight (90–110% of the average seed weight). All seeds were sterilised in 30% H_2O_2 (10002780, Fisher Scientific) for 10 min and rinsed six times with sterile deionised water. Then the seeds were soaked in autoclaved deionised water for 24 h prior to pre-germination on wet paper towel in the dark for two days. Fifteen germinated seeds of each genotype with a similar root length were selected and transferred into pots (height: 23, top diameter: 16.5 cm, bottom diameter: 13.5 cm), with one seed per pot. Each pot was filled with 3700–3900 g (equivalent to 3100 g dry soil) moist soil (sieved John Innes No.2, J. Arthur Bower's, UK). A plastic tray was placed beneath each pot.

After transplantation (day 0), the soil was watered to 40%, 40% and 30% (w/w) soil water content in the well-watered, mild and severe drought treatments respectively (Figure 3.1). One-third of the water was added from the top contained 50 ml Hoagland's nutrient solution (pH = 5.8–6.0), and the other two-thirds was added from the bottom in the tray. When all the seedlings were visible (normally on day 3),

the well-watered treatment was watered further to 45% soil water content and then watered daily to the same water content. The other two drought treatments were only watered on day 0 but the pots were weighed several times during the experimental period. Preliminary experiments showed that maize crown and nodal roots started to develop when the first and the third leaves were fully expanded respectively (when leaf collars became visible). In all of these 28 experiments, the 1st, 2nd and 3rd leaves were fully expanded between 5–6, 8–10 and 12–14 days after transplantation respectively. The plants were harvested at day 17, 3–5 days after the 3rd leaf became fully expanded and the crown root and the first whorl of the nodal root had developed, so that the angle of the crown and nodal roots could be measured. Four of the five plants were harvested in each treatment for plant measurements and the last one was used for measuring water content in the top 3 cm soil layer (see below).

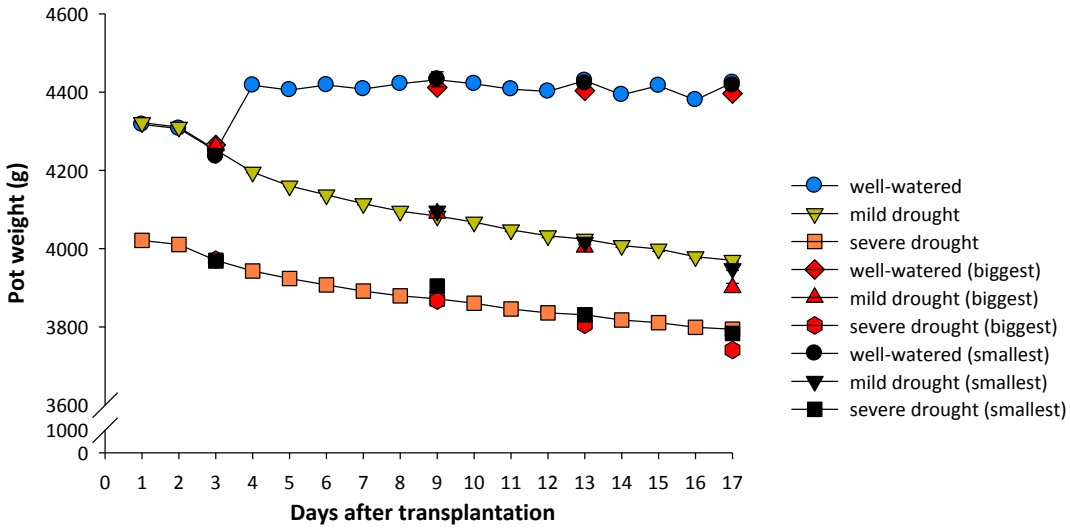


Figure 3.1: Pot weights during 17 d under the three water treatments. On day 0, pots in well-watered, mild and severe drought treatments were watered to 40%, 40% and 30% (w/w) soil water content respectively. After all seedlings were visible (day 3), the pots in well-watered treatment was watered to 45% soil water content (after the weight was recorded) and then

watered daily to the same soil water content level. Pots in mild and severe drought treatments were only watered on day 0. The continuous data from day 1–17 were measured with blank pots (without plants, n = 3). The red and black points indicate the pot weights on four days (day 3, 9, 13 and 17) in experiments with maize genotypes which have the biggest (RootABA1+ × UH007) and the smallest (F7028 × UH007) shoot dry weight respectively (n = 4–10). Points and bars are mean ± standard error.

Soil and shoot water content

In each batch of the experiment, soil water content was measured at three depths, i.e. 0–7, 7–14 and 14–21 cm, in two pots (the 2nd and 4th of four pots) from each treatment after removing the shoots. The soil in each section was well mixed, weighed ($W_{original}$), oven dried at 80°C and weighed again for the dry weight (W_{dry}). The soil water content (%, w/w) was calculated by $[(W_{orignial} - W_{dry}) / W_{dry}] \times 100\%$. Soil water content was also measured on the top 3 cm soil in another pot in each treatment.

After the 3rd and the 5th leaves were harvested for hormone analysis (see below), the rest of the shoot was cut, weighed (W_{shootF}, including the 5th but not the 3rd leaf), oven dried at 80°C and weighed again for the dry weight (W_{shootD}). The shoot water content (%) was calculated by $[(W_{shootF} - W_{shootD}) / W_{shootD}] \times 100\%$.

Root traits

Crown and nodal root angle

After the maize shoot was removed, the root was washed out with tap water from four pots in each water treatment. All seminal and primary roots were cut out but the crown and nodal roots remained with only the top part (ca. 1.5 cm away from the root base vertically) (Figure 3.2A). The basal part of a root was then fixed in the

centre of a square stainless steel mesh with 4 mm aperture (Figure 3.2B, C). Then the locations that crown and nodal roots intersected with the stainless steel mesh were marked with different symbols on a paper that was printed with lines identical to size as the mesh. The distances from the centre of the mesh to the marked root intersections were measured (L_c for crown root and L_n for nodal root). The vertical height from the mesh surface to the basal point of the whole whorl of crown root (H_c) and nodal root (H_n) were measured respectively. The H_c, L_c and the crown root itself or the H_n, L_n and the nodal root itself constitute a right-angled triangle, and the angle between the crown root (θ_c) or the nodal root (θ_n) and the central vertical root axis is the acute crown/nodal root angle (Figure 3.2D). The tangent of these angles can be calculated with $\tan(\theta) = L/H$. The degree of each angle can be obtained by arc tangent transformation. When certain genotypes were under drought stress, some crown and nodal roots were so short that a ruler was used directly to measure the height (H_c and H_n) and length (L_c and L_n).

Total root length, surface area and dry weight

Two plants from each treatment (the 1st and 3rd of four pots) were harvested for root ethylene incubation (see below), then these roots (unused for angle measurements) were scanned and analysed for total root length and root surface area with the WinRHIZO Pro system (with a STD4800 scanner, Regent Instruments Inc., Quebec, Canada). Finally, those root samples were oven dried at 80°C for dry weight.

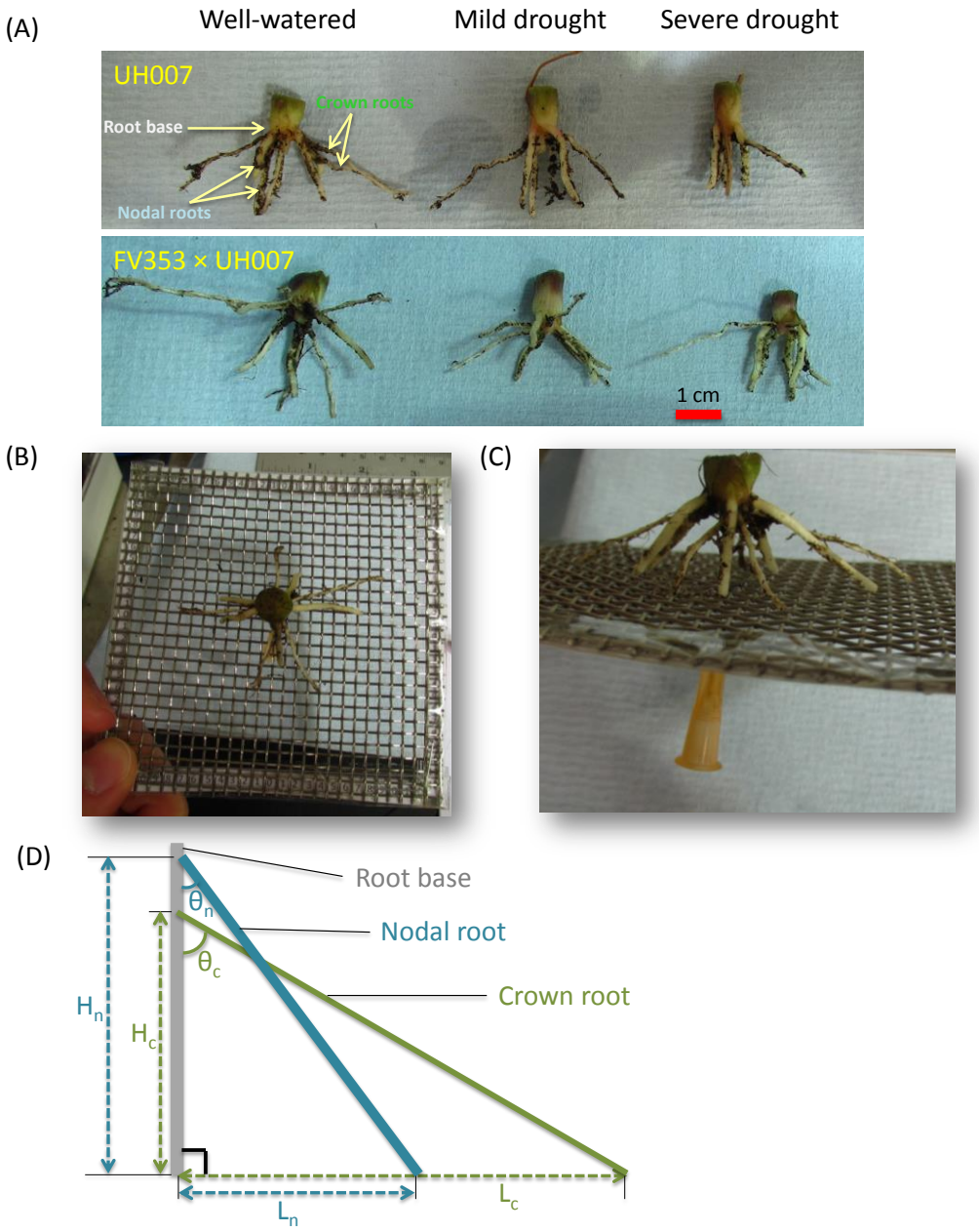


Figure 3.2: Root angle measurements. (A) Examples of root angle samples of two maize genotypes under three water treatments. It showed that under well-watered treatment, UH007 inbred line displayed steep nodal root but shallow crown root; FV353 × UH007 F1 hybrid displayed shallow nodal and crown roots. The seedlings were pictured 17-day after transplantation. The shoot above the soil surface and all seminal and primary roots were pruned and the root base, crown and nodal roots remained (ca. 1.5 cm vertically). The view from (B) the top and (C) side when a root sample was perpendicularly fixed in the centre of the stainless steel mesh (9.3 cm × 9.3 cm) with a needle. (D) The simulated diagram for the root angle measurement. The crown (θ_c) or nodal (θ_n) root axis angle was in a right-angled triangle. The hypotenuse was the crown or nodal root, the needle was the adjacent side (H_c or H_n) and the line from the centre of the mesh to the marked intersection between the root and mesh was the opposite side (L_c or L_n). The tangent of these root angles can be calculated with $\tan(\theta) = L/H$. Then the degree of each angle can be obtained by arc tangent transformation.

Hormones

Leaf and root hormone profile analysis

The leaf and root hormone profile were determined in this study, including 1-aminocyclopropane-1-carboxylic acid (ACC), *trans*-zeatin (*t*Z), zeatin riboside (ZR), isopentenyladenine (iP), gibberellin A1 (GA1), GA3, GA4, indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA). To ensure enough leaf and root tissue for hormone profile analysis, the 3rd leaves from two plants in the same treatment were cut and mixed as one sample, and the entire root system from one pot was washed out (within 0.5 h after removing tops). The sample was put into a 15 ml centrifuge tube, which was immersed into liquid nitrogen immediately and stored in −20°C before the sample was freeze-dried for 48 h. The dried sample was ground and weighed (100 mg) out for a double extraction with 1.5 ml extraction buffer (methanol:water 80:20 v/v) for 0.5 h at 4°C. The extract (3 ml) was purified by passing through a Chromafix C18 column and then concentrated in SpeedVac for at least 3 h. The concentrated sample was dissolved in 1 ml buffer (methanol:water 20:80 v/v) and then 100 µl was loaded to a 96-well plate together with 5 standard solutions (0, 0.001, 0.005, 0.01, 0.1 µM). Samples were analysed on an HPLC-MS system by Dr. Alfonso Albacete in CSIC (Murcia, Spain) (Albacete *et al.*, 2008). Four out of the 11 hormones (*t*Z, ABA, JA and SA) were detected in both the leaf and root of all 14 maize genotypes. Data of ABA and *t*Z were presented in Figure 3.7 and 3.9. Data of JA, SA and IAA (detected in the root samples of 13 genotypes) can be found in Appendix 2 Figure 1.

Leaf and root ethylene release rate

To detect the ethylene release rate from maize leaf and root, the 5th leaf and the entire root system of two plants (the 1st and the 3rd) in each treatment were collected. The sample was incubated in a sealed glass tube (24 ml) for 1.5 h. Then 10 ml gas sample was taken using a syringe and injected into a 20 ml storage bottle. The ethylene concentrations in the samples were measured with a laser-based ethylene detection system, which mainly consists of a catalyser, a valve control box and an ethylene detector (Sensor Sense, Netherlands). A 20-ppm ethylene/nitrogen standard gas (BOC Limited, Surrey, UK) was used to check the accuracy of the ethylene detection system frequently. After incubation, the root samples were used for scanning (see above) before oven drying and the leaf samples were directly oven dried at 80°C. Finally, the leaf and root ethylene release rates were calculated separately.

Statistical analysis

The data from the two batches for the same genotype were combined. Thus, there were eight replicates for shoot dry weight and shoot water content and four replicates for total root length, root surface area, root dry weight, leaf and root ethylene release rates and other hormone measurements. Every crown or nodal root angle from the same treatment was treated as one replicate. The replication number for crown and nodal root varied from 27–43 and 13–41 respectively.

The statistical software SPSS 21.0 (IBM, USA) was used to perform the linear mixed-effects models procedure to test the impact of genotype and water treatment for all the measurements by setting them as the two fixed factors and batch number of experiments was set as the random factor. Except for the relative shoot dry weight

(in every experiment, the mean of well-watered treatment was set as 100%), all the other measurements were significantly affected by the interaction between the two fixed factors (genotype and water treatment) (Appendix 2 Table 1–3). Therefore, for most of the measurements presented here, the effects of those two fixed factors cannot be interpreted without considering their interaction effect. Then all the results in the figures were analysed by two-way ANOVA with Tukey's *post hoc* test at the $P < 0.05$ level (the batch number was one factor) or by using correlation analysis.

3.3 Results

Soil and shoot water contents

To evaluate the intensities of the drought stress levels in drought treatments, the soil water content (w/w) and the shoot water content in the three water treatments were compared at the harvest day (day 17). Figure 3.3A displays the average soil water content for all maize genotypes in three layers of each pot, i.e. 0–7, 7–14 and 14–21 cm, which showed significantly different soil water content under the three water treatments in each soil layer ($P < 0.05$). The topsoil was the driest part in all treatments, especially in the two drought treatments without watering for 17 days. To further assess the drought severity in the driest layer, the water content in the top 3 cm soil was measured also, which showed an even larger difference among treatments than in the 0–7 cm layer (Figure 3.3A, B). More detailed data of individual genotypes is presented in Appendix 2 Table 4.

Plant tissue water content is an indicator of its water status, which can be effectively reduced by drought stress (Hsiao, 1973). On average, the mild and severe

drought in this study reduced shoot water contents in all 14 maize genotypes by 12% and 24% respectively (Figure 3.3C).

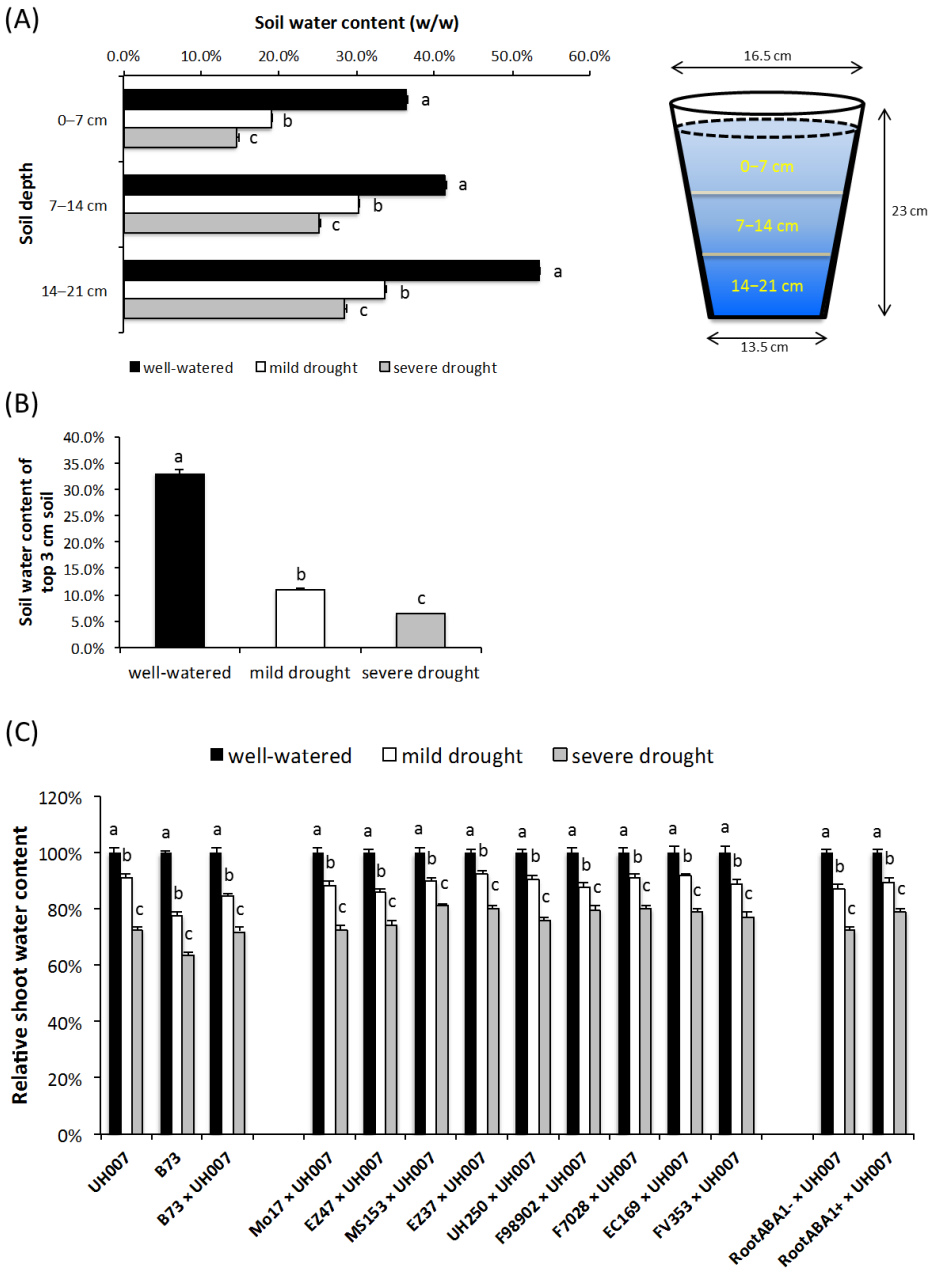


Figure 3.3: Soil water content in a pot (A) at three layers (i.e. 0–7, 7–14 and 14–21 cm), (B) at top 3 cm. (C) Relative shoot water content (as a percentage of that in well-watered plants). The seedlings were 17-day after transplantation. The detailed water treatments were described in Figure 3.1. In one batch of experiment only one maize genotype was used. There were 28 batches of experiments with all 14 genotypes, which were conducted consecutively in July–November 2015. There were five plants for each treatment and four of the five plants were harvested. Data from each batch was analysed separately and then combined for the same maize genotype from two replicated experiments. Two of the four harvested pots were

used to measure the soil water contents in three layers. The fifth pot in each treatment was used to measure the soil water content in the top 3 cm. The soil water content data presented here is the combined result from all 28 experiments. The shoot water content (excluding the 3rd leaf) presented is the combined result from all the harvested plants of the two experiments for the same maize genotype. Columns and bars are means ± standard error. Different letters indicate significant differences between water treatments at $P < 0.05$.

Genetic variation in root angle and plasticity of root angle under drought

The genetic variations in the crown and nodal root angles in 14 maize genotypes when well-watered were observed. In general, the crown root angle varied from 47.5º to 69.6º and it was larger (shallower) than the nodal root, which ranged from 36.8º to 52.4º (Table 3.1). Furthermore, the crown and nodal root angles of the 14 genotypes were positively correlated ($R^2 = 0.483$, $P = 0.006$, Figure 3.10A). Thus a genotype with a shallower crown root angle tended to have a shallower nodal root angle, except for two inbred lines UH007 and B73 (Table 1, Figure 3.10A). UH007 showed a relatively large (shallow, 58.7º) crown root angle but a small (steep, 37.2º) nodal root angle (Table 3.1). In contrast, B73 showed a relatively steep crown root angle (47.5º) and a medium nodal root angle (43.9º) among the 14 genotypes (Table 3.1). Interestingly, both crown and nodal root angles of the hybrid line B73 × UH007 were within the range of their parent lines (Table 3.1).

Table 3.1: Genetic variations in root and shoot traits (mean ± standard error) between 14 genotypes. * indicates the decrease of nodal root angle under mild drought (as a percentage of nodal root angles in well-watered plants). ** indicates the decrease of crown root angle under mild drought (as a percentage of crown root angles in well-watered plants). Different letters indicate significant differences between maize genotypes at $P < 0.05$.

Genotypes	Nodal root angle (°, well-watered)	Nodal root angle decrease* (%)	Crown root angle (°, well-watered)	Crown root angle decrease** (%)	Total root length (m)	Root surface area (cm^2)	Shoot dry weight (g)	Root dry weight (g)
					Well-watered			
UH007	37.2 ± 1.5 e	16.4 ± 5.4 d	58.7 ± 1.9 bcdef	13.9 ± 5.5 c	11.0 ± 0.8 f	128.6 ± 5.4 g	0.3790 ± 0.0211 f	0.1057 ± 0.0078 f
B73	43.9 ± 1.5 bcd	14.9 ± 5.1 d	47.5 ± 1.5 h	16.1 ± 4.1 c	32.3 ± 2.2 cd	308.2 ± 14.6 cd	0.6256 ± 0.0260 cd	0.2479 ± 0.0151 ab
B73 × UH007	40.0 ± 1.0 de	26.3 ± 3.7 bcd	53.1 ± 1.1 fgh	19.4 ± 4.9 bc	36.1 ± 7.6 c	311.71 ± 49.6 cd	0.7104 ± 0.0675 bc	0.1962 ± 0.0257 bcd
Mo17 × UH007	36.8 ± 1.4 e	37.2 ± 3.1 bc	51.7 ± 1.0 gh	35.7 ± 5.0 ab	20.1 ± 1.2 ef	182.1 ± 12.6 efg	0.3993 ± 0.0279 f	0.1264 ± 0.0083 ef
EZ47 × UH007	39.0 ± 1.3 de	23.9 ± 3.2 bcd	52.5 ± 1.5 gh	29.3 ± 4.3 abc	18.1 ± 2.0 ef	188.2 ± 17.1 efg	0.3526 ± 0.0391 f	0.1463 ± 0.0162 def
MS153 × UH007	42.1 ± 1.0 cde	21.0 ± 3.1 cd	55.5 ± 0.9 efg	22.5 ± 3.4 bc	38.7 ± 3.8 bc	351.9 ± 25.5 bc	0.7370 ± 0.0422 bc	0.2407 ± 0.0182 abc
EZ37 × UH007	42.6 ± 1.1 bcde	39.8 ± 4.3 ab	56.5 ± 1.1 defg	32.5 ± 4.1 abc	16.8 ± 2.8 ef	169.7 ± 21.2 fg	0.4329 ± 0.0422 ef	0.1250 ± 0.0100 ef
UH250 × UH007	48.0 ± 1.4 abc	29.9 ± 4.6 bcd	62.5 ± 0.8 bc	15.0 ± 2.9 c	17.6 ± 1.3 ef	197.3 ± 12.4 efg	0.4688 ± 0.0242 def	0.1476 ± 0.0142 def
F98902 × UH007	48.0 ± 1.5 abc	25.8 ± 2.7 bcd	63.4 ± 0.9 b	17.6 ± 3.1 bc	25.5 ± 2.0 de	257.7 ± 16.4 de	0.6006 ± 0.0456 cde	0.1798 ± 0.0109 cde
F7028 × UH007	48.3 ± 1.3 ab	55.8 ± 4.3 a	56.7 ± 1.5 cdefg	42.6 ± 5.3 a	14.3 ± 1.6 f	143.5 ± 16.1 g	0.2982 ± 0.0219 f	0.1056 ± 0.0109 f
EC169 × UH007	52.1 ± 1.5 a	36.1 ± 4.2 bc	61.2 ± 1.2 bcde	21.8 ± 4.1 bc	24.2 ± 2.1 de	236.9 ± 18.1 def	0.6172 ± 0.0471 cd	0.1511 ± 0.0194 def
FV353 × UH007	52.4 ± 1.0 a	24.4 ± 3.6 bcd	69.6 ± 1.4 a	17.7 ± 2.9 bc	35.6 ± 3.1 c	359.4 ± 27.1 bc	0.8087 ± 0.0495 ab	0.2646 ± 0.0245 a
RootABA1- × UH007	44.2 ± 1.2 bcd	16.7 ± 2.5 d	60.6 ± 1.5 bcde	16.9 ± 4.0 bc	51.3 ± 5.1 a	443.9 ± 50.0 a	0.8545 ± 0.0699 ab	0.2970 ± 0.0455 a
RootABA1+ × UH007	46.6 ± 1.0 abc	21.5 ± 2.6 cd	62.3 ± 0.9 bcd	14.0 ± 3.6 c	46.9 ± 2.3 ab	415.3 ± 15.6 ab	0.9207 ± 0.0530 a	0.2762 ± 0.0120 a

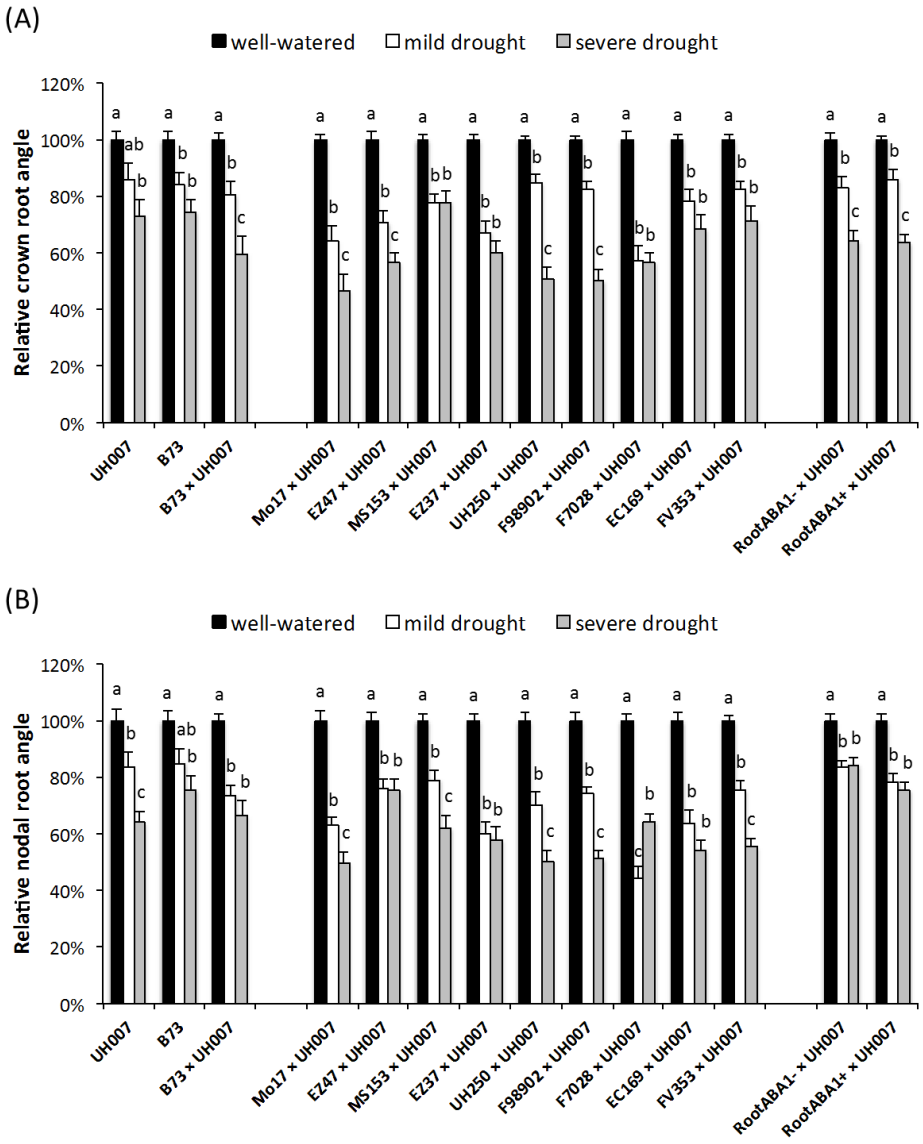


Figure 3.4: (A) Relative crown root angle, (B) relative nodal root angle (as a percentage of that in well-watered plants). The seedlings were 17-day after transplantation. All 14 maize genotypes were grown consecutively in 28 experiments with three water treatments as described in Figure 3.1. Every crown and nodal root angle from the four harvested plants in each treatment was measured as one replication. The mean crown or nodal root angle of well-watered plants were set as 100%. Columns and bars are means ± standard error. Different letters indicate significant differences between water treatments at $P < 0.05$.

Under the mild and severe drought stress, both the crown and nodal root angles decreased (plasticity) in all maize genotypes when compared with well-watered conditions (Figure 3.4), which indicated the plasticity of root angle under drought.

The crown and nodal root angles decreased under mild drought by 14–42% and 15–56% respectively, and under severe drought by 22–50% and 16–50% respectively (Figure 3.4 and Table 3.1). Although most genotypes showed larger decrease in nodal or crown root angle under severe than mild drought, some genotypes had similar decrease in root angles under both drought intensities (Figure 3.4). The latter response suggests that the root angles in these genotypes were sensitive to even a mild drought stress, but root angle not change further under further drying (e.g. crown root of F7028 × UH007 and nodal root of EZ47 × UH007 in Figure 3.4). It might also imply that an even more severe drought (than the current 'severe drought') is needed for these genotypes to show significant decrease in root angles compared to the mild drought (e.g. crown root of B73 and nodal root of EC169 × UH007 in Figure 3.4). The plasticity in crown and nodal root angles (as indicated by the relative decrease of root angle as a percentage of that in well-watered plants) were correlated under mild (R^2 = 0.686, P = 0.0003, Figure 3.10C) but not severe drought (data not shown). Similar to the well-watered condition, positive correlation was seen between the crown and nodal root angle in mild drought (R^2 = 0.669, P < 0.001, Figure 3.10B) but not severe drought condition (data not shown). It should be noted that under severe drought, some crown and nodal roots were not able to grow long enough to allow an accurate angle measurement. Therefore, the plasticity of root angles under mild drought is a more reliable measurement than under severe drought in this study, and further discussions on root angle plasticity will only include the mild drought treatment.

The crown root angle showed smaller plasticity than the nodal root angle as indicated by a slope of 0.67 in Figure 3.10C. This may be due to the fact that the

crown root developed earlier than the nodal root, which may have sensed higher stress levels since the drought stress gradually became stronger due to evaporation in this study. Therefore, the changes in nodal root angle under the mild drought treatment could be a more reliable parameter representing plasticity of root angle under drought. The F7028 × UH007, EZ37 × UH007, Mo17 × UH007 and EC169 × UH007 had the highest root angle plasticity with decreases in root angle by 55.8%, 39.8%, 37.2% and 36.1% respectively (Table 3.1). By contrast, the B73, UH007 and RootABA1- × UH007 had the lowest root angle plasticity and the decreases were only 14.9%, 16.4% and 16.7% respectively (Table 3.1).

Genetic variation of other root traits and their plasticity under drought

Similar to the root angle under well-watered condition, genetic variation in other root traits, i.e. total root length, surface area and dry weight, was observed also (Table 3.1). In addition, these root traits also showed plasticity under mild and severe drought (Figure 3.5). Firstly, the total root length of well-watered maize after 17 days ranged from 11.0–51.3 m/plant in 14 genotypes (Table 3.1). When averaged across the mild and severe drought treatments, the total root length of three genotypes (UH007, RootABA1- × UH007 and RootABA1+ × UH007) significantly increased by 62%, 43% and 36% respectively (Figure 3.5A). In contrast, the total root length was significantly inhibited in FV353 × UH007 under severe drought (Figure 3.5A).

Secondly, the root surface area varied from 128.6–443.9 cm^2/plant in the 14 well-watered maize genotypes, which showed similar pattern as the total root length (Table 3.1). The root surface area of the above-mentioned three genotypes (UH007,

RootABA1- × UH007 and RootABA1+ × UH007) plus Mo17 × UH007 significantly increased under drought by 52%, 37%, 28% and 31% respectively (Figure 3.5B). In MS153 × UH007, the mild but not the severe drought treatment showed significant promotion of root surface area (Figure 3.5B). By contrast, the severe drought caused significant inhibition of total root surface area of FV353 × UH007 by 22% (Figure 3.5B).

Thirdly, the root dry weight of well-watered maize varied from 0.11–0.30 g/ plant among 14 genotypes (Table 3.1). The root dry weight for eight out of 14 genotypes was significantly stimulated under drought (Figure 3.5C). The highest increase in root dry weight was in UH007 by 70% and 123% under mild and severe drought respectively (Figure 3.5C). The root dry weight was only significantly inhibited in one genotype, EC169 × UH007, under severe drought stress by 42% (Figure 3.5C).

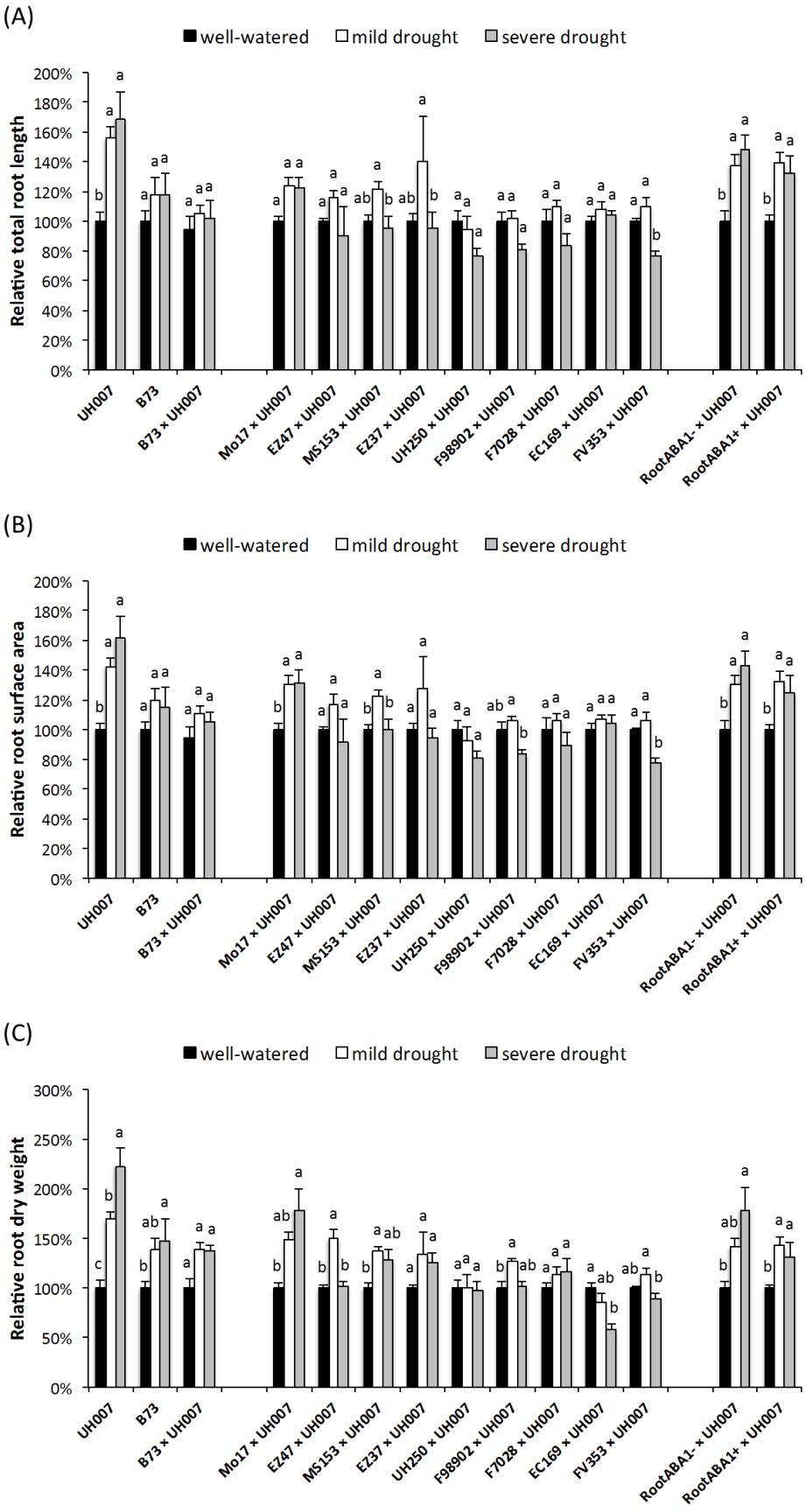


Figure 3.5: (A) Relative total root length, (B) relative root surface area, (C) relative root dry weight (as a percentage of that in well-watered plants). The seedlings were 17-day after

transplantation. All 14 maize genotypes were grown consecutively in 28 experiments with three water treatments as described in Figure 3.1. Two of the four harvested plants in each treatment were harvested for root ethylene (see Figure 3.8B), root scanning and dry weight. After the incubation for ethylene measurement, root samples were scanned and anaylsed with the WinRHIZO Pro system for root length and surface area. Then those samples were oven dried for dry weight. The mean value of well-watered plants was set as 100% in each experiment. Data presented here is the combined result from two experiments with the same maize genotype. Columns and bars are means ± standard error. Different letters indicate significant differences between water treatments at $P < 0.05$.

Genetic variation in drought resistance (indicated by shoot biomass change)

Drought stress can cause shoot biomass and seed yield reductions in plants, and a plant with less inhibition or even promotion can indicate better drought resistance (Huang *et al.*, 1997; Yang *et al.*, 2007). Reductions in shoot dry weight to different extents were observed in the tested genotypes under mild and severe drought in this study (Figure 3.6), indicating genetic variations in drought resistance among the 14 maize genotypes. The mild drought only significantly inhibited shoot dry weight accumulation in EC169 × UH007, FV353 × UH007, UH250 × UH007 and MS153 × UH007 by 24%, 21%, 20% and 11% respectively (Figure 3.6). This result indicated that these four genotypes might be susceptible to even a mild drought stress. On the other hand, the severe drought treatment significantly inhibited shoot dry weight in all genotypes (Figure 3.6). The FV353 × UH007, EZ47 × UH007 and UH250 × UH007 showed the greatest reduction in shoot dry weight by 53%, 50% and 49% respectively, while the UH007, RootABA1- × UH007 and RootABA1+ × UH007 displayed the smallest reduction by 22%, 31% and 31% respectively (Figure 3.6).

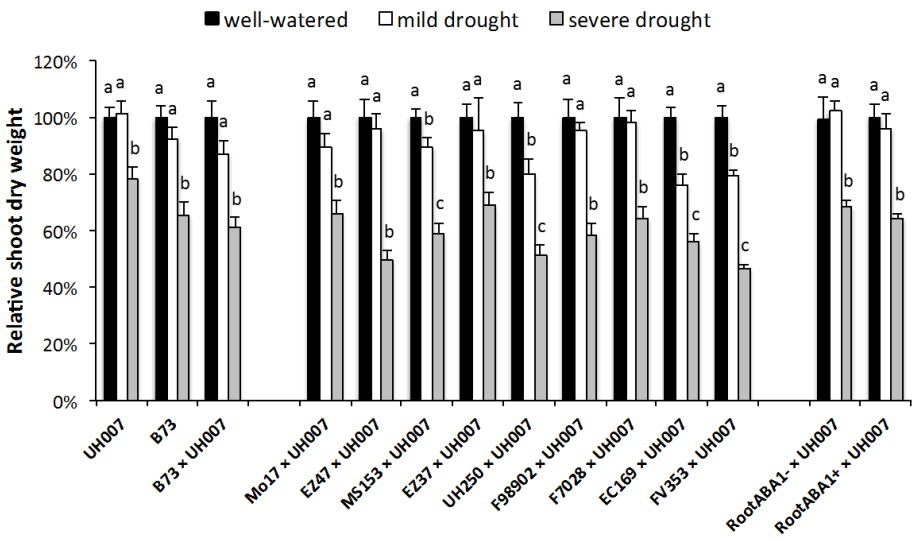


Figure 3.6: Relative shoot dry weight (as a percentage of that in well-watered plants). The seedlings were 17-day after transplantation. All 14 maize genotypes were grown consecutively in 28 experiments with three water treatments as described in Figure 3.1. The mean value of well-watered plants was set as 100% in each experiment. The shoot dry weight (excluding the 3rd leaf) presented is the combined result from all the harvested plants of two experiments with the same genotype. Columns and bars are means ± standard error. Different letters indicate significant differences between water treatments at $P < 0.05$.

Leaf and root hormones in responses to drought

ABA

ABA is a well-known drought stress hormone in plants and its concentration in the root can indicate the soil water availability to the plant (Zhang and Davies, 1989). In this study, the ABA concentrations in the 3rd leaf (expanded) and the entire root system were measured. The ABA concentration was lower in the root than in the leaf in all genotypes under all water treatments (Figure 3.7). In well-watered maize, the ABA concentration in leaf was 2.7–391.8 ng/g DW and 0.3–1.0 ng/g DW in root (Figure 3.7). Moreover, the leaf ABA levels were highest in four genotypes, i.e. F98902 × UH007 (391.8 ng/g DW), UH250 × UH007 (306.3 ng/g DW), and EC169 ×

UH007 (302.1 ng/g DW) and FV353 × UH007 (234.4 ng/g DW) (Figure 3.7A), of which three also showed highest ABA concentrations in the roots, i.e. FV353 × UH007 (1.0 ng/g DW), F98902 × UH007 (0.8 ng/g DW) and EC169 × UH007 (0.7 ng/g DW) (Figure 3.7B). Although RootABA1+ × UH007 (2.7 ng/g DW) and RootABA1- × UH007 (10.9 ng/g DW) had the lowest leaf ABA concentration (Figure 3.7A), their root ABA concentrations were in the middle range of all genotypes (both were 0.6 ng/g DW) (Figure 3.7B). The lowest root ABA level was found in MS153 × UH007 (0.3 ng/g DW), UH250 × UH007 (0.4 ng/g DW) and F7028 × UH007 (0.4 ng/g DW) (Figure 3.7B).

ABA concentrations in leaf and root were elevated in most genotypes under both drought treatments with a larger effect under severe drought (Figure 3.7). For instance, the leaf ABA concentration in 13 genotypes significantly increased under severe drought treatment by 1.1–184.1 folds (Figure 3.7A), while only significantly increased in EZ47 × UH007 under mild drought by 1.4 folds (Figure 3.7B). Furthermore, the root ABA concentration in ten genotypes significantly increased under severe drought by 0.2–1.5 folds (Figure 3.7B), but only significantly increased in B73 × UH007 and B73 under mild drought by 38% and 67% respectively (Figure 3.7B). However under mild drought, there was an increase of 14–202% in the leaf ABA concentration in 11 genotypes (Figure 3.7A) and an increase of 13–59% in the root in 7 genotypes (Figure 3.7B) respectively, though this was not statistically significant ($p > 0.05$).

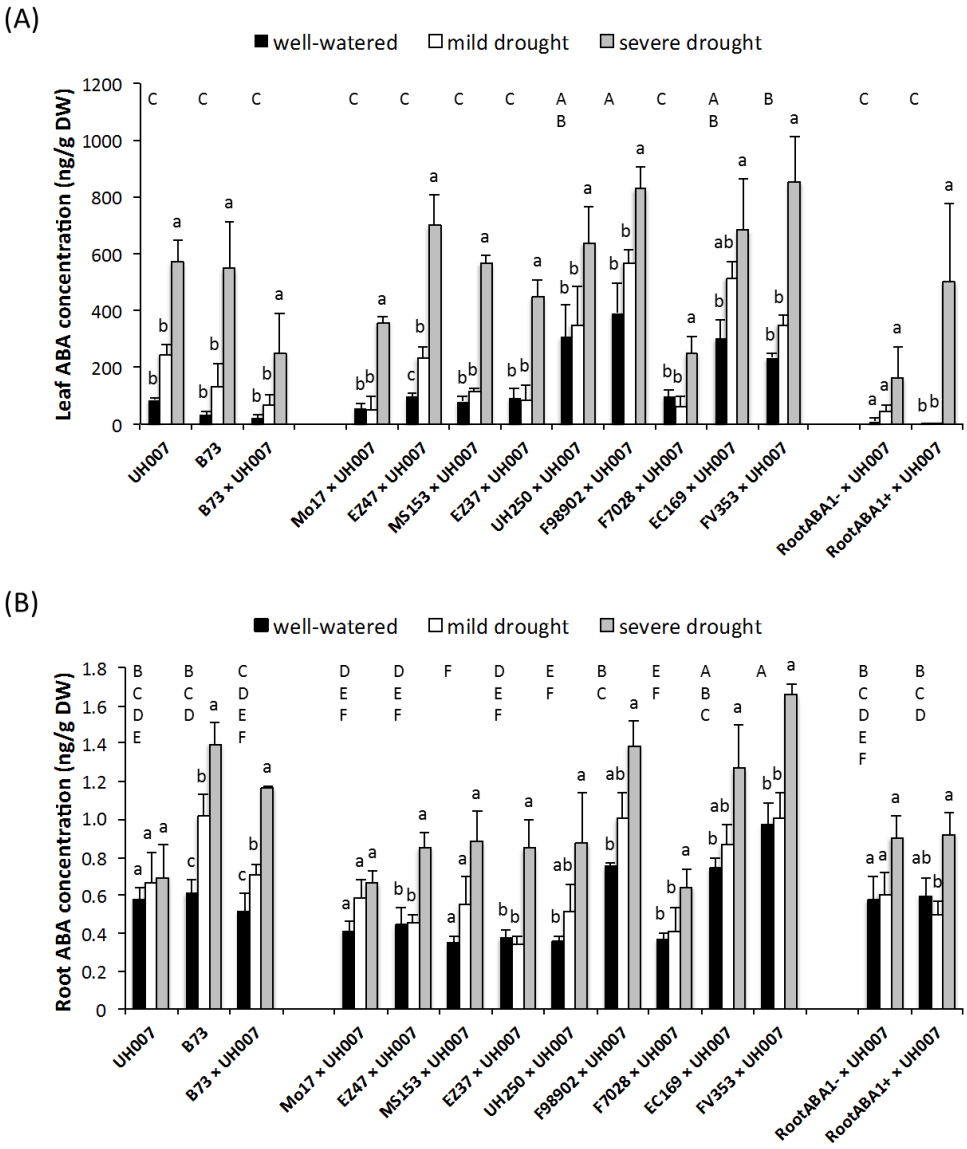


Figure 3.7: Genetic variation in (A) leaf and (B) root ABA concentrations and their changes under different drought treatments. The seedlings were 17-day after transplantation. All 14 maize genotypes were grown consecutively in 28 experiments with three water treatments as described in Figure 3.1. The 3rd leaves from two plants in the same treatment were cut and mixed as one sample for leaf hormone profile analysis. The entire root systems (without the basal part) of them were washed out. The plant used for leaf ethylene incubation was also used for root ethylene incubation, while the root of the other plant was used as a sample for root hormone profile analysis. Data presented here is the combined result from two batches of experiments with the same maize genotype. Columns and bars are means ± standard error. Different lowercase letters indicate significant difference among water treatments in the same genotype at $P < 0.05$. Different uppercase letters indicate significant difference between maize genotypes under well-watered condition at $P < 0.05$.

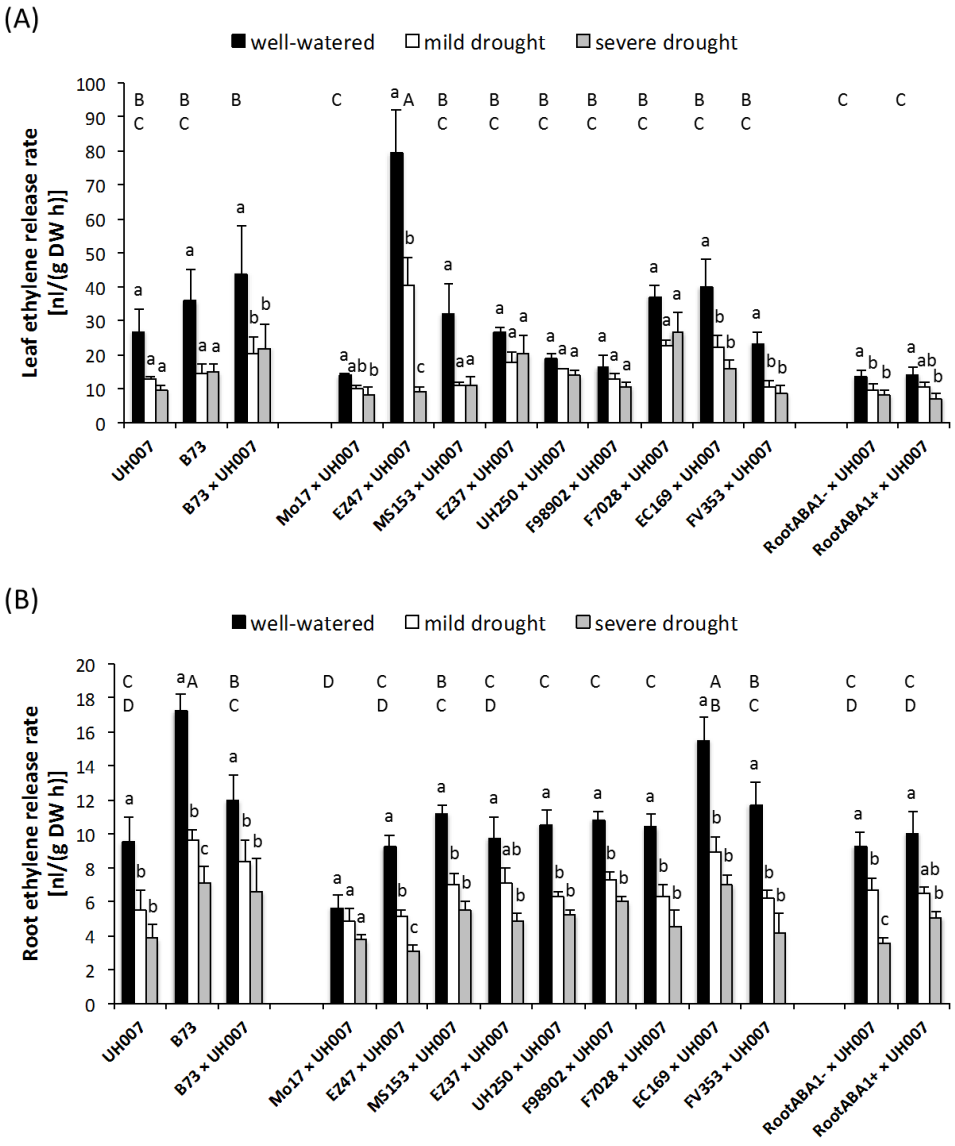


Figure 3.8: Genetic variation in (A) leaf and (B) root ethylene release rates and their changes under different drought treatments. The 5th leaf of one of these two plants that were described in Figure 3.7 was cut for leaf ethylene incubation. The entire root systems (without the basal part) of them were washed out. The plant used for leaf ethylene incubation was also used for root ethylene incubation. Data presented here is the combined result from two batches of experiments with the same maize genotype. Columns and bars are means ± standard error. Different lowercase letters indicate significant difference among water treatments in the same genotype at $P < 0.05$. Different uppercase letters indicate significant difference between maize genotypes under well-watered condition at $P < 0.05$.

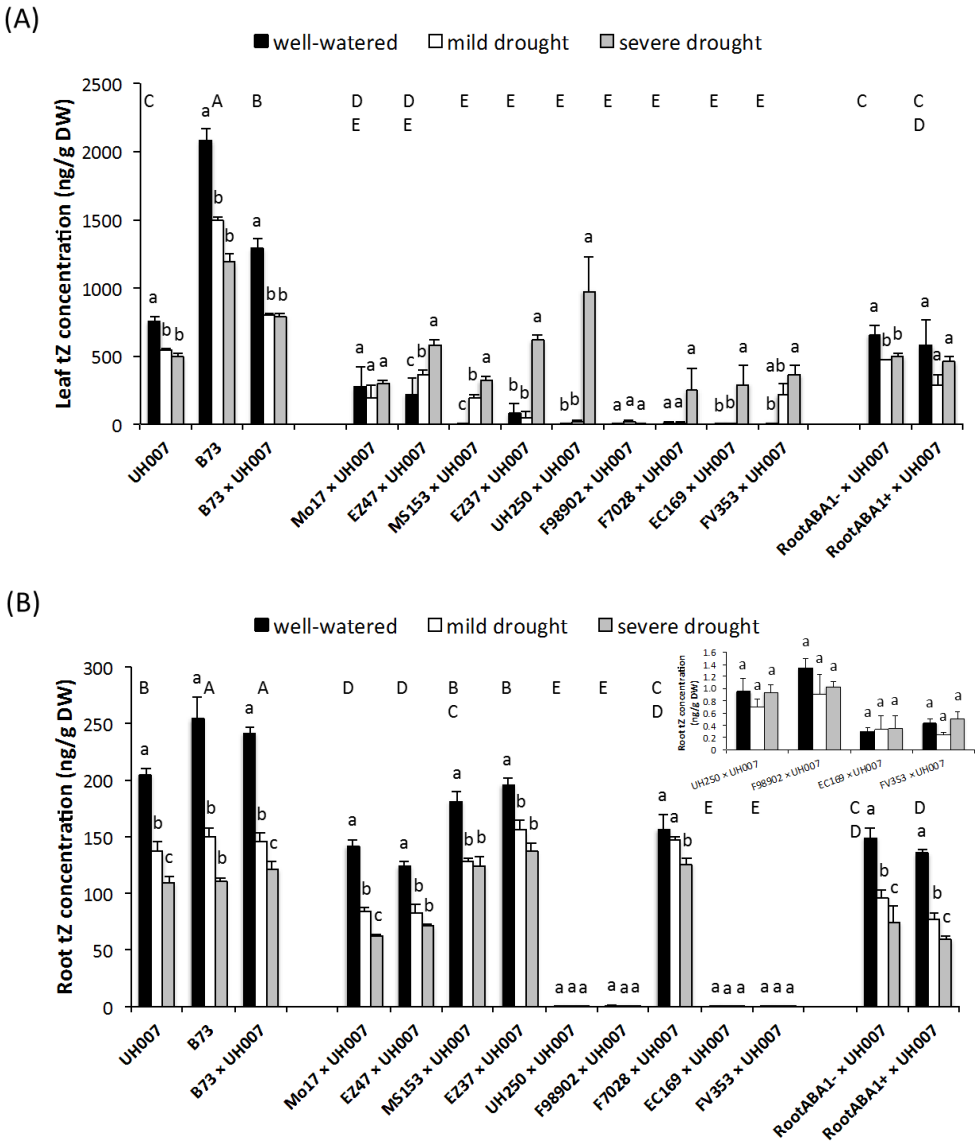


Figure 3.9: Genetic variation in (A) leaf and (B) root *t*Z concentrations and their changes under different drought treatments. The samples are the same as those used for hormone profile analysis in Figure 3.7. Data presented here is the combined result from two batches of experiments with the same maize genotype. Columns and bars are means ± standard error. Different lowercase letters indicate significant difference among water treatments in the same genotype at $P < 0.05$. Different uppercase letters indicate significant difference between maize genotypes under well-watered condition at $P < 0.05$.

Ethylene

The ethylene levels in the 5th leaf and the entire root system were determined to examine the genetic variation in this hormone under well-watered and drought

conditions. In well-watered maize, the ethylene release rate from the leaf ranged from 13–79 nl/(g DW h), which was higher than that from the root, 6–17 nl/(g DW h) (Figure 3.8). Additionally, the highest ethylene release rate from the leaf was in EZ47 × UH007 (79 nl/(g DW h)), while the highest from the root was in B73 × UH007 and EC169 × UH007 (17 and 16 nl/(g DW h), respectively) (Figure 3.8B). The lowest rate from the root was in Mo17 × UH007 (6 nl/(g DW h)) and together with RootABA1- × UH007 and RootABA1+ × UH007 they showed the lowest rates from the leaf (14, 13 and 14 nl/(g DW h) respectively) (Figure 3.8).

Generally, the ethylene production in leaf and root were inhibited under drought and similar to the ABA response, the inhibitory effect became stronger when the drought became more severe (Figure 3.8). The severe drought treatment significantly inhibited ethylene release rate from the leaf in seven genotypes by 40–89% (Figure 3.8A) and by 45–66% from the root in 13 genotypes respectively (Figure 3.8B). Under mild drought, the leaf and root ethylene release rates decreased by 17–66% and 14–47% respectively although they were not all statistically significant (Figure 3.8). Interestingly, Mo17 × UH007 showed insignificant reductions in leaf and root ethylene production under drought, which may be related to the low emission rate even when plants were well-watered (Figure 3.8).

tZ (cytokinin)

tZ is one of the major active cytokinins in plants (Atanassova *et al.*, 1996). In this study, it was found that the *tZ* concentration ranged from 0.9–2078.9 ng/g DW in the leaf (Figure 3.9A), and from 0.3–254 ng/g DW in the root (Figure 3.9B) under well-watered treatment. In addition, most genotypes showed higher *tZ* concentrations in

the leaf than in the root, except for MS153 × UH007, EZ37 × UH007 and F7028 × UH007 (Figure 3.9). It was found that B73 and B73 × UH007 showed the greatest *tZ* concentrations in both the leaf (2078.9 and 1284.6 ng/g DW respectively) and the root (254.3 and 241.4 ng/g DW respectively) (Figure 3.9). Six genotypes showed very low *tZ* concentrations in the leaf (i.e. < 11 ng/g DW) (Figure 3.9A), but only four of them showed very low *tZ* levels in the root with < 3 ng/g DW (Figure 3.9B).

The concentration of *tZ* in the leaf and root responded to drought treatments differently. Four genotypes (UH007, B73, B73 × UH007 and RootABA1- × UH007) showed significant decrease in leaf *tZ* concentration under both mild and severe drought by 24–42% (Figure 3.9A). However, EZ47 × UH007 and MS153 × UH007 showed significant increase in the leaf *tZ* concentration under both drought treatments by 0.7–62.0 fold (Figure 3.9A), and another four genotypes (EZ37 × UH007, UH250 × UH007, EC169 × UH007 and FV353 × UH007) only significantly increased the leaf *tZ* concentration under severe drought by 6.6–362.2 fold (Figure 3.9A). Additionally, no significant change was seen in another four genotypes (Mo17 × UH007, F98902 × UH007, and F7028 × UH007 and RootABA1+ × UH007) under the two drought treatments (Figure 3.9A). On the other hand, the root *tZ* concentrations in nine genotypes significantly decreased under both mild and severe drought, and in F7028 × UH007 it only significantly decreased in the severe drought treatment (Figure 3.9B). Furthermore, there was no significant change in root *tZ* levels among the three water treatments for four genotypes, i.e. UH250 × UH007, F98902 × UH007, EC169 × UH007 and FV353 × UH007 (Figure 3.9B).

3.4 Discussion

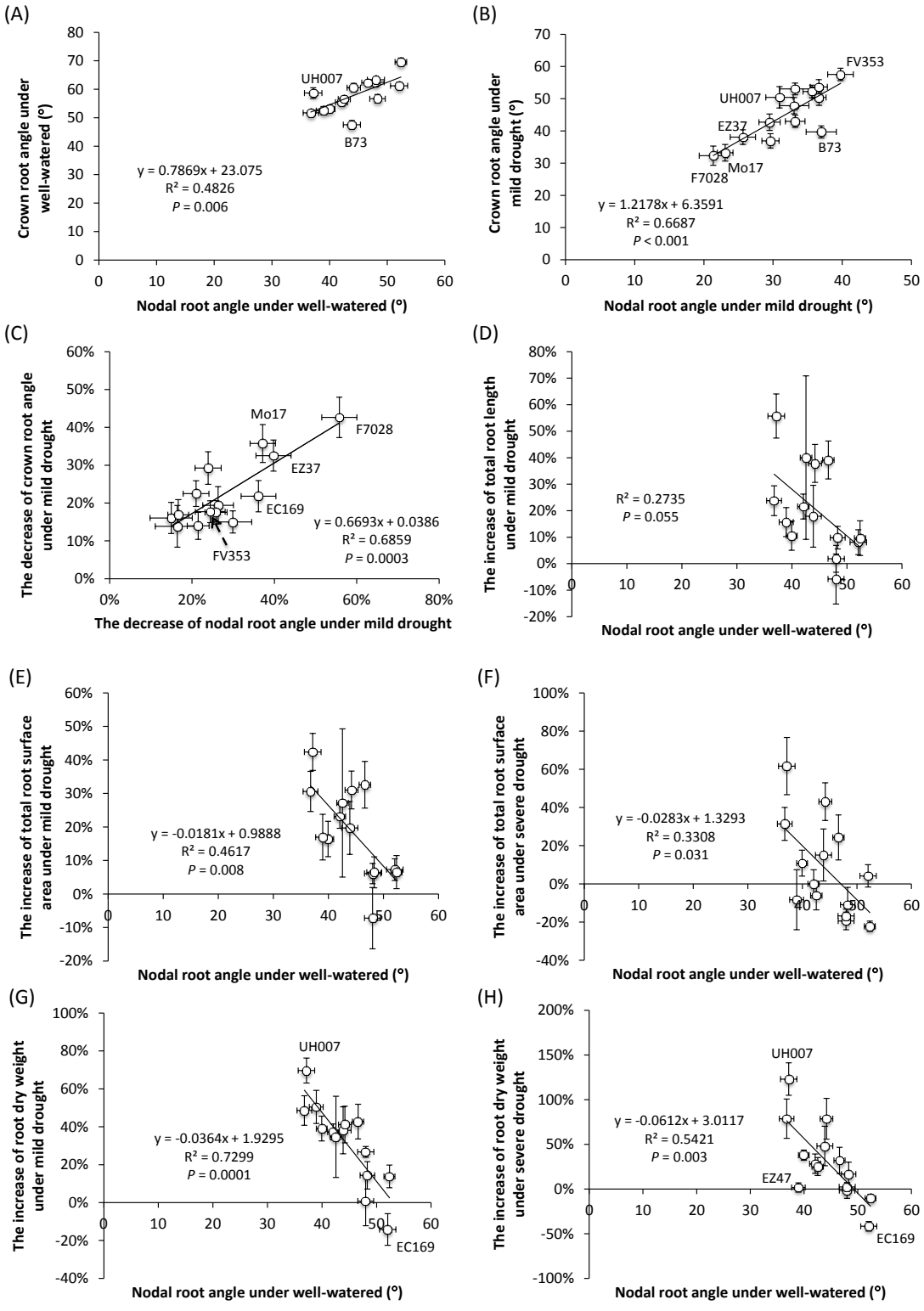
Correlation between root angle and other root traits

In this study, genetic variation was observed in angles of two types of maize shoot-borne roots, i.e. the crown and nodal roots (Hochholdinger *et al.*, 2004; Hochholdinger and Tuberosa, 2009), which were positively correlated across fourteen maize genotypes (except for UH007 and B73). This result is consistent with previous reports that genetic variation was seen in the angles of maize seminal roots and different whorls of nodal roots, which were also correlated (Omori and Mano, 2007; Trachsel *et al*, 2011; Ali *et al.*, 2015). Moreover, the present study showed for the first time the plasticity under drought stress in both crown and nodal root angles, which was also variable among genotypes and was correlated between the two root types. The reduced root angle (steeper roots) under drought stress can be seen as an example of adaptive plasticity because it should improve the accessibility of roots to water.

In this study, it was found that maize root angle under well-watered was related to root system size (as indicated by the total root length, surface area and dry weight) changes under drought. Different changes of root system size were exhibited among the 14 maize genotypes under drought, including stimulation, inhibition and no change (Figure 3.5). The nodal root angle of watered plants was negatively correlated with the increase in the root surface area (mild drought: $R^2 = 0.462$, $P = 0.008$; severe drought: $R^2 = 0.331$, $P = 0.031$, Figure 3.10E, F) and dry weight (mild drought: $R^2 = 0.730$, $P = 0.0001$; severe drought: $R^2 = 0.542$, $P = 0.003$, Figure 3.10G, H) under drought. Similar correlation was also seen between the nodal root angle and the

increase in the total root length under drought though not statistically significant ($P = 0.055$, Figure 3.10D and data not shown).

The total root length, surface area and dry weight are important traits for plant performance under drought because they determine the root system size and thus the plant's capacity for water and nutrient uptake (Kamoshita *et al.*, 2000; Kamoshita *et al.*, 2004; Kano *et al.*, 2011). Some studies report that plants with larger root systems under drought showed better drought resistance (Werner *et al.*, 2010; Kano *et al.*, 2011). On the other hand, some other studies reported that wheat and sorghum plants with steeper root angles display greater drought-resistance, which might be attributed to the fact that they allocate biomass to roots in deeper soil (Oyanagi, 1994; Manschadi *et al.*, 2006; Singh *et al.*, 2012). However, the results in this study showed combined responses to drought in both root size and angle. Genotypes with steeper root angles tended to show larger root sizes (more stimulation or less inhibition) under drought. This result suggested that the maize genotypes with steeper root angles showed less yield penalty than those with shallower root angles under drought in the field (e.g. Ali *et al.*, 2015) may relate to their capacity to maintain a large root under drought.


(A)
Crown root angle under well-watered (°)
Nodal root angle under well-watered (°)
UH007
B73
y = 0.7869x + 23.075
R² = 0.4826
P = 0.006
(B)
Crown root angle under mild drought (°)
Nodal root angle under mild drought (°)
FV353
UH007
EZ37
B73
F7028
Mo17
y = 1.2178x + 6.3591
R² = 0.6687
P < 0.001
(C)
The decrease of crown root angle under mild drought
The decrease of nodal root angle under mild drought
Mo17
F7028
EZ37
EC169
FV353
y = 0.6693x + 0.0386
R² = 0.6859
P = 0.0003
(D)
The increase of total root length under mild drought
Nodal root angle under well-watered (°)
R² = 0.2735
P = 0.055
(E)
The increase of total root surface area under mild drought
Nodal root angle under well-watered (°)
y = -0.0181x + 0.9888
R² = 0.4617
P = 0.008
(F)
The increase of total root surface area under severe drought
Nodal root angle under well-watered (°)
y = -0.0283x + 1.3293
R² = 0.3308
P = 0.031
(G)
The increase of root dry weight under mild drought
Nodal root angle under well-watered (°)
UH007
EC169
y = -0.0364x + 1.9295
R² = 0.7299
P = 0.0001
(H)
The increase of root dry weight under severe drought
Nodal root angle under well-watered (°)
UH007
EZ47
EC169
y = -0.0612x + 3.0117
R² = 0.5421
P = 0.003

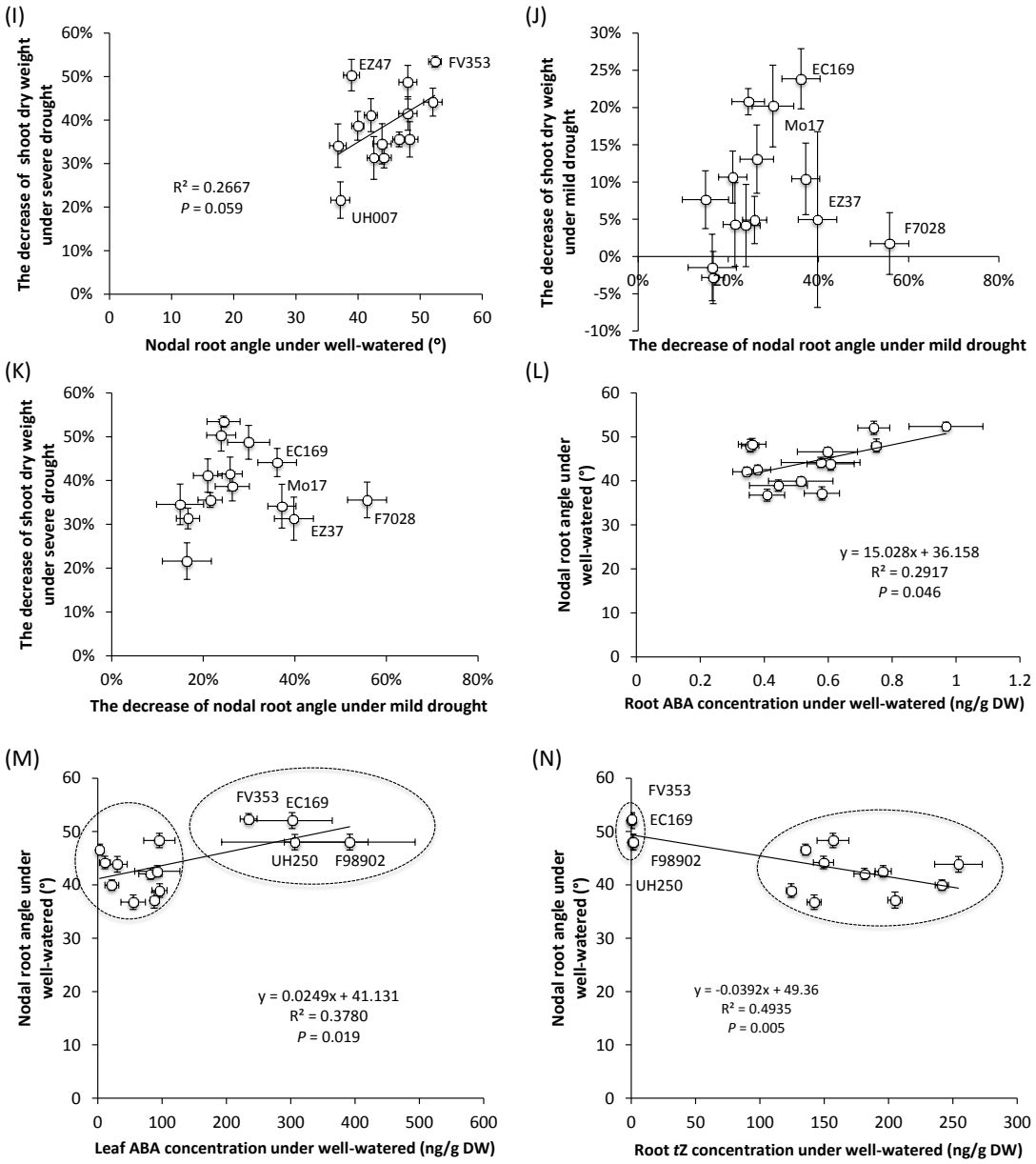


Figure 3.10: The correlations between (A) nodal root angle and crown root angle under well-watered treatment; (B) nodal root angle and crown root angle under mild drought treatment; (C) the decrease of nodal root angle and the decrease of crown root angle under mild drought; (D) nodal root angle under well-watered and the increase of total root length under severe drought; (E) nodal root angle under well-watered and the increase of root surface area under mild drought; (F) nodal root angle under well-watered and the increase of root surface area under severe drought; (G) nodal root angle under well-watered and the increase of root dry weight under mild drought; (H) nodal root angle under well-watered and the increase of root dry weight under severe drought; (I) nodal root angle under well-watered and the decrease of shoot dry weight under severe drought; (J) the decrease of nodal root angle under mild drought and the decrease of shoot dry weight under mild drought; (K) the decrease of nodal root angle under mild drought and the decrease of shoot dry weight under severe drought; (L) the concentration of ABA in root and nodal root angle under well-watered; (M) the

concentration of ABA in leaf and nodal root angle under well-watered; (N) the concentration of *t*Z in root and nodal root angle under well-watered.

Correlation between root traits and drought resistance

The relative change in shoot dry weight under drought was used to indicate the capacity of plant drought resistance in this study. The decrease in shoot dry weight under drought showed significant and negative correlation with the increase in the total root length (mild drought: $R^2 = 0.650$, $P < 0.05$; severe drought: $R^2 = 0.791$, $P < 0.01$), surface area (mild drought: $R^2 = 0.639$, $P < 0.05$; severe drought: $R^2 = 0.795$, $P < 0.01$) and dry weight (mild drought: $R^2 = 0.730$, $P < 0.01$; severe drought: $R^2 = 0.824$, $P < 0.01$) (Table 3.2). Therefore, larger sizes of root system in these genotypes under drought were correlated with higher drought resistance, which agrees with the results of Kano *et al.* (2011), showing that the root system size is crucial for drought resistance.

In contrast, no significant correlation between shoot biomass reduction under drought and the nodal root angle when well-watered was seen ($P = 0.059$, Figure 7I and data not shown), or the angle plasticity under drought (Figure 3.10J, K). Similar results were seen with crown root angle (data not shown). However, there was a trend in the data, suggesting that the genotypes with steeper angles exhibited smaller decreases in shoot biomass under severe drought, with one exception that EZ47 × UH007 showed steep root angle but large decrease in shoot biomass (Figure 3.10I). The general result here is in accordance with previous studies in rice, wheat and maize in which the steeper root angles were associated with better drought

resistance (Manschadi *et al.*, 2008; Uga *et al.*, 2013; Ali *et al.*, 2015). Furthermore, a more interesting phenomenon was that the plasticity of nodal root angle to drought was positively correlated with the shoot biomass reduction when the plasticity was low (i.e. <36 %, Figure 3.10J, K). The correlation became negative if the plasticity was high (i.e. >36 %, Figure 3.10J, K), which means that the higher the plasticity, the less the shoot biomass reduction under drought. This may be related to the fact that those genotypes with steep root angles tend to show lower plasticity (i.e. less potential in root angle reduction) and maintain their steep angles under drought. Kato *et al.* (2006) reported that rice genotypes with steep nodal angles under well-watered condition also showed steep nodal angles under drought stress. On the other hand, genotypes with shallower root angles have higher potentials to show higher plasticity under drought, which is prerequisite for them to reach a steep root angle, similar as that shown by steep genotypes in well-watered soil. Thus, our results indicated when the plasticity was high enough to change a shallow root angle into a much steeper one, it would benefit the drought resistance of the genotype (Figure 3.10J, K). Therefore, compared with the other root traits (i.e. the total length, surface area and dry weight), root angle and its plasticity to drought appear less effective in predicting maize drought resistance.

Table 3.2: The correlations between the relative increase of root length, surface area and root dry weight and the relative decrease of shoot dry weight under drought treatments. * indicates significant difference at 0.05 level. ** indicates significant difference at 0.01 level.

	Total root length increase	Root surface area increase	Root dry weight increase
A			
Mild drought			
Shoot dry weight decrease	-0.650*	-0.639*	-0.730**
B			
Severe drought			
Shoot dry weight decrease	-0.791**	-0.795**	-0.824**

Nevertheless, it is also implicated that the root angle and its plasticity might be important when changes in maize root size under drought are similar. For example, UH250 × UH007 and FV353 × UH007 showed similar shallow root angles when well-watered and similar root size change under drought as F7028 × UH007 (Table 3.1, Figure 3.5 and 3.6), which showed the highest root angle plasticity among the 14 genotypes (Table 3.1). However, F7028 × UH007 showed lower shoot biomass reduction than the other two under drought stress. To some extent, these results were compatible with a previous study that a genetically modified rice genotype (with steep root angles) showed a more vertical root distribution in deep soil and higher yield under drought than the parental line (with shallow root angles), although there was no difference in total root biomass (Uga *et al.*, 2013).

Therefore, these results suggested that a 'better' root system to support plant drought resistance might include combined traits, namely, a steep root angle, and/or high angle plasticity to drought if the angle is shallow in itself, and the ability to grow a root system with large size (e.g. increased length, surface area, and dry weight) under drought conditions. Similarly, deep rooting is a complex trait and combines both root growth and angle (Abe and Morita, 1994; Araki *et al.*, 2002; Uga *et al.*,

2011). Thus, a genotype like UH007 shows the best root traits for drought resistance among all 14 genotypes, including the steepest nodal root angle under well-watered condition (Table 3.1) and the largest promotion in root size under drought (Figure 3.5 and 3.10G, H). Obviously, UH007 presented the smallest shoot biomass reduction (Figure 3.6 and 3.10I). The EZ47 × UH007 and EC169 × UH007 with steep root angles and relatively high angle plasticity respectively showed relatively high shoot biomass reduction under drought (Table 3.1, Figure 3.6). This may be because their root growth were either less stimulated or even inhibited under drought compared with other genotypes with similar steep root angle or angle plasticity (Figure 3.5 and 3.10G–K). FV353 × UH007 showed the worst root traits for drought resistance, which exhibited the shallowest root angle (Table 3.1 and Figure 3.10B), relatively low angle plasticity (Table 3.1 and Figure 3.10C) and no significant promotion of root growth under mild drought and even significant inhibition of root growth under severe drought (Figure 3.5). Because of these combined traits, it is not surprising to see the highest shoot biomass reduction under drought in the FV353 × UH007 (Figure 3.6, 3.10I).

Correlation between root angle and hormone level

Hormones are important regulators for plant responses to drought stress (Santner *et al.*, 2009). In the present study, we observed genetic variation in the endogenous ABA, *t*Z and ethylene levels in the leaf and root tissues and their changes in response to drought among 14 maize genotypes (Figure 3.7). Arabidopsis triple mutant *snrk2.2/2.3/2.6* is insensitive to ABA and showed reduced root growth compared to the wild-type (Fujii and Zhu, 2009). Both ethylene and cytokinins were reported to be

negative regulators of root growth (Riefler *et al.*, 2006; Alarcón *et al.*, 2009). The increase of endogenous ethylene was coupled with root elongation decreases in maize (Alarcón *et al.*, 2009). Cytokinin receptor double mutant *ahk2 ahk3* displayed an enhanced root system through faster growth of primary root and increased root branching (Riefler *et al.*, 2006). However, the correlation between hormone levels and root angles in maize has not been studied before. It was found that the nodal or crown root angle showed significant but weak positive correlation with the ABA concentration in the root (R^2 = 0.292, P = 0.046, Figure 3.10L and data not shown) and the leaf under well-watered condition (R^2 = 0.378, P = 0.019, Figure 3.10M and data not shown). Additionally, four genotypes (i.e. UH250 × UH007, F98902 × UH007, EC169 × UH007 and FV353 × UH007) with relatively higher leaf ABA concentrations and lower root *tZ* concentrations tended to show shallower nodal (Figure 3.10M, N) and crown (data not shown) root angles. It also showed that the nodal root angle was negatively correlated with root *tZ* concentration (R^2 = 0.4935, P = 0.005, Figure 3.10N). By contrast, root angles were not correlated with the leaf or root ethylene level (data not shown). Therefore, the data suggested that the levels of endogenous ABA and *tZ*, but not the ethylene in well-watered maize might be involved in determining root angles.

Under drought condition, root angles or their plasticity did not show any correlation with ABA, *tZ* and ethylene levels in this study, although the root angles decreased in all genotypes under drought. ABA levels in maize leaf and root tissues were elevated under drought, which was also reported previously (Zhang and Davies, 1989; Voisin *et al.*, 2006). The ABA concentration in the leaf of 13 genotypes and in the root of ten genotypes increased under severe drought (Figure 3.7). Although the

mild drought showed significant effect on the root angle (Figure 3.4), it had little effect on the ABA concentration in most genotypes (Figure 3.7). However, the ABA concentration measured in the entire root system may have obscured the local changes in root ABA levels under mild drought. The EZ47× UH007 was an example in which the ABA level increased in the leaf but not in the root under mild drought (Figure 3.7), while such changes can normally be observed first in the root (see Chapter 2). For the ethylene, it was found that the release rates decreased under drought in all 14 genotypes, especially in the root. This is similar to the previous finding in Norway spruce under drought (Eklund *et al.*, 1992). Ethylene was believed to be a negative regulator of plant growth under drought and plants with suppressed ethylene action showed enhanced drought resistance (reviewed by Pierik *et al.*, 2007). As for the *t*Z concentration, it was found to be decreased in the root under drought, except for four genotypes with a relatively low *t*Z level when well-watered (Figure 3.9B). Such reduction in the root *t*Z level is consistent with the study of Nishiyama *et al.* (2011) that drought and salt stresses significantly reduced the levels of *t*Z-type cytokinins in Arabidopsis, but not the *i*P- and *c*Z-type cytokinins. Another study also found reduced *t*Z concentrations in the xylem sap of drought-stressed maize (Alvarez *et al.*, 2008). However, no clear trend was seen for the *t*Z concentration in the leaf (Figure 3.9A). The inconsistent responses of *t*Z level in the leaf and root may be related to the different roles of cytokinins in regulating shoot and leaf growth, since cytokinins were negative regulators in root growth, but required for shoot growth (Werner *et al.*, 2008; Werner *et al.*, 2010). Thus, the increase in ABA levels and the decrease in ethylene and *t*Z levels in root under drought may be adaptive

strategies of plants. Furthermore, the changes in hormone levels were related to the changes in root angles under drought to some extent.

3.5 Conclusion

By studying 14 maize genotypes under well-watered, mild drought and severe drought for 17 days, it was found that 1) there was significant genetic variation in crown and nodal root angles; 2) there was also significant genetic variation in the plasticity of root angle (reduced angle) to drought; 3) the crown and nodal root angles were positively correlated in 14 maize genotypes; 4) the plasticity of the crown and nodal root angles were also positively correlated in these maize genotypes; 5) maize genotypes with steep nodal root angles tended to show more stimulation or less inhibition in the size of the root system under drought (indicated by root length, surface area and dry weight); 6) combined root traits, including the size-related root traits, the root angle and its plasticity may be important predictors for plant ability of drought resistance; 7) under well-watered condition, root angle was positively and negatively correlated with ABA and *t*Z levels respectively, especially with the root ABA level.

Chapter 4 Roles of Ethylene and Auxin in the Biphasic Root Growth Responses to Abscisic Acid

4.1 Introduction

Drought stress is globally the most important environmental factor limiting plant productivity. It can reduce carbon fixation, inhibit cell and organ growth and impact assimilated carbon partitioning to different plant structures (Boyer, 1982; Pugnaire *et al.*, 1999). A root system that is able to efficiently take up water and nutrients from the soil is crucial for drought resistance (Hammer *et al.*, 2009; Hodge *et al.*, 2009; Hodge, 2010). Previous studies have reported that mild soil drying stimulated root growth, but when it became severe, it inhibited root growth (Sharp and Davies, 1979; Watts *et al.*, 1981). However, there is no consensus on the mechanisms underlying these root responses.

Plant hormones, particularly abscisic acid (ABA), have been extensively studied in drought-stressed plants (Hsiao, 1973; Schachtman and Goodger, 2008; Cutler *et al.*, 2010). The endogenous concentration of ABA of a plant can be an indicator of soil water availability (Zhang and Davies, 1989). Generally, ABA is known as an inhibitor of shoot and root growth of plants without water stress (Sharp *et al.*, 1994; Sharp and LeNoble, 2002) and previous studies have shown that ABA also acts as a growth inhibitor of plants under water deficit (Bensen *et al.*, 1988; Creelman *et al.*, 1990). On the other hand, maize plants that had been genetically altered or chemically treated to reduce endogenous ABA content showed enhanced inhibition of primary root

elongation at a low water potential, which indicates that ABA plays a role in maintaining root elongation under low water potentials (Saab *et al.*, 1990). It was further shown that accumulated ABA restricts ethylene production in plants at a low water potential and prevented root inhibition caused by excess ethylene (Spollen *et al.*, 2000). However, other studies have indicated more complex biphasic effects of exogenous ABA where relatively low concentrations of ABA stimulated root growth while high concentrations inhibited root growth (Watts *et al.*, 1981; Xu *et al.*, 2013). This is analogous to the biphasic effects of soil drying: mild water deficit stimulates root growth while more severe deficit inhibits root growth (Sharp and Davies, 1979; Watts *et al.*, 1981; Creelman *et al.*, 1990).

Ethylene and its precursor 1-aminocyclopropane-l-carboxylic acid (ACC) have been reported to inhibit root cell elongation, thus inhibiting root growth (Le *et al.*, 2001; Růžička *et al.*, 2007). Under water-stressed conditions, the greater ethylene emission caused shoot growth reduction in *Vicia faba* L. (El-Beltagy and Hall, 1974). Ethylene is also reported to antagonise ABA induced stomatal closure (Wilkinson and Davies, 2010; Chen *et al.*, 2013b). The involvement of ethylene in ABA-regulated root growth was investigated in more detail by Beaudoin *et al.* (2000) and Ghassemian *et al.* (2000) who found that root growth in a number of ethylene signalling mutants was less sensitive to high ABA concentrations but that the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) did not reduce sensitivity to ABA and even enhanced sensitivity to ABA (Ghassemian *et al.*, 2000). These results indicated a positive role of ethylene signalling, but not *de novo* ethylene biosynthesis, in the inhibitory effect of high ABA concentrations on root growth. In partial contradiction, a recent study found that ethylene biosynthesis is

necessary for the inhibitory effect of high ABA concentration (Luo *et al.*, 2014). Any role for ethylene in the stimulatory effect of low ABA concentrations has not been explored.

Auxin is another important regulator of root development (Mockaitis and Estelle, 2008). Crosstalk between auxin and ABA signalling in root has been reported (Rock and Sun, 2005). Mutants that are resistant to both auxin and ABA also provided genetic evidence for the auxin and ABA interaction (Pickett *et al.*, 1990; Wilson *et al.*, 1990; Tian and Reed, 1999). A recent study reported a role for ABA in modulating auxin transport in the root apex to maintain root growth under moderate water stress (Xu *et al.*, 2013). However, the role of auxin in root responses to ABA is still not well understood, especially under conditions where root growth is inhibited, either by high exogenous ABA concentrations or by severe water deficit.

In this study, the roles of ethylene and auxin in the root responses to both low and high concentrations of ABA were investigated by using five chemical inhibitors and twelve mutant lines that are relevant to ethylene and auxin.

4.2 Materials and methods

Plant materials

The wild-type accession of *Arabidopsis thaliana* L. used in this study was Col-8 (catalogue no. N60000). Besides, the auxin influx *AUX1* mutants *aux1-T* (N657534), *aux1-7* (N9583); the auxin efflux mutants *pin2/eir1-1* (N8058), *pin3-4* (N9363), *pin3-5* (N9364), *pin4-3* (N9368) and *pin7-2* (N9366); and auxin signalling mutants *iaa7/axr2-1* (N3077) and *tir1-1* (N3798) were obtained from the European

Arabidopsis Stock Centre. The ethylene-insensitive mutants *etr1-1* (ethylene response 1), *ein2-1* (ethylene insensitive 2), and *ein3-1* were kindly provided by Dr. Mike Roberts (Lancaster University, UK). The auxin reporter line *DR5::GFP* was a kind gift from Prof. Thomas Guilfoyle (University of Missouri, USA).

Surface-sterilised seeds were sown on solid medium containing 0.02 x B5 medium, 1 mM KNO_3, 0.5% (w/v) sucrose and 1% agar in 90 mm diameter Petri dishes (Zhang and Forde, 1998). After stratifying the seed in the dark (4˚C) for 2–3 d, the Petri dishes were incubated in a vertical orientation in a growth room at 22˚C with a 16 h light-period and an irradiance of 100 µmol m^{-2} s^{-1}. Four to five days later seedlings with similar root length were transferred to fresh plates containing ABA at different concentrations. Five inhibitors were added to the growth medium as required: ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG, 0.3 or 0.5 µM; A6685, Sigma-Aldrich); ethylene perception inhibitor silver thiosulfate (STS, 10 µM); and auxin efflux inhibitors N-1-naphthylphthalamidic acid (NPA, 10 µM; PS343, Sigma-Aldrich), 2,3,5-triiodobenzoic acid (TIBA, 10 µM; T5910, Sigma-Aldrich); and auxin influx inhibitor 3-chloro-4-hydroxyphenylacetic acid (CHPAA, 10 µM; 224529, Sigma-Aldrich). For the treatment plates, 3–6 seedlings were placed on 9 cm diameter plates (25 ml medium), or 7–9 seedlings on 12 cm square plates (50 ml medium). The top one-fifth of the agar medium was excised so that the shoot was not in direct contact with the medium. ABA (A1296, Sigma-Aldrich) stock solutions were made in 10 mM with 0.03 M KOH. A 60 mM STS solution was freshly prepared by mixing 300 mM silver nitrate with 300 mM sodium thiosulphate in a 1:4 (v/v) ratio. At least three independent experiments were performed and similar results obtained and reported.

Root measurement

Primary root growth was monitored during the 3–6 d after seedlings were transferred to the treatment plates by marking the position of the root tips on the base of the plate at 24 or 48 h intervals. At the end of each experiment, the plates were imaged on a flat-bed scanner and the images were analysed using Optimas Image Analysis software (Version 6.1 Media Cybernetics Inc., USA) for root length.

Confocal microscopy

After three days, ABA-treated *DR5::GFP* seedlings were stained briefly (ca. 50 second) with 10 µM propidium iodide. GFP and propidium iodide fluorescence was then detected using a Leica SP2-AOBS confocal laser scanning microscope and the images were electronically superimposed using LCS Lite software (Leica, Germany). Quantification of the GFP fluorescence signal was performed using ImageJ (National Institutes of Health, USA).

Statistical analysis

The statistical software SPSS 21.0 (IBM, USA) was used to perform one-way or two-way ANOVA with Tukey's *post hoc* test at the $P < 0.05$ level.

4.3 Results

Effect of exogenous ABA on root growth

A detailed comparison of the effects of a range of ABA concentrations on root elongation was performed by transferring 4 d-old Arabidopsis seedlings to vertical agar plates containing 0, 0.1, 1 and 10 µM ABA and measuring the increase in root

length at daily intervals over the following 6 d (Figure 4.1). The results showed that 10 µM ABA inhibited root growth by about 40% while 0.1 µM ABA stimulated growth by almost 20% when measured over the 6 d period (Figure 4.1A). The stimulatory effect of 0.1 µM ABA persisted over the duration of the treatment and by the sixth day the roots were growing at a rate over 30% faster than the control (Figure 4.1B). It appears that the intermediate concentration used (1 µM) is close to the threshold for the transition from stimulation to inhibition as it had little effect on root elongation (Fig 4.1A, B). In subsequent experiments, concentrations less than 1 µM ABA (usually 0.1 µM ABA) were therefore used for studying the stimulatory effect of low ABA concentrations and concentrations greater than 1 µM ABA (usually 10 µM ABA) were used for studying the inhibitory effect of high ABA concentrations.

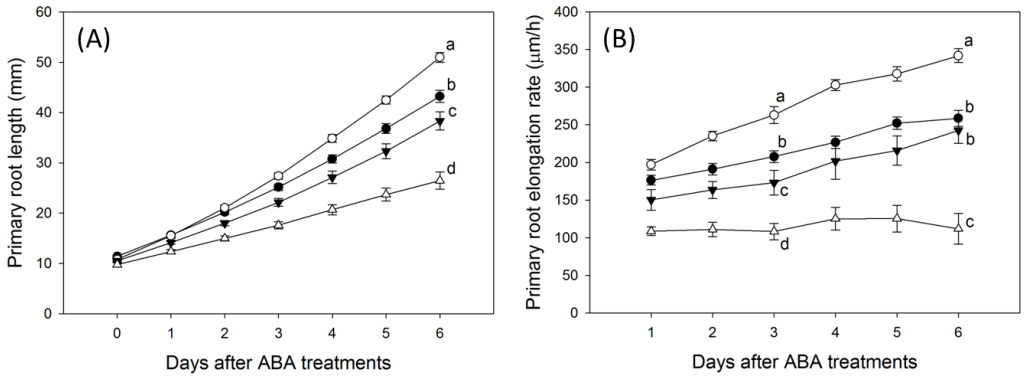


Figure 4.1: Applied ABA showed biphasic effect on primary root elongation rate from the beginning to the end of a six-day treatment. (A) Total primary root length. (B) Primary root elongation rate. Four-day old Arabidopsis wild-type Col-8 seedlings with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5% sucrose) with various ABA concentrations (●, control; ○, 0.1 µM ABA; ▼, 1 µM ABA; △, 10 µM ABA). Primary root at the start point and the increase of primary root were measured every day. The root elongation rate was calculated for each day. The values are means, and the vertical bars represent standard errors. Data analysed using one-way ANOVA and different letters indicate significant differences between ABA treatments in the same day at $P < 0.05$. Seedling numbers: control, n = 14; 0.1 µM ABA, n = 9–14; 1 µM ABA, n = 10–14; 10 µM ABA, n = 11–14.

Investigating the role of ethylene in the root responses to high and low concentrations of ABA

It has previously been established that the inhibitory effect of high ABA concentrations on root growth is an ethylene-dependent process (Ghassemian *et al.*, 2000). To confirm these findings under our experimental conditions and to investigate whether the stimulatory effect of low [ABA] is also ethylene-dependent seedlings were treated with different concentrations of ABA in the presence or absence of either AVG (an ethylene biosynthesis inhibitor) or STS (an ethylene perception inhibitor). The primary root elongation rates were determined over a 4 d period of treatment. When 0.3 or 0.5 µM AVG was included along with the 10 µM ABA treatment, the inhibitory effect was relieved as measured after either 1 d (Figure 4.2A) or 4 d (Figure 4.2B). This result is consistent with recent evidence that stimulation of ethylene biosynthesis by ABA is important for its ability to inhibit root growth (Luo *et al.* 2014). The discrepancy between the present results and an earlier finding (Ghassemian *et al.*, 2000) that AVG did not overcome the inhibitory effect of high [ABA], and even increased the degree of inhibition, could be attributable to the earlier authors' use of higher concentrations of AVG than those used here. These higher concentrations may themselves have been inhibitory to root growth through AVG's reported effects on auxin biosynthesis (Soeno *et al.*, 2010).

In contrast to AVG's ability to interfere with the inhibitory effect of ABA, the presence of either 0.3 or 0.5 µM AVG had no significant influence on the stimulatory effect of 0.1 µM ABA (Figure 4.2A, B). Thus while ethylene biosynthesis is required

for the inhibitory effect of high ABA concentrations it is not required for the stimulatory effect of low ABA concentrations.

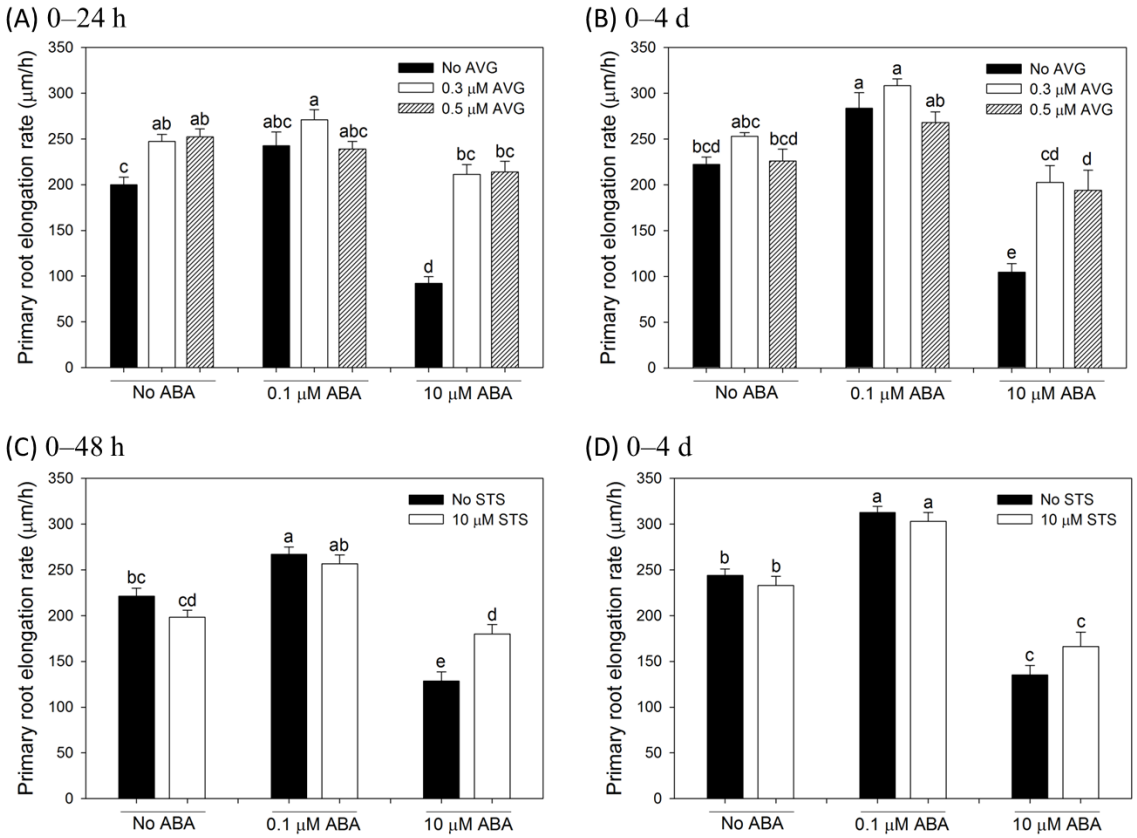


Figure 4.2: Ethylene biosynthesis and signalling inhibitors altered root responses to ABA treatments. AVG: ethylene biosynthesis inhibitor. STS: ethylene signalling inhibitor. (A) The effects of AVG in the first day. (B) The effects of AVG during four days. (C) The effects of STS during the first two days. (D) The effects of STS during four days. Four-day old Arabidopsis wild-type Col-8 seedlings with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5% sucrose) with various ABA and AVG/STS concentrations (μM). Primary root length at the start point and the increase of primary root were measured every day. The root elongation rate was calculated for the first 1 or 2 days and 4 days in average. The values are means, and the vertical bars represent standard errors of the means. Data analysed using one-way ANOVA and different letters indicate significant differences between treatments at $P < 0.05$. Seedling numbers: (A) n = 14; (B) n = 9–14; (C) n = 9–12; (D) n = 7–12.

When 10 μM STS was used to interfere with ethylene perception it almost completely overcame the inhibitory effect of 10 μM ABA when measured after the

first 2 d of treatment (Figure 4.2C). This antagonistic effect was lost when root growth was measured over a 4 d period (Figure 4.2D), which we attribute to the known instability of STS (Ag^+) when exposed to light. However, when included along with 0.1 μM ABA, the STS did not significantly interfere with the stimulatory effect on root growth as measured after either 2 or 4 d (Figure 4.2C, D). Therefore, the inhibitory effect of high [ABA], but not the stimulatory effect of low [ABA], could be eliminated by interfering with ethylene perception.

To look further into the role of ethylene signalling in the two components of the root response to ABA, seedlings of three ethylene-insensitive mutants (*etr1-1*, *ein2-1* and *ein3-1*) were treated with a range of concentrations of ABA. The results of two-way ANOVA showed that the primary root elongation rate was significantly affected by genotype, ABA treatment and their interaction in the first 24 h and the 4 d after treatment (Appendix 3 Table 1). Thus, four genotypes responded to those seven ABA treatments differently. The pairwise comparisons result of relative primary root elongation rate in four genotypes under each ABA treatment is presented in Appendix 3 Table 2 (in each genotype, the mean root elongation rate of plants without ABA treatment was set as 1).

All three mutants to varying degrees showed a diminished response to the inhibitory effect of high [ABA] compared to the wild-type. This was particularly evident in *etr1-1* and *ein2-1* during the first day of treatment when even the highest concentration of ABA (30 μM) had no significant effect on the root elongation rate, and inhibited root elongation by only 14% in *etr1-1* and even stimulated root elongation by 6% in *ein2-1* compared to 48% inhibition in the wild-type (Figure 4.3A,

C and E). A much less pronounced effect was seen in *ein3-1*, where 30 µM ABA inhibited root elongation by 35% over the first day of treatment (Figure 4.3G). Over the 4 d period of treatment the inhibitory effect of the high ABA concentrations was stronger in all lines, but the same pattern of decreased sensitivity in the mutants was observed (Figure 4.3B, D, F and H). The low ABA concentrations (0.1 and 0.2 µM) stimulated root elongation of the wild-type by ~20% in the first day after treatment and by ~30% over the full 4 d of treatment (Figure 4.3A, B). Similarly, the low ABA concentrations also stimulated root elongation of the three ethylene-insensitive mutants as seen after either 1 d or 4 d (Figure 4.3C–H), confirming the results obtained from chemical disruption of ethylene signalling that ethylene signalling pathway has no significant role in the stimulatory effect of low concentrations. These results confirm the evidence from the STS treatment (Figure 4.2) that ethylene signalling is important for the inhibitory effect of high [ABA], but not for the stimulatory effect of low [ABA].

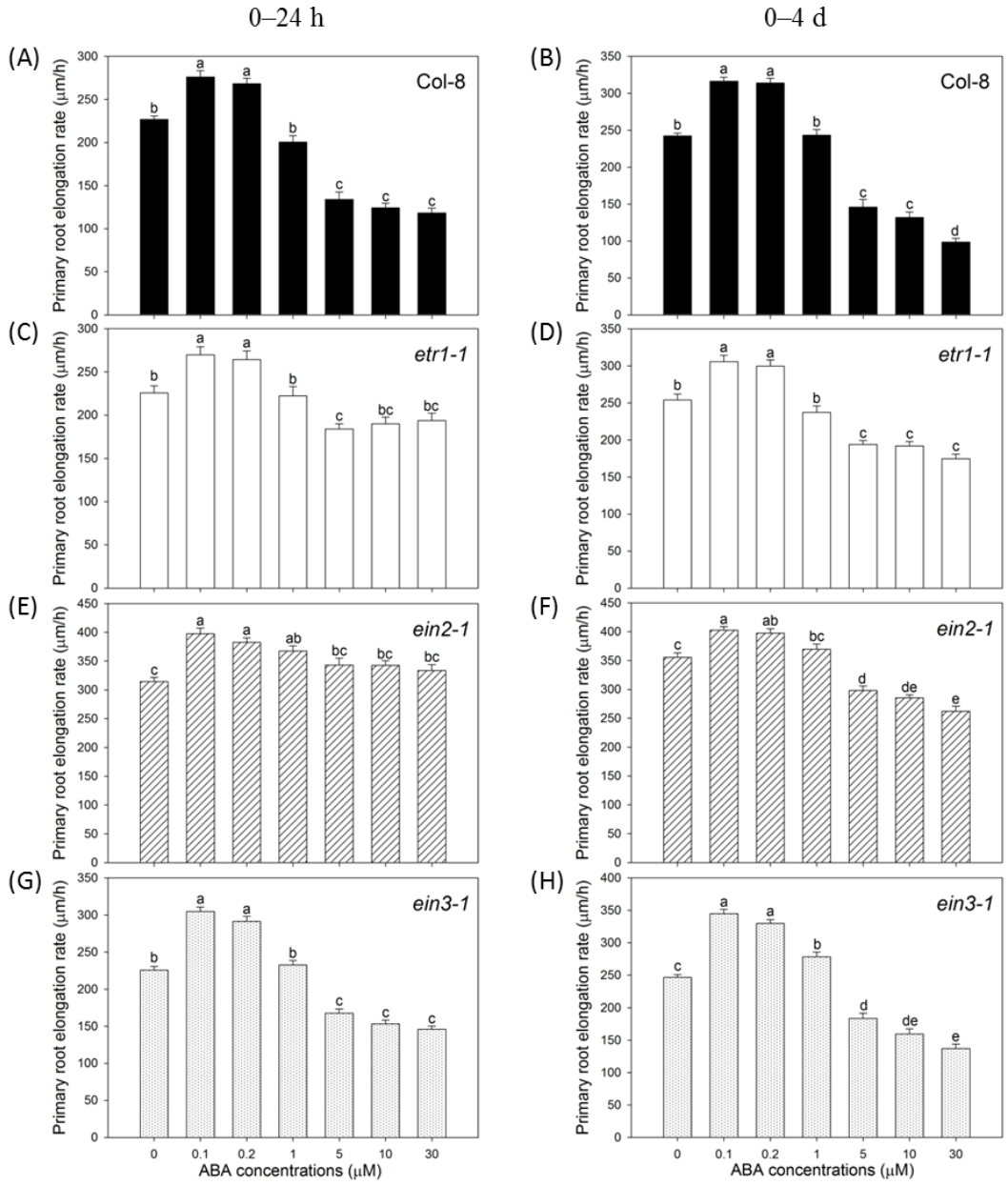


Figure 4.3: Ethylene insensitive mutants showed different sensitivities to ABA treatments in primary root elongation compared to those of wild type plants. Primary root elongation rates during the first day after treatment: (A) Col-8 wild-type; (C) *etr1-1*; (E) *ein2-1*; (G) *ein3-1*, and during the 4 days after treatment: (B) Col-8 wild-type; (D) *etr1-1*; (F) *ein2-1*; (H) *ein3-1*. Four-day old Arabidopsis seedlings of each line with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5%sucrose) with various ABA concentrations (μM). Primary root length at the start point and the increase of primary root were measured every day. The root elongation rate was calculated for the first day and four days in average. Only one line was used in each experiment (n = 14), and results for each genotype comes from combining two set of independent experiments. All 8 experiments were done consecutively from 17-07-2013 (day/month/year) to 26-08-2013. The values are means, and the vertical bars represent standard errors of the means. Data analysed using one-way ANOVA and different letters indicate significant differences between ABA treatments at $P <$

0.05. Seedling numbers: (A) n = 28; (B) n = 21–28; (C) n=28; (D) n=22–28; (E) n=28; (F) n=21–28; (G) n=28; (H) n = 27–28.

Investigating the role of auxin transport and signalling in the root responses to ABA

To investigate the role of auxin transport in the root responses to ABA, two auxin efflux inhibitors (NPA and TIBA) and an auxin influx inhibitor (CHPAA) were firstly employed in this study. In this experiment, the stimulatory effect of the low ABA concentration (0.1 µM) was only seen after 4 d treatment and not after the first day (Figure 4.4). However, when seedlings were grown for 4 d in the presence of either of the auxin efflux inhibitors, the stimulatory effect of 0.1 µM ABA was no longer observed (Figure 4.4B). However, in the presence of CHPAA this concentration of ABA still had a significant positive effect (28% stimulation over CHPAA alone, compared to 34% in the control). Thus, it can be concluded that auxin efflux is necessary for the stimulatory effect of low ABA concentrations but that there is no evidence of a role for auxin influx.

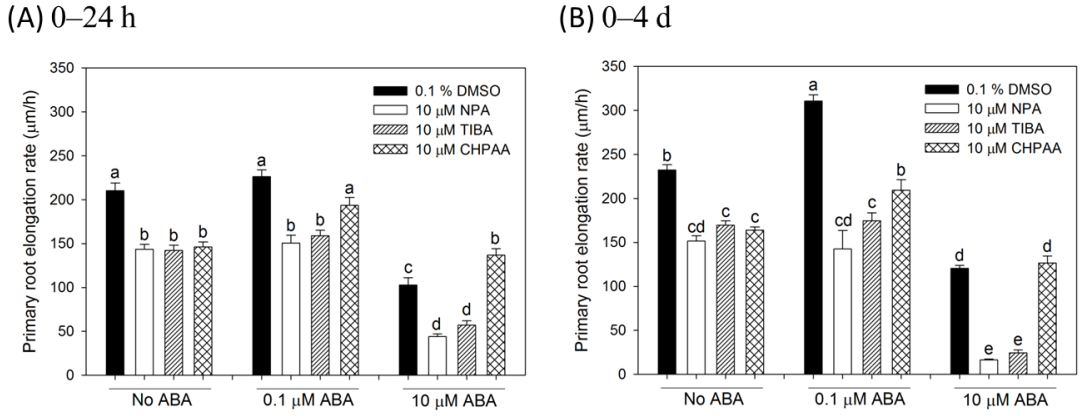


Figure 4.4: Auxin influx and efflux inhibitors altered root responses to ABA. NPA: *N*-1-naphthylphthalamidic acid, auxin efflux inhibitor. TIBA: 2,3,5-triiodobenzoic acid, auxin

efflux inhibitor. CHPAA: 3-chloro-4-hydroxyphenylacetic acid, auxin influx inhibitor. Primary root elongation rates during (A) the first day after treatment and (B) the 4 days after treatment. Four-day old Arabidopsis wild-type Col-8 seedlings with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5% sucrose) with various ABA concentrations and 0.1% DMSO or 10 µM NPA/TIBA/CHPAA. Primary root length at the start point and the increase of primary root were measured every day. The root elongation rate was calculated for the first day and four days in average. The values are means, and the vertical bars represent standard errors of the means. Data analysed using one-way ANOVA and different letters indicate significant differences between treatments at $P < 0.05$. Seedling numbers: (A) n = 10–12; (B) n = 3–12.

Looking at the inhibitory effect of a high ABA concentration, this was surprisingly accentuated in the presence of either of the auxin efflux inhibitors, leading to an 86–89% inhibition of root elongation after 4 d compared to 48% inhibition with 10 µM ABA alone (Figure 4.4). By contrast, the auxin influx inhibitor CHPAA had the effect of reducing the inhibitory effect of 10 µM ABA to 6% and 23% of CHPAA alone after 1 and 4 days of treatment respectively (Figure 4.4). These results indicate that auxin influx is important for the root response to high ABA concentrations and that auxin efflux may play a negative role in the mechanism by which high ABA concentrations inhibit root elongation.

A genetic approach was used to investigate the respective roles of auxin efflux and influx in the root responses to ABA. The allelic auxin influx mutants *aux1-7* and *aux1-T* and five auxin efflux mutants (*pin2/eir1-1*, *pin3-4*, *pin3-5*, *pin4-3* and *pin7-2*) were treated with a range of concentrations of ABA, and their root elongation rates were compared with that of wild-type over the first day and over a 4 d period. The results of three separate experiments are shown in Figure 4.5. Two-way ANOVA was performed for each of those three experiments to test the impact of genotype, ABA treatment and their interaction. In all experiments, irrespective of whether

measurements were made in the first 24 h after treatment or the 4 d after treatment, there were significant effects of genotype and ABA treatment. In the first experiment (wild-type, *pin2/eir1-1*, *aux1-T* and *iaa7/axr2-1*), the results showed that there was significant genotype × ABA treatment interaction effect on the primary root elongation rate in the first 24 h and the 4 d after treatment (Appendix 3 Table 3). In the second experiment (wild-type, *pin4-3*, *pin7-2* and *tir1-1*), the interaction between genotype and ABA treatment significantly affected the average primary root elongation rate in the 4 d of treatment, but not in the first 24 h after treatment (Appendix 3 Table 3). In contrast, the results of the third experiment (wild-type, *aux1-7*, *pin3-4* and *pin 3-5*) suggested that the interaction between genotype and ABA treatment significantly affected the primary root elongation rate in the first 24 h of ABA treatment, but not the average primary root elongation rate during the 4 d treatment (Appendix 3 Table 3). Overall, the different genotypes responded differently to ABA treatment.

In the first one of these experiments (Figure 4.5A, B), it was found that the *aux1-T* knockout mutant was insensitive to both low and high concentrations of ABA in the first day and to the higher concentration of ABA when measured over 4 d, but that a slight positive effect of low concentration of ABA could be detected after 4 d. However, the *aux1-7* missense mutant showed a weaker phenotype, being unaffected in its sensitivity to low [ABA] over either 1 d or 4 d (Fig 4.5E, F) and insensitive to high [ABA] during the first 24 h of treatment (Figure 4.5E) but not during the subsequent 3 d (Figure 4.5F). These results are consistent with a role for AUX1-mediated auxin influx in the inhibitory effect of high [ABA], confirming the results obtained with CHPAA (Figure 4.2). An additional role of AUX1 in the stimulatory

effect of low [ABA] cannot be ruled out but was only detectable in the early stages of treatment and only in the knockout mutant.

Of the five auxin efflux mutants tested, only *pin2/eir1-1* behaved differently to the wild-type, showing less sensitivity to low [ABA], but normal sensitivity to high [ABA] (Figure 4.5A, B). There was one exception that in one of the three repetitions of this experiment, low [ABA] (0.1 μM) showed similar stimulatory effect in root elongation in *pin2/eir1-1* as in wild-type after 4 d treatment (by 15% *vs.* 17%, data not shown). However, *pin3-4*, *pin3-5*, *pin4-3*, *pin7-2* all showed similar ABA responses to the wild-type (Figure 4.5A–F). These results are consistent with the evidence from the auxin efflux inhibitors, NPA and TIBA, that blocking auxin efflux did not alleviate the inhibitory effect of high [ABA]. It also suggests that the role for auxin efflux in the stimulatory effect of low [ABA] indicated by use of these inhibitors might involve PIN2/EIR1-1.

Two auxin insensitive mutants (*tir1-1* and *iaa7/axr2-1*) were used to investigate the role of auxin signalling in the root responses to ABA. While the *iaa7/axr2-1* mutant showed reduced sensitivity to both low and high [ABA] (Figure 4.5A, B), the *tir1-1* mutant did not respond significantly differently from the wild-type (Figure 4.5C, D).

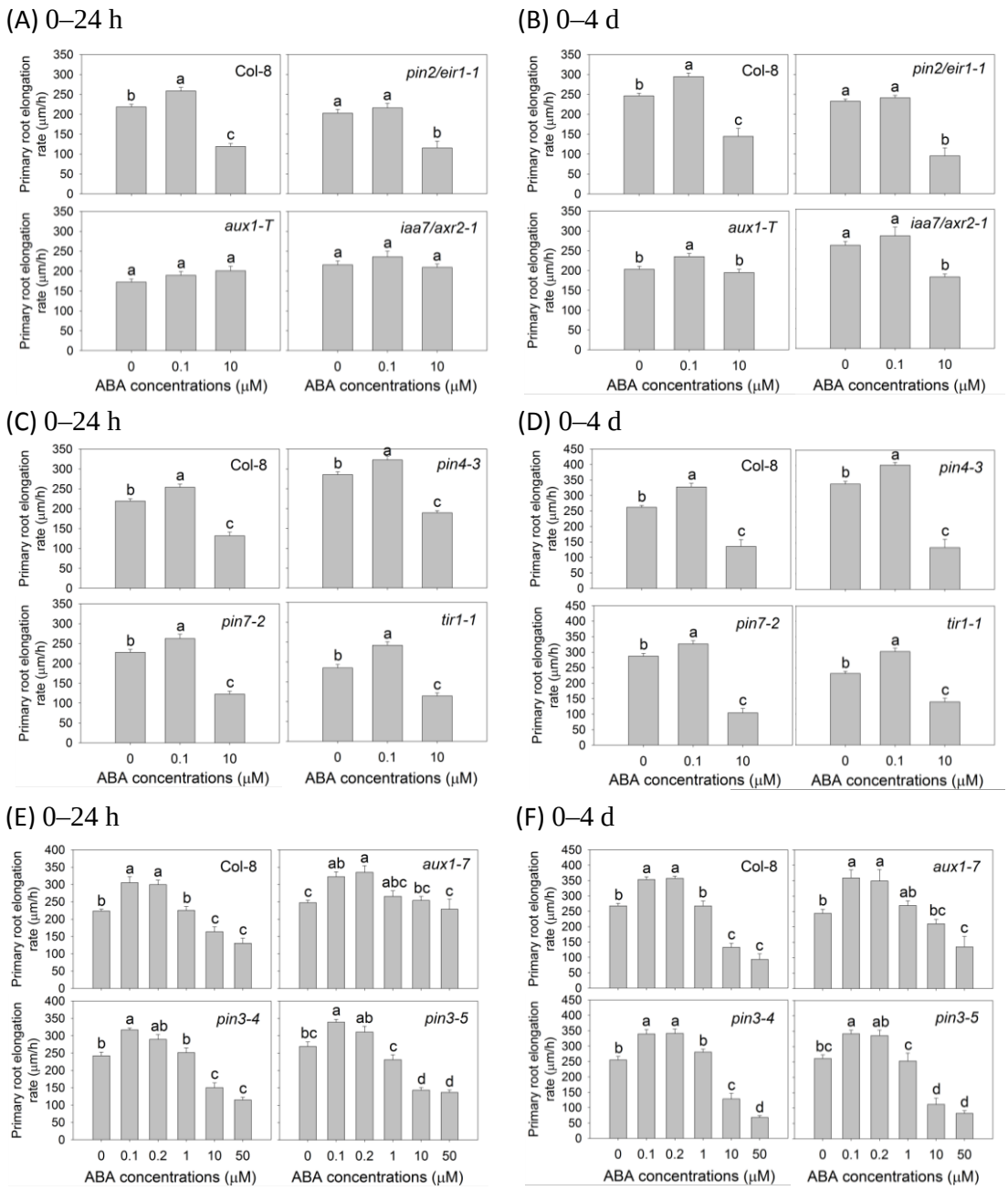


Figure 4.5: Auxin relevant mutants showed auxin signalling and also auxin transport are important for root growth response to ABA treatments. Primary root elongation rate during the first 24 h after treatment: (A) wild-type Col-8, *pin2/eir1-1*, *aux1-T*, *iaa7/axr2-1*; (C) Col-8, *pin4-3*, *pin7-2*, *tir1-1;* (E) Col-8, *aux1-7*, *pin3-4*, *pin3-5*. Average primary root elongation rate during the 4-day treatment: (B) Col-8, *pin2/eir1-1*, *aux1-T*, *iaa7/axr2-1*; (D) Col-8, *pin4-3*, *pin7-2*, *tir1-1;* (F) Col-0, *aux1-7*, *pin3-4*, *pin3-5*. Figures (A) and (B); (C) and (D); (E) and (F) were results from three experiments respectively. In each experiment, 4-day old Arabidopsis seedlings of each line with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5% sucrose) with various ABA concentrations (μM). Primary root length at the start point and the increase of primary root were measured every day. The root elongation rate was calculated for the first day and four days in average. The values are means, and the vertical bars represent standard errors of the means. Data analysed using one-way ANOVA and different letters indicate significant differences between ABA

treatments at $P < 0.05$. Seedling numbers: (A) n = 12; (B) n = 6–12; (C) n = 12; (D) n = 3–12; (E) n = 8; (F) n = 4–8. Similar experiments were done for at least 3 times with different mutant combinations and similar results showed.

Effect of ABA on the spatial pattern of expression of the DR5::GFP auxin reporter line

To investigate whether low and high ABA concentrations have differential effects on auxin distribution in the root tip, seedlings of the *DR5::GFP* auxin reporter line were treated with 0.1 and 10 µM ABA for 3 d. As expected, roots of the *DR5::GFP* line showed the normal growth responses to low and high [ABA] treatments (Appendix 3 Figure 1). When the pattern of GFP expression in the root tips of the ABA-treated seedlings was compared with that of controls using confocal microscopy, it was found that GFP signal was enhanced in the lateral root cap and centralized in the middle of vascular tissue in both ABA treatments (Figure 4.6A). By contrast, the GFP expression was reduced in root cap columella cells but not in the quiescent centre (Figure 4.6B).

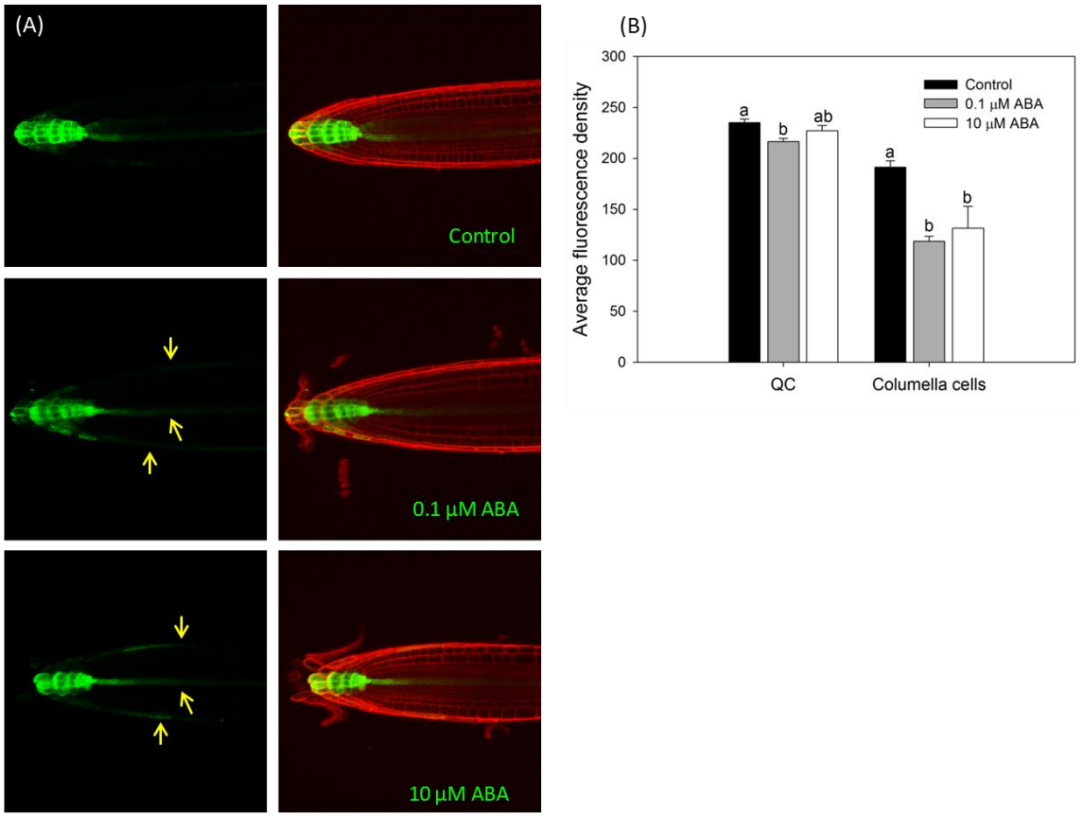


Figure 4.6: ABA treatments induced GFP signalling redistribution in root tips of *DR5::GFP* line. (A) Merged z-stack images of root tips (3-day after ABA treatments). (B) Average GFP fluorescence density in QC and columella cells (per area unit). Seedling numbers: n = 3. Four-day old seedlings with similar root length were chosen and transferred to newly made 0.02 × B5 medium (1 mM KNO_3, 0.5% sucrose) with various ABA concentrations. There were six seedlings per plate and three of them were chosen for imaging. Confocal images were merged from 7 image sections. The interval was 1.1965 µm between every two sequential image sections. The values are means, and the vertical bars represent standard errors of the means. Data analysed using one-way ANOVA and different letters indicate significant differences between ABA treatments at $P < 0.05$. Arrows point out where a changed pattern of GFP signal can be seen in ABA-treated roots (the lateral root cap and the middle of vascular tissue).

4.4 Discussion

The positive and negative effects of ABA on root growth differ in their requirement for ethylene signalling

Previous studies identified the importance of ethylene signalling and ethylene biosynthesis for the inhibition of primary root growth by ABA (Ghassemian *et al.*,

2000; Luo *et al.*, 2014). In the present study the objective was to try to understand how different concentrations of ABA can have opposing effects on root growth and to compare the signalling mechanisms responsible for the positive and negative responses. Use of the ethylene perception inhibitor STS (Figure 4.2C, D) along with three ethylene-insensitive mutants (*etr1-1, ein2-1* and *ein3-1*) (Figure 4.3) confirmed that ethylene signalling was important for the inhibitory effect of high [ABA] under our experimental conditions. Furthermore, the ability of the ethylene biosynthesis inhibitor AVG to completely suppress the inhibitory effect of high [ABA] (Figure 4.2) is consistent with recent evidence that ABA inhibits root growth in Arabidopsis by promoting ethylene biosynthesis (Luo *et al.* 2014).

In contrast to the ethylene-dependence of the inhibitory effect of high [ABA], there was no evidence of any involvement of ethylene biosynthesis or signalling in the stimulatory effect of low [ABA]: neither AVG nor STS blocked the stimulatory effect (Figure 4.2) and the ethylene-insensitive mutants still responded positively to low [ABA] (Figure 4.3). This clearly distinguishes the opposing effects of high and low ABA concentrations and indicates that they operate through distinct signalling pathways.

Among the three ethylene-insensitive mutants tested here, *ein3-1* showed weaker interference to the root response to high [ABA] than the other two did. The alleles of *ein3* were reported to be less insensitive to ethylene than the strong alleles of *etr1* and *ein2* (Roman *et al.*, 1995; Chao *et al.*, 1997). Six members of *EIN3* family have been identified, in which *EIL1* relates to *EIN3* most closely (Alonso *et al.*, 2003b). A complete ethylene-insensitive phenotype has been reported in a double mutant *ein3*

eil1, while the *ein3* and *eil1* single mutants have incomplete ethylene insensitivity (Alonso *et al.*, 2003a, b), which indicates that other *EIN3* members, especially *EIL1*, may be involved in the ABA responses in *ein3-1*.

IAA7/AXR2-dependent auxin signalling is involved in both the positive and negative responses to exogenous ABA

A role for auxin in the inhibitory effect of high [ABA] on Arabidopsis root growth has already been established from a number of studies using mutants defective in auxin transport and signalling (Belin *et al.*, 2009; Wang *et al.*, 2011; Thole *et al.* 2014; Zhao *et al.*, 2015). Here two mutants defective in components of the auxin signalling pathway were used to investigate whether there were differences between the responses to low and high [ABA] in their requirement for auxin signalling. The results showed that the auxin response mutant *iaa7/axr2-1* had significantly reduced sensitivity to both the inhibitory effect of 10 µM ABA and the stimulatory effect of 0.1 µM ABA (Figure 4.5A, B). It has previously been shown that ABA represses the expression of the *IAA7/AXR2* gene, leading to the suggestion that *IAA7/AXR2* is at the nexus of crosstalk between ABA and auxin signalling pathways by acting as a negative regulator of both pathways (Belin *et al.*, 2009). The lack of a similar phenotype in another auxin signalling mutant *tir1-1* (Figure 4.5) is consistent with an earlier report that the *tir1-1* mutant showed normal repression of embryonic axis elongation in response to ABA. This could indicate either that other F-box proteins are involved or it could be explained by genetic redundancy amongst members of this small family of auxin receptors (Dharmasiri *et al.*, 2005b; Parry *et al.*, 2009).

Differences between the positive and negative responses to ABA in their requirements for auxin influx and efflux

A number of previous studies have provided evidence of a role for auxin transport in the inhibitory effect of ABA on root growth. There are two reports that *aux1* auxin influx mutants are less sensitive to high concentrations of ABA (Belin *et al.*, 2009; Thole *et al.*, 2014) and a *pin2* auxin efflux mutant was also found to be insensitive to ABA-dependent repression of both hypocotyl and radicle elongation (Belin *et al.*, 2009). The *aux1* phenotype with respect to high [ABA] was confirmed in the present study (Figure 4.5) and a role for auxin influx in ABA's inhibitory effect on root growth was further supported by the ability of the auxin influx inhibitor CHPAA to antagonise this response to high [ABA] (Figure 4.4). How *aux1* mutations affect the stimulatory effect of low [ABA] was less clear-cut: an absence of stimulation of root growth by low [ABA] was only observed in the *aux1-T* knockout mutant and then only in the first 24 h of treatment. No phenotype was seen in the *aux1-7* missense mutant. Nevertheless CHPAA failed to block the stimulatory effect of low [ABA], indicating that there are differences between the positive and negative responses to ABA in their requirement for auxin influx.

When the positive and negative responses to ABA were compared for their requirement for auxin efflux, a distinct difference was found. No evidence of a positive role for auxin efflux in the inhibitory effect of ABA was obtained, based on the phenotypes of the *pin2/eir1-1, pin3-4, pin3-5* and *pin4-3* mutants (Figure 4.5A–F) and the inability of two auxin efflux inhibitors (NPA and TIBA) to overcome the inhibitory effect (Figure 4.4). On the other hand, the enhanced degree of inhibition

by 10 µM ABA that was seen in the presence of either NPA or TIBA in the latter experiment suggests the possibility that auxin efflux might have a role in counteracting the inhibitory effect of high [ABA]. By contrast, both NPA and TIBA were successful in blocking the stimulatory effect of low [ABA] and the *pin2/eir1-1* mutant (but not the other *pin* mutants tested) was also defective in its response to low [ABA]. This evidence of the importance of auxin efflux in the response to low [ABA] agrees with a previous report that TIBA was able to partially suppress the positive effect of a low concentration of ABA on root growth in rice (Zhao *et al.*, 2015).

Of the four *PIN* genes whose role in the ABA response was tested (*PIN2, PIN3, PIN4* and *PIN7*), it is notable that *PIN2* is the only one that is expressed in the lateral root cap (Blilou *et al.*, 2005; Kleine-Vehn *et al.*, 2010; Band *et al.*, 2014). The *pin2/eir1-1* mutant also shows an altered pattern of distribution of the auxin maximum in the root tip compared to other *pin* mutants (Ottenschläger *et al.*, 2003, Blilou *et al.*, 2005). It is possible that the reduced sensitivity to low [ABA] that is seen in the *pin2/eir1-1* mutant might be related to specific alterations in auxin distribution that arise from loss of PIN2/EIR1's contribution to auxin efflux in the lateral root cap.

Previous studies have demonstrated that exogenous ABA treatments cause alterations in the spatial pattern of auxin distribution/auxin signalling in the root tip. Inhibitory concentrations of ABA were found to cause a reduction in the level of expression of the *IAA2::GUS* auxin reporter gene in Arabidopsis root tips (Wang *et al.*, 2011). In rice roots treated with stimulatory concentrations of ABA there was

also a reduction in the expression of the *DR5::GFP* auxin reporter gene (Zhao *et al*. 2015). In the present study it was found that both high and low [ABA] treatments led to reduced level of *DR5::GFP* expression in the root columella cells, but the expression was enhanced in the lateral root cap and centralized in the middle of vascular tissue (Figure 4.6). Xu *et al*. (2013) observed a very similar pattern of redistribution in the expression of *DR5::GFP* in Arabidopsis roots treated with stimulatory concentrations of ABA. Surprisingly, despite the differences in the growth response to high and low [ABA], and the differences in relative the roles of auxin influx and auxin efflux in the two responses, no difference was detected in the effect of the high and low [ABA] treatments on the spatial pattern of expression of *DR5::GFP*. However, since these expression patterns were observed 3 d after the start of treatment it cannot rule out the possibility that there were short-term differences in the effects of the high and low [ABA] treatments on *DR5::GFP* expression that were missed in these experiments.

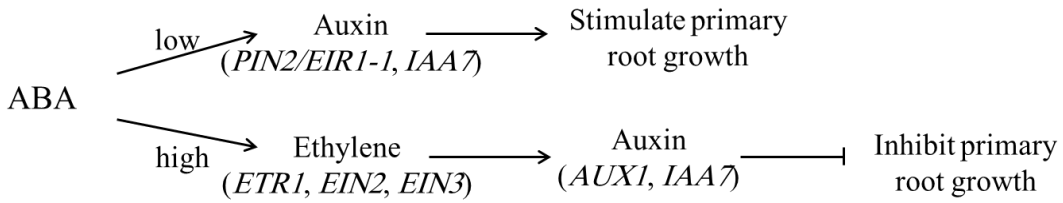


Figure 4.7: A model shows the involvement of ethylene and auxin in root growth responses to different ABA treatments. ABA regulates root growth through two distinct pathways: 1. an ethylene-independent stimulatory pathway that operates at low [ABA] and requires auxin signalling and auxin efflux through PIN2; and 2. an ethylene-dependent inhibitory pathway that operates at high [ABA] and that also requires auxin signalling and auxin influx through AUX1. The auxin pathway working downstream of the ethylene pathway is based on the report that *aux1-T* mutant exhibited ACC-resistant root growth (Růžička *et al*., 2007).

4.5 Conclusion

The results in this study provided evidence that the stimulatory effect of low ABA concentrations on root growth operates through an ethylene-independent pathway, and requires auxin signalling and auxin transport by the PIN2/EIR1-1 auxin efflux carrier (Figure 4.7). However, the inhibitory effect seen at high ABA concentrations is through an ethylene-dependent pathway that requires auxin signalling and auxin influx through AUX1 (Figure 4.7).

Chapter 5 General Discussion

5.1 Methodological issues

Suitable non-lethal drought stress treatments

Drought is an imprecise term and does not have a universal definition (Wilhite and Glantz, 1985). However, it is valuable to use a combination of indices to characterise a specific drought stress event (e.g. onset, severity and duration), which can facilitate comparison and interpretation of specific plant drought responses (Wilhite, 2010; Lawlor, 2013). A non-lethal drought stress is common in the field and it has been argued that effects of this kind of drought are worthy of attention in order to improve plant performance in drought environments (Tuberosa *et al.*, 2007; Skirycz *et al.*, 2011). To establish a non-lethal gradual soil drying process to investigate maize root and shoot physiological responses during this process, several preliminary experiments were conducted. A six-day soil drying treatment was chosen and imposed on maize seedlings that were 15 d after sowing (transplantation) and grown in John Innes No. 2 compost under the controlled environment as described in Chapter 2. Sharp (2002) reported that the shoot elongation of maize, soybean, cotton and squash was completely inhibited when the water potential of the substrate was as low as −0.8 MPa, while the root growth was maintained with a reduced rate at such low water potential. In Chapter 2, the soil water potential declined from −0.2 to −0.8 MPa (Figure 2.1, Appendix 1 Figure 1). Maize plants started to wilt on Day 6 after last watering. Considering the permanent wilting point is around −1.5 MPa (Kramer and

Boyer, 1995), this soil drying episode was a non-lethal drought. The difference of the start points for root and shoot responses to soil drying was seen (Figure 2.2–2.5). However, although the drying process in this study has been described clearly with detailed soil water content values (Figure 2.1C), we still need to be careful when comparing the drought stress level and plant responses with other experiments using different plants, soil types and growth conditions.

Based on the different root and leaf responses that were presented in Chapter 2 and in a few pre-trials, drought intensity in this soil was classified to four categories: well-watered (soil water content at > 32%), mild drought (20–32%), moderate drought (13–20%), and severe drought (< 13%). This classification was used as reference points to set up the drought treatments in Chapter 3 because the growth substrates used in both Chapters showed similar water characteristics curves (Appendix 1 Figure 1).

In the experiment reported in Chapter 3, water contents in the top layer of soil in each pot were carefully regulated because seed germination and nodal root development can be seriously affected by the soil drying in this layer. The experiment was designed to ensure three distinctly different soil water contents (i.e. well-watered, mild and severe drought) on day 17 when the nodal root had just developed and could be used for root angle measurement using the mesh method described in Figure 3.2.

An alternative way to generate an experimental drought stress is to use chemical treatments with polyethylene glycol (PEG), mannitol, melibiose or NaCl to generate low water potentials in growth media (e.g. nutrient solutions and agar plates) to

mimic stress imposed by soil drying (Sands and Clarke, 1977; Verslues *et al.*, 2006). Verslues *et al.* (2006) presented evidence that the high molecular weight PEG might be the best solute to impose a low water potential on solid agar plates to reflect a certain drought level, because other low molecular weight solutes such as mannitol and melibiose showed stronger and non-recoverable toxic effects on plant growth and these could obscure the real drought responses. Moreover, adding PEG to the growth plate can create a steady and uniform stress condition over time, which is difficult to achieve in the soil (Verslues *et al.*, 2006). Nevertheless, there are still debates on the suitability of such osmotic-induced drought stresses because of the chemical toxicity and low oxygen content in the growth media (especially when adding PEG into nutrient solutions) that may be caused by adding those chemicals (Mexal *et al*, 1975; Verslues *et al.*, 2006). Furthermore, some studies found that results from experiments with PEG treatments were inconsistent with results from studies with natural soil drying (e.g. Kano *et al.*, 2011).

Localised root sampling for ABA and root water potential analysis

In Chapter 2, it was found that the root sampling method was important to accurately investigate maize root ABA responses to drought. Water is not homogeneously distributed in the soil and higher water contents were generally recorded in the lower sections of the soil columns (Figure 2.1C). Roots in the lower sections of the columns are probably less affected by soil drying than those in the top layer, which suffers faster soil drying. If the sampling includes roots in the lower column, this may obscure the root response to drought occurring in the upper soil layer, which was observed on many occasions in pre-trials. This is consistent with

the report of Puértolas *et al*. (2015) which showed that roots sampled in the lower wetter part of soil column had a lower ABA concentration than roots in the upper drier soil in a vertical partial root-zone drying system. Additionally, the ABA level started to increase in growing root tips at higher root water content than the other roots that were relatively more mature (Zhang and Tardieu, 1996). However, the growing root tips are thought to have lower root water potential than the whole root under drought, due to greater osmotic adjustment in the tips (Greacen and Oh, 1972; Turner, 1986). In Chapter 2, by sampling only the roots from the top 2/3 of the pot, the changes in root ABA level (only sampled the root tips, ca. 3 cm long for each) and root water potential (samples without the root tip) were effectively detected when the soil water content decreased to 25% on Day 3 after last watering (Figure 2.1C, 2.5 and 2.8A).

An accurate method for root angle measurement

Several methods have been reported for assessing root angle, such as the basket method (Oyanagi, 1994), the 'Shovelomics' method (Trachsel *et al*., 2011), and direct measurement of the angle between the soil surface and the line marked along the direction of root growth with a protractor (Omori and Mano 2007; Ali *et al*., 2015). However, the basket method is not accurate enough to give the exact degree of a root angle. By the other two methods it often measures only one or two readings for a certain type of root angle. In Chapter 3, an accurate method was developed to measure the angle of every crown and nodal root (Figure 3.2). The root base (ca. 1.5 cm long) was pinned using a needle in the centre of a stainless wire mesh, and the axis of the root base (the needle) was kept perpendicular to the mesh surface. And

thus a right-angled triangle is formed with the crown or nodal root as the hypotenuse, the needle as one of the right-angle sides and the other right-angle side on the wire mesh. The arc tangent of the root growth angle can be calculated after measuring the lengths of the two right-angle sides. This mesh method provides an exact measure for each root angle. Therefore, it is possible to investigate the variation in the angle of a certain type of root within a particular genotype (Forde, 2009), which may provide useful information for breeders.

If the root is too short to intersect with the mesh, the lengths of the two right-angle sides can be directly measured with a ruler. However, the error will increase because a small measurement error could account for a relative large portion of these lengths. Using this method, it took about five minutes to measure the growth angles of all roots in one young plant (with 3–6 crown and nodal roots respectively) and this is longer than the 'Shovelomics' method that only needs two minutes to complete (Trachsel *et al.*, 2011). Clearly, it will take a longer time with increased number of roots. In addition, it also takes time to dig the root out from the soil and prepare the root sample for measurement. Therefore, the mesh method can be an accurate and practical method for measuring every root angle from young plants, but may still be laborious for a more mature plant with an intricate root system. However, by dividing such a root system into small sections and developing appropriate image analysis software, it should be possible to simplify and speed up root angle measurement.

5.2 Physiological response in maize root is more sensitive to soil drying than that in the leaf

In Chapter 2, asynchronous physiological responses in maize leaf and root were recorded during a short-term soil drying episode. Consistent with previous findings, maize root showed earlier responses to soil drying than the leaf (Sharp and Davies, 1979). Moreover, root growth was stimulated when the drought was mild but was inhibited when it became severe (Sharp and Davies, 1979; Watts *et al.*, 1981; Creelman *et al.*, 1990). Leaf growth is normally found to be inhibited by drought (Sharp and Davies, 1979; Munns and Sharp, 1993). Stomatal conductance in a younger leaf (the 4th leaf) showed an earlier reduction (by 12%) than that in the older leaf (3rd) during soil drying, which was also prior to any leaf elongation inhibition (Figure 2.6). Generally, leaf growth inhibition and stomatal closure are recognised as the earliest plant responses to drought and the former is earlier than the latter (Hsiao, 1973; Chaves, 1991; Osório *et al.*, 1998). However this study suggested that root growth, root water potential and root ABA level showed even earlier response to soil drying (Figure 2.2–2.5, 2.7–2.9). Additionally, the stomatal closure in the younger leaves rather than in the older leaves showed early reduction in response to soil drying (Figure 2.6, 2.9C). Thus, it is suggested that earlier root physiological responses to soil drying and stomatal closure in younger leaves may be important and better indicators to define the onset and severity of a drought event than leaf growth inhibition and other later responses in leaves. However, the stomatal closure in young leaves will be easier to measure than the responses in the root when plants are grown in soil.

It was found that the root growth was promoted when the root water potential decreased and root ABA increased under soil drying, while the leaf water potential and leaf ABA were not affected (Chapter 2). It has been argued that a promotion of root growth by mild drought may allow increased access to soil water, which in turn will further maintain plant shoot growth as soil dries (Kano *et al.*, 2011). Such responses have been characterised as a drought avoidance strategy (Verslues *et al.*, 2006; Kano *et al.*, 2011). Therefore, it is possible that appropriate crop management techniques that allow such mild drought to promote root growth may be beneficial to plant water uptake and increase plant water use efficiency. Some studies in partial root-zone drying have reported evidences that support this possibility. For instance, Mingo *et al.* (2004) found that the root growth of tomato plants in partial root-zone drying system was promoted remarkably in the drying part and the yield was not affected. It indicates that the promoted root growth may play an important role in delivering improved plant water use efficiency when the localised drying is not severe.

Deficit irrigation strategies involve irrigation management that provides plants with an amount of water that is below the full crop-water requirement (determined by plant size and evaporative demand) (Davies *et al.*, 2011). Water supply below the potential evapotranspiration will sustain a degree of water deficit, save water and can increase crop water use efficiency (Costa *et al.*, 2007; Davies *et al.*, 2011). Partial root-zone drying is one of the main deficit irrigation strategies and it is based on the physiological and developmental regulation via root to shoot signalling (e.g. hormones) (Stoll *et al.*, 2000). Signals from the root in the drying part of the soil can reduce leaf stomatal conductance even when leaf water status is not perturbed (Stoll

et al., 2000). This strategy can minimise the yield penalty that may occur with reduced water availability (Stoll *et al.*, 2000; Costa *et al.*, 2007); however, sometimes it is difficult in practice to reach this aim (Dodd, 2009; Davies *et al.*, 2011). Although the mild drought is thought to be beneficial for plant growth and saving water, it is not easy to determine the degree of soil drying which will promote root growth in the field in a deficit irrigation system (Kang and Zhang, 2004). Sometimes severe droughts arising from deficit irrigation systems caused huge reduction in crop yield (Costa *et al.*, 2007; Davies *et al.*, 2011). The soil water status when root growth was affected (either stimulated or inhibited) in Chapter 2 may provide useful information to adjust the timing of irrigation in a deficit irrigation system, thereby minimising the yield penalty. Because deficit irrigation can result in different yield reductions depending on plant species, genotypes, the climate, plant growth stages and the soil characteristics (Costa *et al.*, 2007), specific trials should be conducted to find out the appropriate soil water status that affects root growth and related physiological responses as required.

5.3 Root phenotyping for drought resistance

Specific root traits that are related to drought resistance are difficult to identify and characterise in root phenotyping studies (Burton *et al.*, 2013). A larger root system has been suggested to be important for better plant drought resistance and been widely studied in different crop species to enhance plant performance under drought (Price *et al.*, 1997; Werner *et al.*, 2010). Root growth angle may play an important role in determining plant drought resistance as it determines the direction of root elongation and the extent of root distribution which have been shown to be key in

determining the plant's access to water (Kamoshita *et al.*, 2000; Hammer *et al.*, 2009; Uga *et al.*, 2015). Plants with a steeper root growth angle may be able to distribute more roots in deeper soil layers and extract more water to maintain a better growth and yield under drought conditions (Manschadi *et al.*, 2006; Hammer *et al.*, 2009). However, root angle plasticity under drought and the potential relationship between the angle and the size of root system has not previously been investigated in detail. In this study, the 14 maize genotypes showed extraordinary genetic variations not only in the crown and nodal root angles (when well-watered), but also in the plasticity of the angles (under the same mild drought condition). Moreover, maize nodal root angle under well-watered condition was correlated with the changes in the root system size under drought (Figure 3.10D–H). That is, genotypes with a steep nodal root angle tended to show more growth stimulation or less inhibition in the size of its root system under drought. It suggests that a genotype with a steep root angle might display better drought resistance, not only because it has deeper roots (Manschadi *et al.*, 2006; Manschadi *et al.*, 2008) but also because it tends to grow more root under drought. More interestingly, it was also found that a genotype with a shallow root angle might still display a high drought resistance as long as it is able to show high plasticity to become a steeper root phenotype under drought (Chapter 3).

Nevertheless, both the root angle and its plasticity to drought showed weaker correlation with plant drought resistance (biomass reduction) compared with the plasticity of root size (Figure 3.10I–K, Table 3.2). Therefore, a better target in phenotyping for improving drought resistance should be combined traits including root system size, root angle and its plasticity to drought. The combination of various

root traits such as root growth angle, diameter, length of lateral root, and numbers of seminal and lateral roots have been suggested to be important to form an ideotype to cope with different water and nutrient deficient environments (Lynch, 2013), especially when the environment is changing significantly and quickly (e.g. in alternate wetting and drying conditions) (Suralta and Yamauchi, 2008; Price *et al.*, 2013). However, root phenotyping in the field is notoriously labour-intensive and time-consuming (Wasson *et al.*, 2012; Araus and Cairns, 2014). However, root phenotyping studies at early growing stages in controlled environment have been shown to be important in the prediction of later yield performance under drought (Nass and Zuber, 1971; Canè *et al.*, 2014; Ali *et al.*, 2015). Thus, it will be valuable to do phenotyping on those combined root traits with young seedlings under controlled environment. In addition, the development of high-throughput root phenotyping techniques may greatly accelerate this process in the future. For example, Atkinson *et al.* (2015) reported a high-throughput root phenotyping system that is based on image segmentation and analysis software, and this system is able to screen more than twenty root traits at one time in a few minutes. With such high-throughput root phenotyping systems, it will also be quicker and easier to conduct quantitative trait loci (QTL) studies (Atkinson *et al.*, 2015) and to identify important QTLs for drought resistance relevant root traits, which can be useful for crop breeders.

5.4 Hormone signalling in regulating root development during drought

The endogenous level of ABA in plants will increase while the ethylene level may decrease under drought (Zhang and Davies, 1989; Sharp, 2002). However, it is unclear from the literature when the hormone levels start to change following the

initiation of a soil drying episode and whether these changes are synchronous with other root or leaf physiological changes. The results in Chapter 2 showed that the increase in ABA levels in maize root was accompanied by a decrease in root water potentials and promoted root growth (Figure 2.9A, B and D). The root ABA started to accumulate before there was any change in the leaf elongation rate and the leaf water potential (Figure 2.9B, D). It was also found that the decrease in root ethylene release rate happened two days later than the increase of root ABA (Figure 2.9D, E). Applied ABA has been reported to both stimulate and inhibit root growth, depending on its concentration (Watts *et al.*, 1981; Xu *et al.*, 2013). Thus, both the stimulated and inhibited root growth during the 6-d soil drying process (Figure 2.9A) may be attributed to the increased ABA concentration in root, while it is not clear what the role of reduced root ethylene level in this regulation was. However, Spollen *et al.* (2000) reported that the increased ABA level is able to restrict extra ethylene production and then maintain maize root and shoot growth at low substrate water potentials. Thus, the reduced root ethylene production which occurred after the root ABA had increased during the soil drying process in this study may illustrate the adaptation of plant to soil drying to maintain its root and shoot growth when the drought becomes more severe (Figure 2.9D, E). To confirm whether ABA regulation is the main cause of those root growth rate changes during soil drying and the role of ethylene in this regulation, genetic (e.g. ABA or ethylene related mutants) and chemical (e.g. ABA or ethylene inhibitors) methods could be useful for further to investigation.

The correlations between root angle or its plasticity to drought and endogenous plant hormone levels (ABA, ethylene and *t*Z) were explored in Chapter 3. The root

angle was positively and negatively correlated with ABA and *tZ* levels respectively (Figure 3.10L–N), implying that ABA and *tZ* levels might be crucial in determining root angles when the plants are well watered. The current findings cannot provide direct evidence on whether the root hormone level changes are involved in the root angle plasticity under drought. Ethylene and cytokinin have been reported to antagonise ABA signalling, for example, by inhibiting ABA-induced stomata closure under drought (Blackman and Davies, 1983; Tanaka *et al.*, 2005, 2006). Ethylene and cytokinin were often found to be negative regulators of root growth (Alarcón *et al.*, 2009; Werner *et al.*, 2010). Additionally, several studies have found that drought normally increases the ABA levels but decreases the ethylene and cytokinin levels in the root (Zhang and Davies, 1989; Spollen *et al.*, 2000; Nishiyama *et al.*, 2011; Chapter 2). Changes of hormone levels under drought stress indicate that cooperation or the cross talk between those hormones may be involved in mechanisms that plants use to fine-tune their growth in response to drought. Similar to the findings in Chapter 2, the increased or decreased root growth in different maize genotypes under drought in Chapter 3 may be attributed to the increased root ABA levels and perhaps the decreased root ethylene levels as well (Figure 3.5, 3.7B and 3.8B). It will be interesting to investigate whether hormone signalling under drought induces the plasticity of various root traits during drought stress (e.g. root angle reduction and root size changes).

Although the increase of ABA level under drought may be responsible for root growth stimulation and inhibition (Watts *et al.*, 1981; Chapter 2), the signalling crosstalk between ABA, ethylene and auxin in such responses is not clear. In Chapter 4, experiments were conducted to elucidate the involvement of ethylene

biosynthesis and signalling, and auxin signalling and transport in Arabidopsis root biphasic response to ABA.

Firstly, the positive and negative effects of applied ABA on root elongation (Watts *et al.*, 1981; Xu *et al.*, 2013) were confirmed in the current experimental condition and the critical ABA concentrations that can induce these effects were identified (Figure 4.1, 4.3A and 4.5).

Secondly, the negative effect of high ABA concentrations was reduced or eliminated when ethylene biosynthesis or perception was inhibited by AVG (ethylene biosynthesis inhibitor) and STS (ethylene perception inhibitor) respectively (Figure 4.2), and also when auxin influx was inhibited by CHPAA (auxin influx inhibitor) (Figure 4.4). This was further confirmed by using mutants with blocked ethylene signalling (*etr1-1*, *ein2-1*, *ein3-1*), auxin signalling (*iaa7/axr2-1*) and a defect in the auxin influx carrier AUX1 (*aux1-7*, *aux1-T*) (Figure 4.3, 4.5). These results indicated that the inhibitory effect of high ABA concentrations is via an ethylene-dependent pathway that requires auxin signalling and auxin transport that through AUX1 auxin influx carrier. This is consistent with the findings that ethylene signalling is required for the inhibitory effect on root growth of high ABA concentrations (Ghassemian *et al.*, 2000; Luo *et al.*, 2014). Moreover, Luo *et al.* (2014) showed that the inhibitory effect of high ABA concentrations is through enhanced ethylene biosynthesis. In addition, ABA was found to repress *IAA7/AXR2* expression, and it is suggested that *IAA7/AXR2* is at the nexus of ABA and auxin signalling crosstalk by acting as a negative regulator of both pathways (Belin *et al.*, 2009). In addition, auxin influx

mutant *aux1* was reported to be less sensitive to high concentrations of ABA (Belin *et al.*, 2009; Thole *et al.*, 2014).

Thirdly, the positive effect of low ABA concentrations was removed when auxin efflux carriers were inhibited by NPA or TIBA (auxin efflux inhibitors) and in a mutant with defective auxin efflux carrier PIN2/EIR1-1 (Figure 4.4, 4.5A). However, ethylene biosynthesis and signalling inhibitors and auxin influx inhibitor did not affect the positive effect of low ABA concentrations (Figure 4.2, 4.4). These results were further confirmed by using mutants with blocked ethylene signalling, auxin signalling (*iaa7/axr2-1*) and a defective auxin efflux carrier PIN2/EIR1-1 (*pin2/eir1-1*) (Figure 4.3, 4.5). Therefore, it is suggested that the stimulatory effect of low ABA concentrations on root growth operates via an ethylene-independent pathway, which requires auxin signalling and auxin transport through PIN2/EIR1-1 auxin efflux carrier. These results further confirmed that *IAA7/AXR2-1* is a crucial regulator for both ABA and auxin signalling pathways (Belin *et al.*, 2009). Results reported here also agree with the findings of Xu *et al.* (2013), which suggested that auxin transport may be important for the stimulatory effect of low ABA concentrations on Arabidopsis and rice root growth. Furthermore, Belin *et al.* (2009) found a *pin2* auxin efflux mutant is insensitive to ABA-induced inhibition of both hypocotyl and radicle elongation.

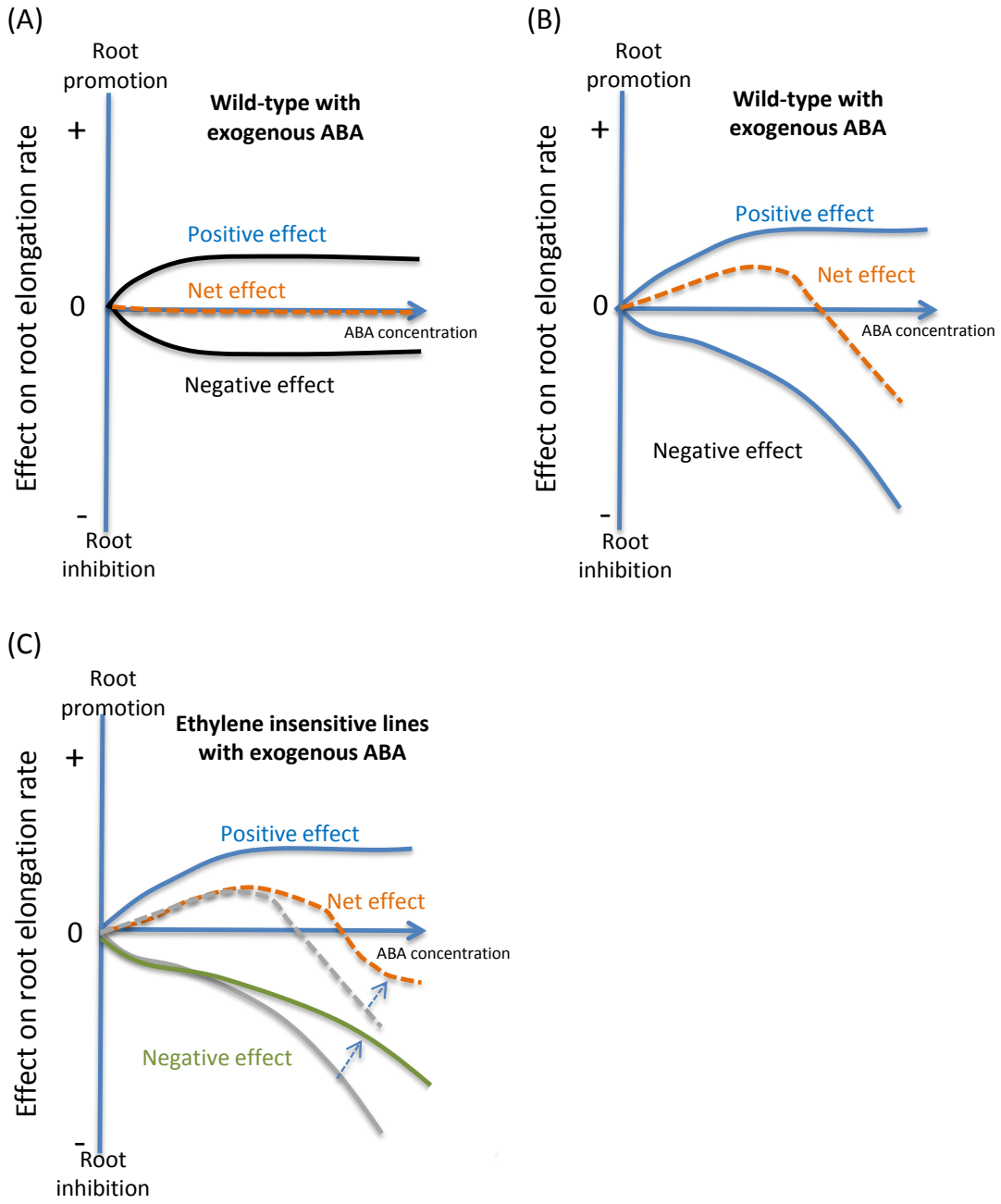


Figure 5.1: Model to explain the biphasic response of Arabidopsis root elongation to the addition of abscisic acid (ABA) at different concentrations. (A) A hypothetical wild-type plant in which exogenous ABA shows no stimulatory or inhibitory net effect. (B) Wild-type plants with exogenous ABA and ABA changes root growth. Both positive and negative effects are modified and the balance between them is broken. The positive effect outweighs the negative effect at low ABA concentrations and shows a net stimulatory effect, while the negative effect outweighs the positive effect at high ABA concentrations and shows a net inhibitory effect. (C) Ethylene signalling insensitive mutants (i.e. *etr1-1*, *ein2-1*, *ein3-1*) with exogenous ABA. The negative effect of ABA is ethylene-dependent and the blocked ethylene signalling pathway modifies the negative effect of ABA. This modification does not change much of the ABA effect when ABA concentrations are low, but reduced the negative effect at high ABA concentrations.

A model to explain the biphasic effect of ABA on root elongation and the involvement of ethylene signalling in these processes is presented in Figure 5.1. Figure 5.1A shows an imaginary wild-type plant where there is no positive or negative effect of ABA on root elongation rate; or the positive and negative effects of ABA are perfectly balanced over the entire range of ABA concentrations. Thus the net effect of ABA on root elongation rate is zero. Figure 5.1B displays the wild-type plant where the positive effect saturates at low ABA concentrations and the negative effect becomes strong only at high concentrations, which has been seen with Col-8 wild-type Arabidopsis in Chapter 4. When ABA is applied to such wild-type plants, it shows both positive and negative effects on the root elongation dependent on the ABA concentrations (Figure 5.1B). The positive effect outweighs the negative effect at low ABA concentrations and shows a net stimulatory effect, while the negative effect outweighs the positive effect at high ABA concentrations and shows a net inhibitory effect (Figure 5.1B). The negative effect of ABA on root elongation is dependent on ethylene, but not the positive effect. Therefore, when the ethylene signalling insensitive mutants are treated with exogenous ABA, the negative effect of ABA is different from the wild-type (Figure 5.1C). This modification does not impact much of the ABA effect at low concentrations, but reduces the negative effect of high ABA concentrations. Thus, the same high concentration of ABA shows less net inhibitory effect in ethylene insensitive lines than in the wild-type (Figure 5.1C). This is only one possible conceptual model, which needs further investigations to validate.

The studies in this thesis have shown that hormone signalling, especially ABA signalling, is important for regulating root growth and this may be useful for

breeders and crop managers. Different aspects of root system architecture, such as the root size under drought, are important for plant drought resistance (Chapter 3). Thus genetically modified or chemically treated plants with elevated endogenous ABA concentrations (or increased ABA sensitivity) could lead to stimulation of root growth and a larger root system than an untreated wild-type. It should be noted that if there is a large increase in endogenous ABA levels (or ABA sensitivity) that can inhibit root growth, a smaller root system may develop. There is one study that may support this speculation. A quantitative trait locus (QTL) *Root-ABA1* was identified in maize and this QTL enhanced the leaf ABA level, root branching, root dry weight and plant root to shoot ratio (Giuliani *et al.*, 2005), but unfortunately, the root ABA level was not reported. In addition, plants with reduced ethylene sensitivity or biosynthesis may exhibit less root growth inhibition caused by accumulated high ABA levels under severe drought, and show better performance under such conditions. For example, transgenic maize plants with down-regulated ethylene biosynthetic pathway exhibited improved grain yield under drought conditions (Habben *et al.*, 2014). As for the farming practice, crop management such as the deficit irrigation strategies may apply the knowledge of hormone signalling regulation gained in this thesis to enhance plant water use efficiency and save water. For example, it is possible to create a drought scenario that is able to increase the ABA level to a range that can promote the root growth and improve plant performance with reduced water supply and increase its water use efficiency.

5.5 Conclusions and perspectives

The work described in this thesis attempted to address the development of plant root systems in response to different levels of drought stress and the involvement of hormones (ABA, ethylene, auxin and cytokinin) in such processes. Several specific methods were developed during this work. These included the precise definition and application of the non-lethal drought stress treatments to maize plants in a controlled environment, the root sampling method and an accurate method to measure maize root angles. In Chapter 2, the synchronisation of changes in maize leaf and root growth and ABA and ethylene levels during soil drying was examined. In Chapter 3, the genetic variation in drought resistance relevant root traits and its possible relationships with hormones (ABA, ethylene and *t*Z) in 14 maize genotypes were explored. In Chapter 4, the involvement of ethylene and auxin in ABA-regulated biphasic root elongation response of Arabidopsis were investigated. Following are the main conclusions drawn from these studies:

(1) Maize root and shoot showed asynchronous physiological responses to soil drying. The root responses were more sensitive than the shoot responses. Root growth can be both stimulated and inhibited by soil drying, depending on the drought severities, while the leaf elongation was inhibited when drought became more severe (Chapter 2).

(2) The increase of root ABA level was synchronous with the root water potential changes during soil drying and it might be responsible for the promoted and inhibited root growth responses (Chapter 2).

(3) Significant genetic variation was seen in maize root traits (root angle, length, surface area and dry weight) and in the plasticity of those traits under drought (Chapter 3).

(4) Root angle under well-watered conditions was negatively correlated with the relative increase in root size under drought, i.e. maize genotypes with smaller (steeper) root angle under well-watered conditions are more likely to show more increase or less decrease in root size under drought (Chapter 3).

(5) Combined root traits, including root angle, its plasticity (reduced angle) to drought and the plasticity of root size under drought (the changes in root length, surface area and dry weight) could be a better parameter to predict maize drought resistance than any one trait alone (Chapter 3).

(6) Maize root angle may be determined by hormone levels under well-watered condition, because it was positively and negatively correlated with ABA and *t*Z concentrations respectively (Chapter 3).

(7) The inhibitory effect on root growth of high ABA concentrations is via an ethylene-dependent pathway and requires auxin signalling and auxin influx through AUX1 (Chapter 4).

(8) The stimulatory effect on root growth of low ABA concentrations is via an ethylene-independent pathway and also requires auxin signalling and auxin efflux through PIN2/EIR1-1 (Chapter 4).

It will be potentially rewarding to use genetic and chemical methods to further investigate whether ABA regulation is the main cause of variation in root growth rates under different degrees of soil drying. If this proves to be the case, it will be important to know what the critical concentrations are. It is also crucial to know

whether ethylene and auxin are involved in ABA-regulated root growth of crops under soil drying as was shown by experiments with Arabidopsis in Chapter 4. In addition, it is necessary to know how ABA and other hormones are involved in the root angle reduction under drought because such changes in root angle can be crucial in the regulation of root system structure and improve plant drought performance. When the hormone cross talk that potentially regulates root system architecture under drought becomes more clear, it may be possible to modify plant root systems artificially by manipulating the accumulation (or sensitivity) of one or a group of plant hormones to create an ideotype for a particular drought environment.

In an application perspective, the universality of these root growth changes under drought in different plant species at various growth stages is important, which can help us to understand the variations in drought effects and to plan water management strategies. Phenotyping of root systems at early growing stages is important in the prediction of later yield performance (Nass and Zuber, 1971; Canè *et al.*, 2014; Ali *et al.*, 2015). Nevertheless, it will still be worthwhile to establish field experiments under well-controlled conditions to test the contribution to crop production under drought of those root traits identified and quantified in Chapter 3.

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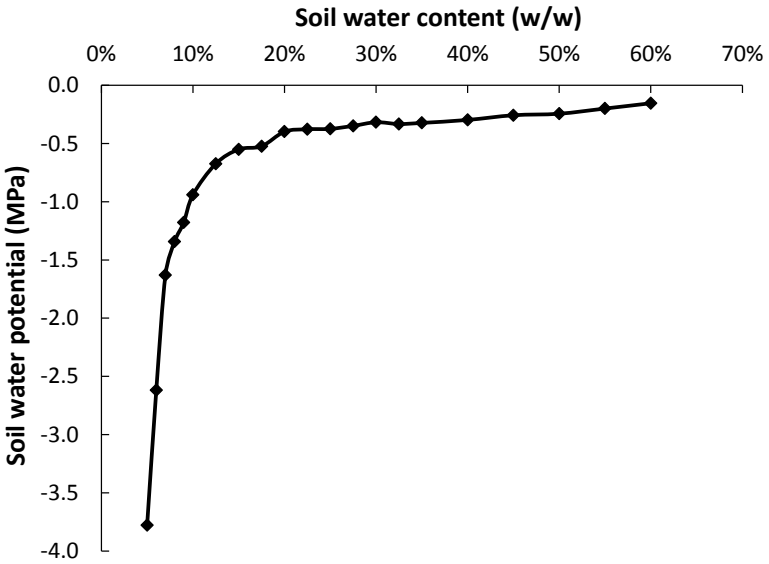
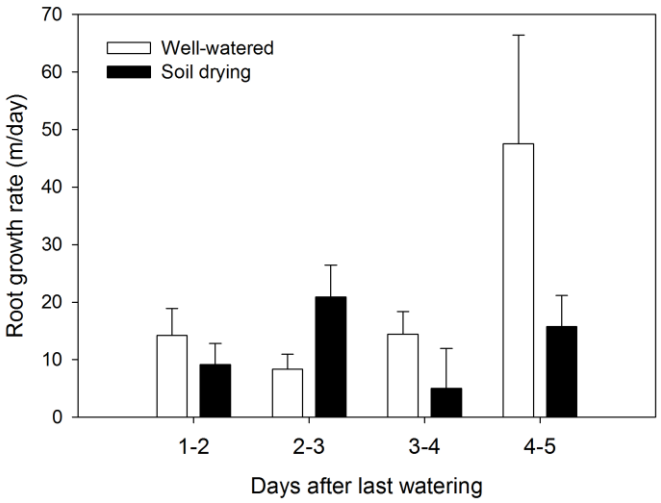
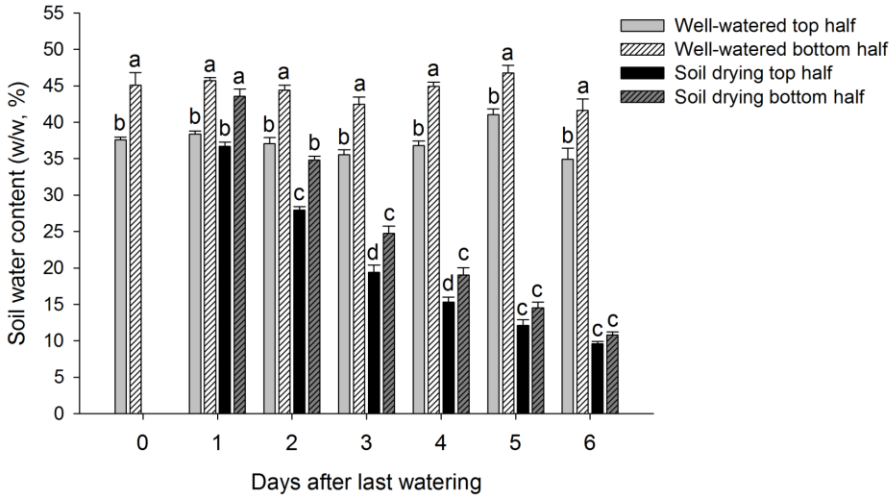
Appendix 1



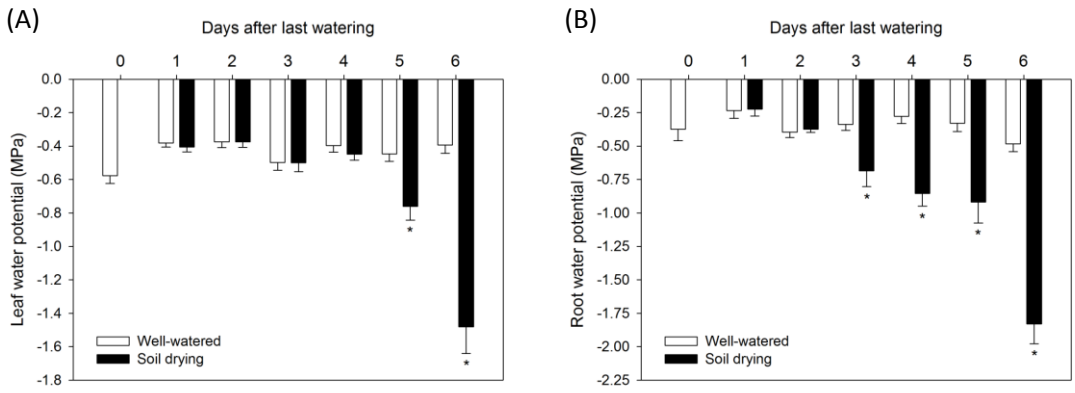
Appendix 1 Figure 1: Soil water characteristic curve: soil water potential against soil water content. (John Innes No.2, Foremost, UK).



Appendix 1 Figure 2: Root growth rate during a 5-day soil drying (a preliminary experiment with John Innes No.2, n = 4). The soil water contents in this experiment were 40%, 33%, 22%, 16% and 12% on each day respectively during Day 1–5 after last watering. During the 5-day soil drying, the total root length in each day were scanned and analysed with the WinRHIZO Pro system. The daily increase rates of root length were then calculated. Columns and bars are means ± standard error.



Appendix 1 Figure 3: Soil water content in top and bottom parts of the well-watered and soil drying treatments (Day 0–6) in the repeat experiment. Columns and bars are means ± standard error. Different letters indicate significant difference on the same day at $P < 0.05$ (n = 5).



Appendix 1 Figure 4: (A) The leaf and (B) root water potentials during Day 0–6 in the repeat experiment. Columns and bars are means ± standard error. Stars indicate significant difference between treatments on the same day at $P < 0.05$ (n = 5).

Appendix 2

Appendix 2 Table 1: Impact of genotype and water treatment on nodal root angle, crown root angle, total root length, root surface area, shoot dry weight, root dry weight and shoot water content. Data analysed by using the linear mixed-effects models procedure in SPSS with genotype and treatment as fixed factors and batch number as random factor. Degrees of freedom (df), *F* values and *P* values are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

	Nodal root angle			Crown root angle			Total root length			Root surface area		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	14.8	***	13	10.0	***	13	17.5	***	13	14.7	***
Treatment	2	435.2	***	2	363.7	***	2	24.6	***	2	27.9	***
Genotype × treatment	26	6.6	***	26	3.3	***	26	4.7	***	26	4.4	***

	Shoot dry weight			Root dry weight			Shoot water content		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	10.1	***	13	10.6	***	13	2.5	*
Treatment	2	269.1	***	2	52.2	***	2	1016.7	***
Genotype × treatment	26	3.8	***	26	5.6	***	26	3.9	***

Appendix 2 Table 2: Impact of genotype and water treatment on relative shoot water content, relative nodal root angle, relative crown root angle, relative total root length, relative root surface area and relative shoot dry weight (in every batch of experiment, the mean of well-watered treatment is set as 100%). Data analysed by using the linear mixed-effects models procedure in SPSS with genotype and treatment as fixed factors and batch number as random factor. Degrees of freedom (df), *F* values and *P* values are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

	Relative shoot water content			Relative nodal root angle			Relative crown root angle			Relative total root length		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	5.2	**	13	7.4	***	13	2.6	*	13	2.8	*
Treatment	2	1016.6	***	2	409.3	***	2	360.8	***	2	21.8	***
Genotype × treatment	26	3.6	***	26	5.2	***	26	3.3	***	26	3.3	***

	Relative root surface area			Relative root dry weight			Relative shoot dry weight		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	3.4	*	13	4.7	**	13	1.3	0.33
Treatment	2	23.5	***	2	50.8	***	2	269.0	***
Genotype × treatment	26	3.4	***	26	5.8	***	26	1.4	0.08

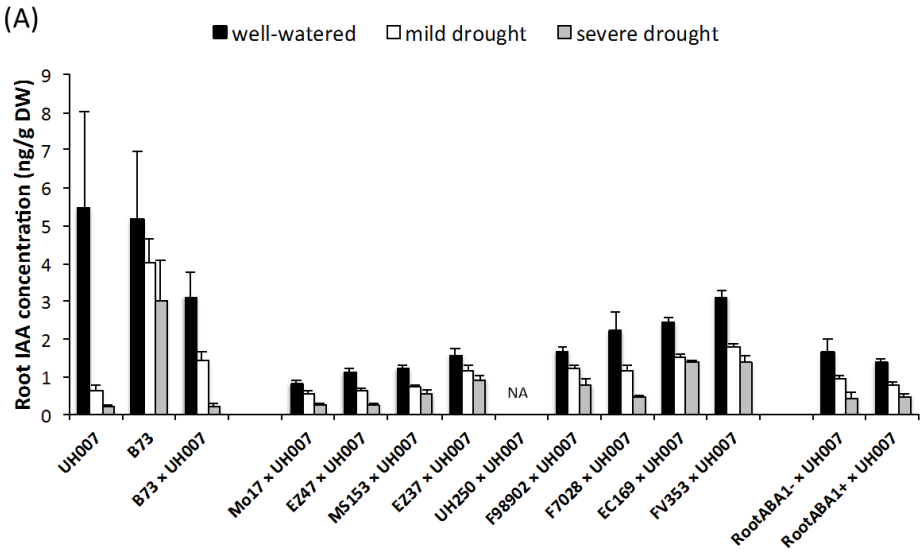
Appendix 2 Table 3: Impact of genotype and water treatment on hormone levels (ABA, ethylene and *t*Z) of leaf and root tissues. Data analysed by using the linear mixed-effects models procedure in SPSS with genotype and treatment as fixed factors and batch number as random factor. Degrees of freedom (df), *F* values and *P* values are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

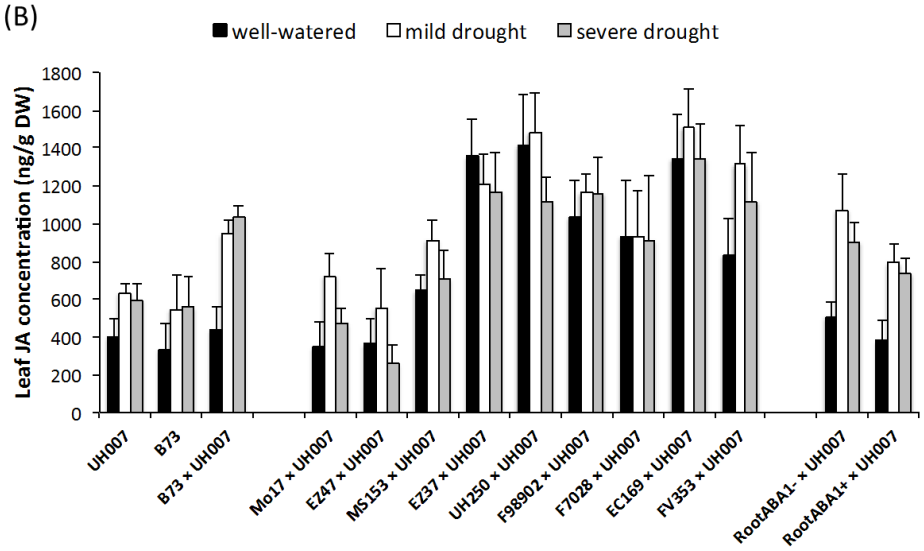
	Leaf ABA concentration			Root ABA concentration			Leaf ethylene release rate		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	2.5	*	13	3.1	*	13	3.2	*
Treatment	2	134.6	***	2	101.6	***	2	67.0	***
Genotype × treatment	26	1.6	*	26	1.6	*	26	4.9	***

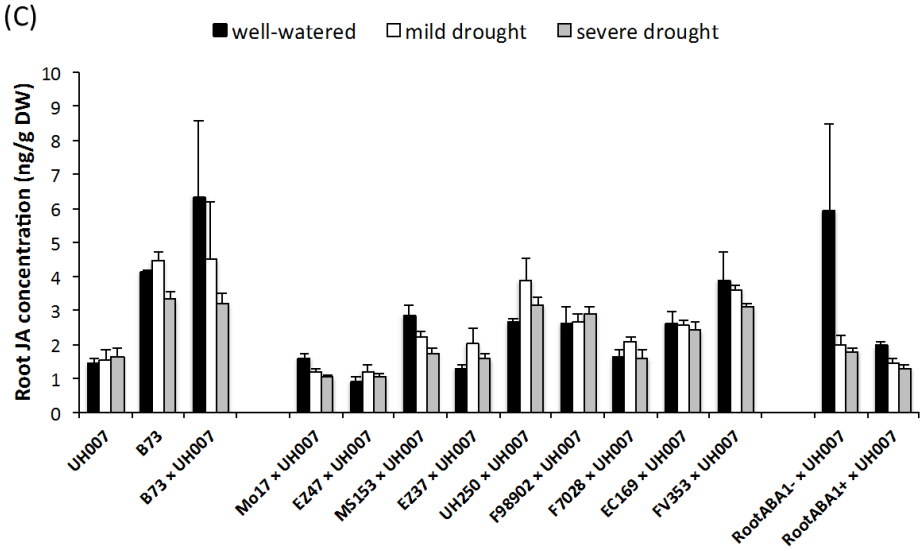
	Root ethylene release rate			Leaf *t*Z concentration			Root *t*Z concentration		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Genotype	13	2.4	0.06	13	32.8	***	13	108.1	***
Treatment	2	309.8	***	2	23.1	***	2	421.1	***
Genotype × treatment	26	2.7	***	26	12.7	***	26	20.8	***

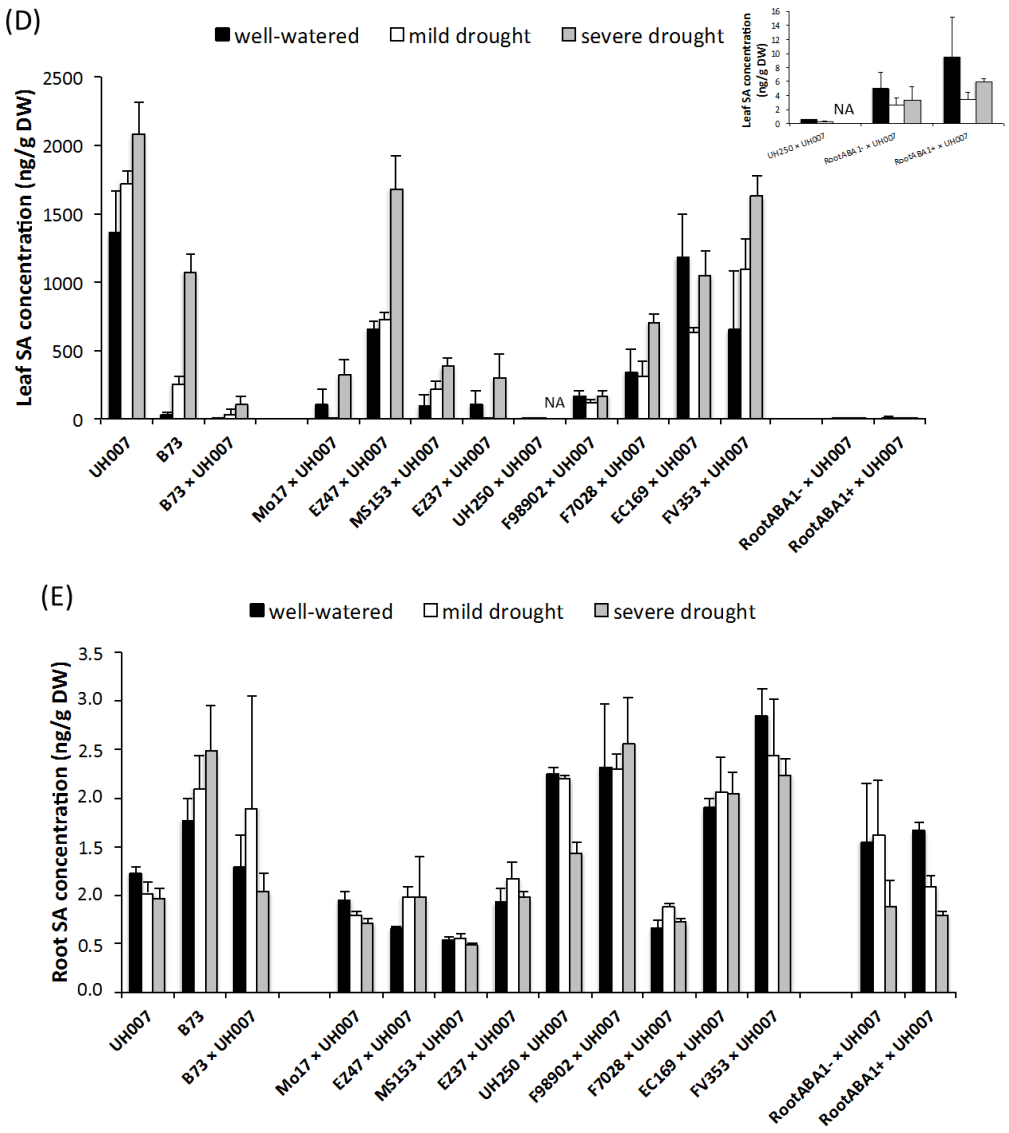
Appendix 2 Table 4: Soil water contents (mean ± standard error) in three soil layers for all genotypes (data from two batches were combined for each genotype).

Genotypes	0–7 cm			7–14 cm			14–21 cm		
	well-watered	mild drought	severe drought	well-watered	mild drought	severe drought	well-watered	mild drought	severe drought
UH007	34.3 % ± 0.4 %	19.2 % ± 0.5 %	14.6 % ± 0.2 %	41.3 % ± 0.6 %	30.5 % ± 0.5 %	24.8 % ± 0.5 %	53.4 % ± 1.2 %	33.6 % ± 0.2 %	27.9 % ± 0.6 %
B73	34.8 % ± 0.4 %	19.4 % ± 0.3 %	15.5 % ± 0.7 %	40.3 % ± 0.8 %	29.5 % ± 0.6 %	23.8 % ± 0.2 %	53.5 % ± 2.1 %	32.5 % ± 0.4 %	27.0 % ± 0.5 %
B73 × UH007	36.2 % ± 1.0 %	20.2 % ± 0.7 %	13.9 % ± 0.4 %	41.1 % ± 0.5 %	30.0 % ± 0.8 %	24.5 % ± 0.6 %	54.9 % ± 0.3 %	31.9 % ± 0.6 %	27.9 % ± 0.5 %
Mo17 × UH007	34.1 % ± 0.5 %	19.1 % ± 0.4 %	13.1 % ± 0.4 %	40.7 % ± 0.5 %	31.0 % ± 0.4 %	24.3 % ± 0.4 %	52.7 % ± 0.4 %	34.0 % ± 0.5 %	28.9 % ± 0.4 %
EZ47 × UH007	36.2 % ± 1.3 %	19.0 % ± 0.7 %	13.7 % ± 0.4 %	40.7 % ± 0.7 %	29.6 % ± 0.3 %	24.4 % ± 0.4 %	52.7 % ± 0.4 %	33.8 % ± 0.4 %	28.8 % ± 0.2 %
MS153 × UH007	35.5 % ± 1.5 %	18.8 % ± 1.0 %	15.1 % ± 1.0 %	41.8 % ± 1.4 %	29.8 % ± 0.9 %	25.2 % ± 0.8 %	53.1 % ± 1.6 %	33.6 % ± 0.5 %	28.9 % ± 1.2 %
EZ37 × UH007	36.7 % ± 0.8 %	18.1 % ± 0.3 %	15.0 % ± 0.4 %	40.9 % ± 0.2 %	30.2 % ± 0.6 %	24.9 % ± 0.2 %	53.3 % ± 0.7 %	35.3 % ± 0.4 %	28.7 % ± 0.7 %
UH250 × UH007	39.1 % ± 0.9 %	19.4 % ± 0.4 %	15.2 % ± 0.8 %	41.9 % ± 0.7 %	29.7 % ± 0.5 %	26.7 % ± 0.8 %	53.5 % ± 0.7 %	33.0 % ± 0.8 %	29.6 % ± 0.6 %
F98902 × UH007	39.8 % ± 1.4 %	20.1 % ± 0.2 %	16.0 % ± 0.5 %	44.7 % ± 1.0 %	32.8 % ± 0.5 %	27.9 % ± 0.9 %	54.7 % ± 1.2 %	36.6 % ± 1.0 %	30.5 % ± 0.6 %
F7028 × UH007	37.0 % ± 1.1 %	18.4 % ± 0.6 %	13.7 % ± 0.4 %	39.8 % ± 0.6 %	29.7 % ± 0.3 %	24.9 % ± 0.4 %	51.9 % ± 1.1 %	34.4 % ± 0.4 %	28.4 % ± 0.2 %
EC169 × UH007	39.7 % ± 0.6 %	19.5 % ± 1.2 %	15.0 % ± 0.9 %	42.1 % ± 0.6 %	29.8 % ± 0.9 %	25.5 % ± 0.9 %	55.0 % ± 1.3 %	36.6 % ± 1.3 %	30.5 % ± 1.1 %
FV353 × UH007	38.1 % ± 1.2 %	18.3 % ± 0.9 %	14.3 % ± 0.3 %	42.1 % ± 0.6 %	29.8 % ± 0.9 %	25.5 % ± 0.9 %	52.7 % ± 1.7 %	32.8 % ± 1.4 %	27.6 % ± 0.7 %
RootABA1- × UH007	33.8 % ± 0.4 %	18.3 % ± 0.5 %	13.1 % ± 0.6 %	39.8 % ± 0.2 %	28.7 % ± 0.3 %	23.3 % ± 0.3 %	53.4 % ± 0.9 %	30.9 % ± 0.2 %	26.8 % ± 0.7 %
RootABA1+ × UH007	33.9 % ± 0.7 %	17.8 % ± 0.8 %	14.3 % ± 0.8 %	40.3 % ± 0.4 %	28.5 % ± 0.7 %	24.2 % ± 0.6 %	52.8 % ± 0.9 %	31.2 % ± 1.7 %	26.8 % ± 0.7 %


(A)
well-watered
mild drought
severe drought
Root IAA concentration (ng/g DW)
0
1
2
3
4
5
6
7
8
9
NA
UH007
B73
B73 × UH007
Mo17 × UH007
EZ47 × UH007
MS153 × UH007
EZ37 × UH007
UH250 × UH007
F98902 × UH007
F7028 × UH007
EC169 × UH007
FV353 × UH007
RootABA1- × UH007
RootABA1+ × UH007


(B)
well-watered
mild drought
severe drought
Leaf JA concentration (ng/g DW)
0
200
400
600
800
1000
1200
1400
1600
1800
UH007
B73
B73 × UH007
Mo17 × UH007
EZ47 × UH007
MS153 × UH007
EZ37 × UH007
UH250 × UH007
F98902 × UH007
F7028 × UH007
EC169 × UH007
FV353 × UH007
RootABA1- × UH007
RootABA1+ × UH007


(C)
well-watered
mild drought
severe drought
Root JA concentration (ng/g DW)
0
1
2
3
4
5
6
7
8
9
10
UH007
B73
B73 × UH007
Mo17 × UH007
EZ47 × UH007
MS153 × UH007
EZ37 × UH007
UH250 × UH007
F98902 × UH007
F7028 × UH007
EC169 × UH007
FV353 × UH007
RootABA1- × UH007
RootABA1+ × UH007



Appendix 2 Figure 1: Genetic variation in hormone contents from maize leaf and root tissues and their changes under different drought treatments. Root (A) IAA concentration; (C) JA concentration; (E) SA concentration. Leaf (B) JA concentration; (D) SA concentration. The samples are the same as those used for hormone profile analysis in Figure 3.7. Data presented here is the combined result from two batches of experiments with the same maize genotype. Columns and bars are means ± standard error.

Appendix 3

Appendix 3 Table 1: Impact of genotype (wild-type, *etr1-1*, *ein2-1* and *ein3-1*) and ABA treatment on primary root elongation rate. (A) Absolute values of primary root elongation rate. (B) Relative primary root elongation rate (in each genotype, the mean root elongation rate of plants without ABA treatment is set as 1). Data analysed by using two-way ANOVA with genotype and treatment as main factors. Degrees of freedom (df), sums of squares (SS), *F* values and *P* values from ANOVA are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

	Primary root elongation rate							
	0–24 h				**0–4 d**			
	SS	**df**	***F* value**	***P* value**	**SS**	**df**	***F* value**	***P* value**
A								
Absolute values of primary root elongation rate								
Genotype	3154476.8	3	628.6	***	1652142.1	3	409.5	***
Treatment	1485213.6	6	148.0	***	3024066.2	6	374.7	***
Genotype × treatment	338018.3	18	11.2	***	181012.9	18	7.5	***
Residuals	1264605.6	756			956263.5	711		
B								
Relative primary root elongation rate (the root elongation rate of wild-type without ABA treatment is set as 1)								
Genotype	7.7	3	94.5	***	0.9	3	15.4	***
Treatment	27.2	6	167.6	***	43.8	6	382.7	***
Genotype × treatment	7.2	18	14.7	***	4.9	18	14.3	***
Residuals	20.4	756			13.6	711		

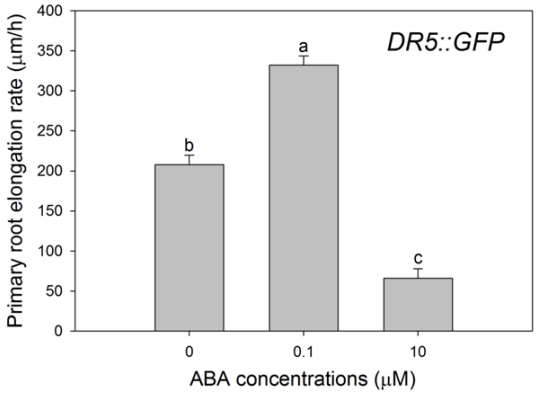
Appendix 3 Table 2: Pairwise comparisons of relative root elongation rates between four genotypes under seven ABA treatments (in each genotype, the mean root elongation rate of plants without ABA treatment is set as 1). Mean differences between genotypes and *P* values are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

			0–24 h		0–4 d	
ABA treatment (μM)	**Genotype (a)**	**Genotype (b)**	**MD (a – b)**	***P* value**	**MD (a – b)**	***P* value**
0	wild-type	*etr1-1*	0.00	1.00	0.00	1.00
		ein2-1	0.00	1.00	0.00	1.00
		ein3-1	0.00	1.00	0.00	1.00
	etr1-1	wild-type	0.00	1.00	0.00	1.00
		ein2-1	0.00	1.00	0.00	1.00
		ein3-1	0.00	1.00	0.00	1.00
	ein2-1	wild-type	0.00	1.00	0.00	1.00
		etr1-1	0.00	1.00	0.00	1.00
		ein3-1	0.00	1.00	0.00	1.00
	ein3-1	wild-type	0.00	1.00	0.00	1.00
		etr1-1	0.00	1.00	0.00	1.00
		ein2-1	0.00	1.00	0.00	1.00
0.1	wild-type	*etr1-1*	0.02	0.61	0.10	**
		ein2-1	-0.05	0.29	0.17	***
		ein3-1	-0.13	**	-0.09	*
	etr1-1	wild-type	-0.02	0.61	-0.10	**
		ein2-1	-0.07	0.12	0.07	*
		ein3-1	-0.16	***	-0.19	***
	ein2-1	wild-type	0.05	0.29	-0.17	***
		etr1-1	0.07	0.12	-0.07	*
		ein3-1	-0.09	*	-0.27	***
	ein3-1	wild-type	0.13	**	0.09	*
		etr1-1	0.16	***	0.19	***
		ein2-1	0.09	*	0.27	***
0.2	wild-type	*etr1-1*	0.01	0.78	0.12	**
		ein2-1	-0.03	0.45	0.18	***
		ein3-1	-0.11	*	-0.04	0.26
	etr1-1	wild-type	-0.01	0.78	-0.12	**
		ein2-1	-0.05	0.30	0.06	0.09
		ein3-1	-0.12	**	-0.16	***
	ein2-1	wild-type	0.03	0.45	-0.18	***
		etr1-1	0.05	0.30	-0.06	0.09
		ein3-1	-0.07	0.09	-0.22	***
	ein3-1	wild-type	0.11	*	0.04	
		etr1-1	0.12	**	0.16	***
		ein2-1	0.07	0.09	0.22	***
1	wild-type	*etr1-1*	-0.10	*	0.07	0.08
		ein2-1	-0.28	***	-0.04	0.34
		ein3-1	-0.15	***	-0.13	***
	etr1-1	wild-type	0.10	*	-0.07	0.08
		ein2-1	-0.18	***	-0.10	**
		ein3-1	-0.05	0.30	-0.19	***

			0–24 h		0–4 d	
ABA treatment (μM)	**Genotype (a)**	**Genotype (b)**	**MD (a – b)**	***P* value**	**MD (a – b)**	***P* value**
1	*ein2-1*	wild-type	0.28	***	0.04	0.34
		etr1-1	0.18	***	0.10	**
		ein3-1	0.14	**	-0.09	*
	ein3-1	wild-type	0.15	***	0.13	***
		etr1-1	0.05	0.30	0.19	***
		ein2-1	-0.14	**	0.09	*
5	wild-type	*etr1-1*	-0.22	***	-0.16	***
		ein2-1	-0.50	***	-0.24	***
		ein3-1	-0.15	***	-0.14	***
	etr1-1	wild-type	0.22	***	0.16	***
		ein2-1	-0.28	***	-0.07	0.07
		ein3-1	0.07	0.10	0.02	0.59
	ein2-1	wild-type	0.50	***	0.24	***
		etr1-1	0.28	***	0.07	0.07
		ein3-1	0.35	***	0.10	*
	ein3-1	wild-type	0.15	***	0.14	***
		etr1-1	-0.07	0.10	-0.02	0.59
		ein2-1	-0.35	***	-0.10	**
10	wild-type	*etr1-1*	-0.29	***	-0.21	***
		ein2-1	-0.54	***	-0.26	***
		ein3-1	-0.13	**	-0.10	**
	etr1-1	wild-type	0.29	***	0.21	***
		ein2-1	-0.25	***	-0.05	0.23
		ein3-1	0.16	***	0.11	**
	ein2-1	wild-type	0.54	***	0.26	***
		etr1-1	0.25	***	0.05	0.23
		ein3-1	0.41	***	0.16	***
	ein3-1	wild-type	0.13	**	0.10	**
		etr1-1	-0.16	***	-0.11	**
		ein2-1	-0.41	***	-0.16	***
30	wild-type	*etr1-1*	-0.34	***	-0.28	***
		ein2-1	-0.54	***	-0.33	***
		ein3-1	-0.13	**	-0.15	***
	etr1-1	wild-type	0.34	***	0.28	***
		ein2-1	-0.20	***	-0.05	0.23
		ein3-1	0.21	***	0.13	***
	ein2-1	wild-type	0.54	***	0.33	***
		etr1-1	0.20	***	0.05	0.23
		ein3-1	0.42	***	0.18	***
	ein3-1	wild-type	0.13	**	0.15	***
		etr1-1	-0.21	***	-0.13	***
		ein2-1	-0.42	***	-0.18	***

Appendix 3 Table 3: Impact of genotype and ABA treatment on primary root elongation rate. Data analysed by using two-way ANOVA with genotype and treatment as main factors. Degrees of freedom (df), sums of squares (SS), *F* values and *P* values from ANOVA are presented. Significance: *, 0.05; **, 0.001; ***, <0.0001.

	0–24 h				0–4 d			
	SS	df	*F* value	*P* value	SS	df	*F* value	*P* value
A								
Genotypes are wild-type, *pin2/eir1-1*, *aux1-T* and *iaa7/axr2-1*; treatments are 0, 0.1 and 10 µM ABA								
Genotype	36369.6	3	9.1	***	43767.2	3	11.3	***
Treatment	100398.8	2	37.7	***	234130.6	2	90.3	***
Genotype × treatment	104481.0	6	13.1	***	60270.5	6	7.7	***
Residuals	175928.4	132			143934.8	111		
B								
Genotypes are wild-type, *pin4-3*, *pin7-2* and *tir1-1*; treatments are 0, 0.1 and 10 µM ABA								
Genotype	146644.6	3	63.3	***	53389.0	3	15.8	***
Treatment	428337.3	2	277.3	***	637138.3	2	282.0	***
Genotype × treatment	4695.1	6	1.0	0.42	32301.4	6	4.8	***
Residuals	101945.5	132			117485.4	104		
C								
Genotypes are wild-type, *aux1-7*, *pin3-4* and *pin3-5*; treatments are 0, 0.1, 0.2, 1, 10 and 50 µM ABA								
Genotype	79017.2	3	18.1	***	20080.4	3	3.9	*
Treatment	738930.0	5	101.7	***	1320216.0	5	153.1	***
Genotype × treatment	79765.9	15	3.7	***	38902.4	15	1.5	0.11
Residuals	244173.2	168			248275.3	144		



Appendix 3 Figure 1: Primary root elongation rate of *DR5::GFP* line during the 3-day treated with ABA. Seedling numbers: n = 5–6. The values are means, and the vertical bars represent standard errors of the means. Different letters indicate significant differences at $P < 0.05$.