ABSCISIC ACID: INTERACTIONS WITH AUXIN IN THE REGULATION OF ROOT GROWTH UNDER WATER DEFICIT CONDITIONS

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

ABSCISIC ACID: INTERACTIONS WITH AUXIN IN THE REGULATION OF ROOT GROWTH UNDER WATER DEFICIT CONDITIONS

Presented by Danté O. Smith

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
1-MCP	1-methylcyclopropane
ABA	Abscisic acid
aba2	*abscisic acid 2* mutant (*Arabidopsis*)
abi3	*abscisic acid insensitive 3* mutant (*Arabidopsis*)
ABC	ATP-binding cassette proteins
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC-oxidase
ACS	ACC-synthase
AIBA	2-aminoisobutyric acid
$AgNO_3$	Silver nitrate
AOA	(Aminooxy) acetic acid
AtMDR1	*Arabidopsis thaliana multidrug resistance 1* mutant
AtPGP1	*Arabidopsis thaliana P-glycoprotein 1* mutant
AUX1	Auxin 1 influx carrier

AVG	Aminoethoxyvinyl glycine
C_2H_4	Ethylene
CO^{2+}	Cobalt
cpm	Counts per minute
dpm	Disintegrations per minute
etr1	Ethylene-insensitive mutant (*Arabidopsis*)
FLU	Fluridone
GACC	γ-glutamyl-ACC
GC-MS	Gas chromatography-mass spectrometry
GGT	γ-glutamyl-transferase
H_2DCFDA	5-(and 6)-carboxy-2',7'-dichlorodyhidrofluorescein diacetate
H_2O_2	Hydrogen peroxide
IAA	Indole-3-acetic acid
IPyA	Indole-3-pyruvic acid
LC-MS	Liquid chromatography-mass spectrometry
lrd2	*lateral root development 2 mutant* (Maize)

MACC	Malonyl-ACC
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance protein family
MPa	Megapascal
NCED	9-*cis*-epoxycarotenoid dioxygenase
NMT	N-malonyl-transferase
NPA	Naptalam/ N-1-Naphthylphthalamic acid
O_2^-	Superoxide
·OH	Hydroxyl radical
PA	1,10-phenanthroline
PCD	Programmed cell death
PEG	Polyethylene glycol
PIN	PIN-Formed auxin efflux carriers
PLP	Pyridoxal phosphate
RIA	Radioimmunoassay
ROS	Reactive oxygen species

SAM	S-adenosyl-L-methionine
STS	Silver thiosulfate
TAA	Tryptophan aminotransferase
TAA-1	Tryptophan aminotransferase-1
TIBA	2,3,5-Triiodobenzoic acid
Trp-D	Tryptophan-dependent
Trp-I	Tryptophan-independent
vp5	*viviparous 5* mutant (Maize)
vp14	*viviparous 14* mutant (Maize)
WS	Water-stressed
wt	wild-type
WW	Well-watered
XET	Xyloglucan endotransglycosylase
YUC	YUCCA
ZmACS6	ACC synthase mutant (Maize)
Ψ_w	Water potential

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ABSTRACT

Water stress has been shown to inhibit shoot growth more than root growth (Sharp *et al*., 1988). Abscisic acid (ABA) has been shown to be involved in this differential growth response in maize (*Zea mays* L.*)* seedlings (Saab *et al*., 1990; Sharp *et al*., 1994). ABA accumulates in the elongation zone of the primary root at low water potentials, and this response is required for root growth maintenance under water deficit conditions (Saab *et al*., 1990; Sharp *et al*., 1994). Previous studies have shown that under severe water deficit in the ABA-deficient mutant *viviparous 14* (*vp14*), plants produce high levels of endogenous ethylene (Spollen *et al*., 2000) and intracellular reactive oxygen species (ROS) in the root growth zone (Cho, 2006). Excess ethylene and ROS have been shown to have negative effects on plant growth and development. Therefore, it was hypothesized that these two factors were interconnected in this system. However, evidence presented in this study demonstrates that using a specific inhibitor of ethylene perception, 1-methylcyclopropane (1-MCP), neither ethylene nor ROS are the primary cause of root growth inhibition caused by ABA-deficiency, suggesting an alternate function of ABA in the regulation of root growth maintenance under water deficit conditions. The hypothesis was tested that an interaction of ABA with auxin could potentially play a key role in regulating maize root growth maintenance under water deficit conditions by using the

ABA-deficient mutant *vp14* to determine whether altered auxin levels are associated with primary root growth inhibition. The results show that auxin levels are decreased in the growth zone of ABA-deficient roots under water stress. To test whether the decrease in auxin is casually related to root growth inhibition, inhibitors of auxin transport and addition of auxin were administered during the growth of ABA-deficient roots at low water potentials. These treatments demonstrated that both inhibition of auxin transport and addition of auxin can completely restore endogenous auxin levels and root elongation in ABA-deficient water-stressed seedlings.

These findings indicate that there is an important interaction between ABA and auxin in the regulation of maize primary root growth under water stress conditions. Further analysis with this system will lead to a greater understanding of the primary mechanisms involved in the regulation of root growth by ABA under water deficit conditions.

CHAPTER 1

LITERATURE REVIEW

Drought is one of the most limiting factors affecting plant growth and development. Among environmental factors, drought is the leading cause of crop yield loss in the US and globally (Boyer, 1982). Under water deficit conditions, plants suffer from cellular dehydration and this is often accompanied by an increase in temperature (Henckel, 1964). Many land plants typically grow in unfavorable conditions and are termed "stressed" (Boyer, 1982). As a result of being stressed, plants have developed many adaptations in order to maintain the highest productivity possible. Gaining an understanding of these adaptive mechanisms is a vitally important goal to assist with breeding and biotechnological efforts to help bring plant productivity closer to the existing genetic potential (Boyer, 1982).

Drought adaptation and avoidance are complex traits. Currently, there are many studies being done at the molecular and whole-plant levels to understand the processes underlying plant responses to drought. Approaches include but are not limited to the investigation of plant hormones and secondary metabolites, and the utilization of altered gene expression patterns through transgenic manipulation (Chaves *et al*., 2003). However, the overall complexity and mechanisms of drought adaptation are still poorly understood and require additional research.

Root growth and water deficits

Root growth arises from new cell production and expansion in the apical meristem. The maize (*Zea mays* L.) root system forms a network of embryonic and postembryonic roots. For the purpose of this dissertation, the focus will be on the primary root, which is part of the embryonic system. The primary root is formed within the embryo and this region

becomes noticeable 10-15 days after pollination (Yamashita and Uneo, 1992). After germination, cell expansion in the primary root covers a 12-mm region beginning from the apex (Burstrom, 1953; Erickson and Sax, 1956). The primary root of maize has the ability to remain active throughout the entire life cycle of the plant (Vu and De Smet, 2016).

Water uptake is essential for plant growth and development and is necessary for cell expansion. Turgor pressure and cell wall extensibility are primary factors determining the rate of cell expansion. Cellular water uptake results from the absorption and synthesis of solutes inside the cell, which decreases the cell water potential (ψ_w) below that of the apoplast, thereby driving water uptake. Turgor pressure is generated because of the constraining cell wall, and is required to provide the physical force to drive cell wall expansion. Under drought conditions, plant tissues can experience a decrease in ψ_w and water content, resulting in cellular damage and loss of turgor that adversely affect growth.

When water availability is limited, roots have adaptive mechanisms in order to sustain growth, including osmotic adjustment (accumulation of solutes in the cells; Sharp and Davies, 1979) and changes in cell wall extension properties (Wu *et al*., 1996). The maize primary root has the ability to continue elongation at very low ψ_w compared to the shoot (Fig. 1), which helps to maintain adequate plant water status during seedling establishment (Sharp *et al*., 2004). The physiological mechanisms underlying maintenance of primary root growth at low ψ_w are important to understand, and have been reviewed by Sharp and co-workers (Sharp, 2002; Ober and Sharp, 2007; Ober and Sharp, 2013). In the maize primary root growing under low soil ψ_w conditions (-1.6 MPa), cell elongation is completely maintained in the apical 1-3 mm region. However, in the 4-7 mm region, which exhibits maximum elongation in well-watered roots, cell elongation is progressively

inhibited in water-stressed roots, resulting in a shortened growth zone. These responses involve complex and differential changes in the cell wall extension properties in the apical and basal regions of the root growth zone (Wu *et al*., 1996).

ABA and root growth

Accumulation of the phytohormone abscisic acid (ABA) in water-stressed plant tissues was discovered almost 50 years ago (Wright and Hiron, 1969). ABA synthesis in plants is linked to cellular dehydration (Wright, 1977), and has been shown in root and shoot tissues. Cellular dehydration caused by soil water shortages has been shown to dramatically increase ABA concentrations in plant tissues, correlating with stomatal closure. The role of ABA in regulating stomatal closure has been studied in detail and is well understood (Assmann, 1994; Hamilton *et al*., 2000; Hetherington, 2001; Schroeder *et al*., 2001; Wilkinson *et al*., 2002; Zhang *et al*., 2002; Outlaw, 2003).

In contrast, the involvement and role of ABA in growth regulation of water-stressed plants is less well defined. Traditionally, ABA has been viewed as a growth inhibitor in water-stressed plants. This view arose because when the hormone was applied to well-watered plant tissues to simulate the accumulation of ABA under water stress there was often a positive correlation between application of ABA and decreases in cell division, inhibition of leaf initiation, and decreases in cell expansion (Trewavas and Jones, 1991). Similarly, in well-watered maize seedlings, application of ABA to well-watered plants inhibits primary root growth (Sharp *et al*., 1994). However, a potential issue in interpreting these results is the assumption that both water-stressed and non-stressed plants behave similarly in response to increased concentrations of ABA (Takahashi, 1973; Reid, 1990).

An alternative approach that avoids this concern is the use of chemical inhibitors of ABA biosynthesis or mutants impaired in ABA biosynthesis to reduce endogenous ABA accumulation in water-stressed plants. This approach allows direct investigation of the role of ABA in plant responses to water deficit.

A concern with utilizing inhibitors of ABA synthesis or ABA-deficient mutants to study effects of ABA on plant growth is that, because of the role of ABA in stomatal regulation, a decrease in endogenous ABA levels can lead to impaired water status within the plant (Quarrie, 1987). As a result, ABA-deficiency often causes a "wilty" phenotype even under well-watered conditions because of the inability to close stomata (Tal and Nevo, 1973; Jones *et al*., 1987). This effect could result in growth inhibition independently of direct effects of ABA on cell division and expansion.

Previous studies by Sharp and co-workers have utilized both inhibitors of ABA biosynthesis and ABA-deficient mutants to address the role of ABA in root and shoot growth in maize seedlings growing under water deficit conditions (reviewed in Sharp, 2002). To combat the problem of increased transpirational water loss between ABA-deficient and normal plants, the seedlings were grown at near-saturation relative humidity in darkness (Sharp *et al*., 1988). When growth measurements were taken, plants were exposed to green light, because in maize, white light inhibits root elongation by affecting both cell division activity and the ability of cells to elongate (Wilkins *et al*., 1974). In contrast with the traditional view that ABA is a plant growth inhibitor, the use of this system determined that ABA accumulation plays a dual role in determining the growth response of seedlings to low ψ_w. ABA-deficiency, caused either by genetic or chemical means, resulted in severe inhibition of primary root elongation and promotion of shoot

growth, indicating that the normal increase in ABA levels in water-stressed seedlings is in fact required for maintenance of primary root growth, but also causes inhibition of shoot growth (Saab *et al*., 1990; Sharp *et al*., 1994). In initial studies, endogenous ABA levels were modified in two ways. First, by chemical inhibition of ABA biosynthesis using fluridone (FLU), and second, by genetic manipulation using the ABA-deficient mutant of maize *viviparous 5* (*vp5*). Fluridone inhibits the conversion of phytoene to phytofluene in the carotenoid biosynthetic pathway, thereby decreasing ABA synthesis from carotenoid precursors; the *vp5* mutant is blocked at the same step (Fig. 2). Results with the two methods were very comparable. ABA application to both FLU-treated and *vp5* seedlings to restore normal ABA levels in the root growth zone resulted in restoration of root elongation, providing compelling evidence that ABA accumulation is required for root growth maintenance in maize seedlings growing at low ψ_w (Sharp *et al*., 1994).

However, since in both FLU-treated and *vp5* seedlings ABA biosynthesis is inhibited via impairment of carotenoid biosynthesis, it remained possible that the physiological basis behind impaired root growth was not solely due to ABA-deficiency but also involved an effect on carotenoid metabolism. To address this possibility, Cho (2006) re-examined the effects of ABA deficiency on root growth at low ψ_w using an alternate ABA-deficient mutant of maize, *viviparous 14* (*vp14*), which was identified in a Robertson's Mutator strain as a viviparous mutant with a weak penetrance (Tan *et al*., 1997). *vp14*-2274 and *vp14*-3250 mutant lines were outcrossed to Wisconsin 22 (W22) and maintained by self-pollination of heterozygous plants (Tan *et al*., 1997). *vp14* was shown to be impaired in one of the 9-cis-epoxydioxygenase (NCED) genes; NCED catalyzes the oxidative cleavage of epoxy-carotenoids to xanthoxin, which represents the first committed

step in ABA biosynthesis (Fig. 2). Accordingly, *vp14* is a useful resource, because inhibition of ABA biosynthesis takes place downstream of the carotenoid pathway, thus not interfering with carotenoid metabolism. Similar to the previous studies with FLU-treated and *vp5* seedlings, the results showed that primary root growth was inhibited in water-stressed *vp14* and could be restored with exogenous ABA application, confirming that ABA accumulation is required for maize primary root growth maintenance at low ψ_w (Cho, 2006).

Interactions of ABA and ethylene

The studies described above were important to demonstrate that ABA accumulation is required for root growth maintenance at low ψ_w. However, hormonal regulation of plant development is typically more complex than the isolated action of a single hormone. Different hormones can regulate the same developmental processes, and interactions within hormone signaling pathways are commonly involved in regulating plant development and responses to environmental stimuli (Gazzarrini and McCourt, 2001).

In particular, it has become increasingly recognized that many interactions take place between ABA and ethylene (Sharp, 2002). For example, ABA can trigger ethylene biosynthesis, and thereby has been shown to play a crucial role in tomato fruit maturation and senescence (Chernys and Zeevaart, 2000). However, in the developmental process of fruit ripening, ethylene also induces NCED gene expression and ABA accumulation, which results in post-ripeness (Zhang *et al*., 2009). Genetic analysis also suggests that ABA and ethylene closely interact in the modulation of carbon status during early seedling growth and development. For example, in *Arabidopsis* seeds, ethylene-insensitive mutants showed

increased ABA responsiveness, leading to the conclusion that ethylene is a negative regulator of ABA signaling in plants (Gazzarrini and McCourt, 2001).

In the case of maize primary root growth at low ψ_w, ABA and ethylene have been shown to have an antagonistic relationship. In the above-described experiments in which ABA levels in water-stressed roots were decreased by treatment with FLU or in the *vp5* and *vp14* mutants, it was observed that as root tip ABA content decreased, ethylene evolution rates increased in correlation with root growth inhibition (Fig. 3; Spollen *et al*., 2000; Sharp, 2002). ABA-deficiency under water stress was also associated with root tip swelling (Fig. 4; Sharp *et al*., 1993), which is a typical symptom of excess ethylene production (Moss 1988). When root tip ABA levels were restored with exogenous ABA, ethylene evolution rates decreased to that of the control plants.

To determine whether the increase in ethylene evolution was the cause of inhibition of primary root growth in ABA-deficient seedlings under water stress, FLU-treated seedlings were also treated with inhibitors of ethylene synthesis (aminooxyacetic acid [AOA] and aninoethoxyvinylglycine [AVG]) and ethylene action (silver thiosulfate [STS]) (Spollen *et al*., 2000). All three of the ethylene inhibitor treatments resulted in almost complete restoration of root elongation without altering root tip ABA contents, demonstrating that the plants remained ABA-deficient and indicating that root growth recovery was attributable to the inhibition of ethylene synthesis or action. These results indicated that an important role of ABA accumulation in root growth maintenance at low ψ_w is to prevent excess ethylene production (Spollen *et al*., 2000).

To confirm this result genetically, similar experiments using ethylene synthesis inhibitors were attempted with the *vp5* (Spollen *et al*., 2000) and *vp14* (Cho, 2006) mutants,

but in these cases only partial recovery of root growth was obtained. A possible reason for the incomplete recovery of root growth in the mutant experiments is that the ethylene inhibitors were supplied only as a pre-treatment during germination (as was FLU in the non-mutant experiments). This procedure was followed because uptake of compounds from the dry vermiculite in which the seedlings were grown is very limited. Thus, the effectiveness of the inhibitor treatments may have diminished over time after transplanting to the low ψ_w condition, whereas the new root tissue produced during the mutant experiments was consistently ABA-deficient due to the genetic impairment in ABA biosynthesis. For this reason, a hydroponic culture system was utilized to allow continuous treatments with inhibitors of ethylene synthesis in studies of ABA-ethylene interactions using the *vp14* mutant system (Smith, 2011; Chapter 1-Supplement). These studies demonstrated that using chemicals inhibitors of ethylene synthesis in the hydroponic culture system, the increase in ethylene production was fully prevented and primary root growth was restored in ABA-deficient roots growing under low ψ_w conditions.

Interactions of ABA and reactive oxygen species

Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), are highly reactive by-products of the normal metabolism of oxygen. ROS can have potentially damaging effects on cellular components when present in excess amounts, but can also play vital roles in cellular signaling and in mediating plant responses to environmental stresses (Torres *et al*., 2005; Miller *et al*., 2009). ABA can interact with both the production and signaling functions of ROS. Examples of the interaction between ABA and ROS production include a study by Hu *et*

al. (2006), who determined that ABA is a key inducer of H_2O_2 production in leaves of maize plants under water stress, and several studies showing that ABA-induced H_2O_2 production is involved in stomatal closure (Pei *et al*., 2000; Zhang *et al*., 2001; Kwak *et al*., 2003; Desikan *et al*., 2004). Research has also shown that ABA signal transduction interacts with ROS metabolism both upstream and downstream of ROS production (Kwak *et al*., 2006).

Studies of maize seedlings under water stress have shown that ABA-deficiency not only leads to an increase in ethylene production, as detailed above, but also causes an increase in cytosolic ROS levels in the primary root growth zone (Cho, 2006). This was demonstrated using the membrane-permeable dye 5-(and-6)-carboxy-2´,7´-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) to stain for intracellular ROS levels in the *vp14* mutant and wild-type roots. Additional experiments demonstrated that the increase in ROS levels preceded, and presumably was causally related to, loss in plasmamembrane integrity (assessed by propidium iodide staining of cell nuclei), ultimately leading to cell death. Restoration of root tip ABA levels by exogenous application prevented the high ROS and cell death phenotypes, confirming that ABA accumulation functions to prevent excess ROS production in water-stressed roots (Cho, 2006). The role of ROS in programmed cell death (PCD) has become an important topic in recent years (Breusegum *et al*., 2008). It has been speculated that ROS-dependent PCD is not only caused by "indiscriminative oxidation", but also through interaction with other signaling pathways and plant growth regulators (Breusegum *et al*., 2008).

Interactions of ethylene and reactive oxygen species

Both ethylene and ROS can have positive and negative effects on plant growth and

development, and there is much evidence to support their interaction. For example, ethylene and ROS both play a positive role in lateral root nodule formation of the semiaquatic legume *Sesbania rostrata* (Haeze *et al*., 2003). Additionally, both ethylene and ROS regulate the formation of lysigenous aerenchyma in *Arabidopsis* (Muhlenbock *et al*., 2007). However, under stress conditions, both ethylene and ROS can increase to excessive levels, having negative consequences for plant growth and metabolism. For example, under osmotic stress, up-regulation of the ethylene biosynthesis enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) was shown to trigger an increase in intracellular ROS, resulting in cellular damage (Ke and Sun, 2004). In addition, ROS production has been shown to be tightly associated with ethylene production resulting from chilling stress (Ke *et al*., 2002, 2003).

As detailed above, in ABA-deficient primary roots at low ψ_w there are increases in both ethylene production and intracellular ROS levels, both of which are hypothesized to be involved in root growth inhibition. Studies by Smith (2011) addressed whether the increase in ethylene production causes, or is caused by, the increase in ROS levels in ABA-deficient roots. To investigate these interactions, the chemical inhibitors of ethylene synthesis AOA (Smith, 2011) and AVG (as described in Chapter 1 Supplement) were used to demonstrate that decreasing endogenous ethylene production prevented the increase in intracellular ROS and completely restored primary root growth in ABA-deficient roots. These findings indicated that the interaction of ABA and ethylene in water-stressed roots is upstream of the production of ROS and suggest that primary root growth inhibition in this system is due to excess ethylene and/or ROS (Smith, 2011).

Off-target effects of ethylene synthesis and action inhibitors

Recent studies discovered that the inhibitors of ethylene synthesis AOA and AVG can directly affect auxin biosynthesis by blocking the conversion of indole-3-pyruvic acid (IPyA) to indole-3-acetic acid (IAA) via inhibition of a pyridoxal phosphate-dependent (PLP) enzyme, tryptophan aminotransferase-1 (TAA-1), in the tryptophan-dependent portion of the auxin biosynthetic pathway. Studies in *Arabidopsis* showed that when comparing the effects of AOA and AVG, AVG had the strongest anti-auxin activity and reduced IAA levels in roots and shoots by approximately 60% after a 24 h treatment compared with control plants (Soeno *et al*., 2010). In the same experiment, ACC was added to AVG-treated plants and the results showed that IAA levels failed to increase, suggesting that AVG inhibits auxin biosynthesis independently of ethylene. The effects of AOA and AVG on auxin activity were repeated in other monocots and dicots, including wheat, rice and tomato, with similar results (Soeno *et al*., 2010). In addition to inhibitors of ethylene synthesis, the inhibitors of ethylene action silver thiosulfate (STS) and silver nitrate ($AgNO_3$) have been shown to increase auxin efflux independently of the effects of an ethylene response (Strader *et al*., 2009).

Since the chemical inhibitors of ethylene synthesis (AOA, AVG) and action (STS) used to establish conclusions in the Spollen *et al*. (2000) and Smith (2011) studies can simultaneously affect auxin biosynthesis or efflux, it is possible that the recovery of primary root growth under water-stressed conditions observed using these compounds could have been due to interactions with auxin rather than, or in addition to, ethylene and ROS. Therefore, it is critical to re-evaluate the casual interrelationships between ABA, ethylene, ROS and growth in ABA-deficient roots under low ψ_w conditions. Additionally,

it is necessary to investigate a possible interaction of ABA and auxin in the response of primary root growth to water stress in the same system.

Auxin biosynthesis

The importance of auxin in plant growth and development has been extensively reviewed (Berleth *et al*., 2000; Doerner, 2000; Hamann, 2001, Muday, 2001; Benfey, 2002). Auxins are organic compounds that promote or inhibit cell elongation at low concentrations. In addition to indole-3-actetic acid (IAA), there are other natural auxins found in plants as free acids and conjugated forms. In the majority of plant tissues, the conjugated forms of auxin are the most abundant. Auxin can be converted to ester conjugates (with sugars involving UDP-glucose transferases) or amide conjugates (with amino acids by IAA amino acid conjugate synthetases) (Bandurski *et al*., 1995; Slovin *et al*., 1999). IAA production can be in a tryptophan-dependent (Trp-D) or tryptophan-independent (Trp-I) manner. For the purpose of this dissertation, the focus will be on tryptophan-dependent IAA synthesis, because it has been established that the inhibitors of ethylene synthesis (AOA, AVG) used to establish conclusions in Spollen *et al*. (2000) and Smith (2011) can inhibit the enzyme tryptophan aminotransferase (TAA), a rate-limiting step of the IPyA pathway, which is the main IAA biosynthesis pathway in plants (Soneo *et al*., 2010; Mashiguchi *et al*., 2011) (Fig. 5).

The tryptophan-dependent portion of the auxin pathway was revealed by radiotracer labeling studies and biochemical assays. Studies from Bartel (1997) assisted in gaining a clear understanding that IAA is converted to tryptophan or a precursor of tryptophan. The most linear path to IAA synthesis first involves the compound tryptophan being converted to IPyA via the enzyme TAA. The enzyme YUCCA (YUC) then converts

IPyA to the most abundant and active auxin form, IAA. IAA catabolism involves the modification of the side chain or indole nucleus that brings about a loss in auxin activity. Modification of either the side chain or indole nucleus is the only irreversible step that regulates IAA levels.

As previously mentioned, it has been well documented that plant hormones often do not act singly, and the auxin metabolic response has been tied to various signals including light, temperature and other plant hormones including ethylene and ABA. With genetic mutant approaches, research involving auxin cross-talk with other signaling molecules will continue to gain additional attention (Sawrup *et al*., 2002).

Auxin transport and root growth

Understanding the transport of chemical signals and messengers in plants has been of great importance for many years. Movement of one particular chemical signal was discovered by the scientist Peter Boysen-Jensen in the 1900's (Boysen-Jensen, 1911; Boysen-Jensen, 1913). Boysen-Jensen demonstrated the flow of this chemical signal by removing the tip from the coleoptile of cereal seedlings and placing the tip on gelatin blocks. By removing the tip, the remaining coleoptile could not bend in response to light. By re-applying the tip and associated gelatin block back on cut coleoptiles, the phtototropic response of the plant was restored. The chemical signal in question was later identified as the naturally-occurring auxin, IAA. Regulated transport of auxin is based on polarity, an active process that moves the hormone in a cell-to-cell manner.

Studies in *Arabidopsis* have shown that auxin is transported towards the root tip in a cell-to-cell manner, and this transport was sufficient to create an auxin maxima and

gradient that can guide the direction of root growth (Grieneisen *et al*., 2007; Robert and Friml, 2009). Involved in these processes are the AUXIN 1 (AUX1) influx and PIN-FORMED (PIN) efflux family of carriers. PIN proteins in the root are aligned in an asymmetrical direction in the plasma membrane of protophloem cells that correlates with the direction of auxin flow (Gaelweiler *et al*., 1998; Mueller *et al*., 1998; Friml *et al*., 2002a; Friml *et al*., 2002b). Specifically, PIN 1 and PIN 4 control the efflux of auxin, thus maintaining the auxin gradient towards the root tip. Re-distribution of auxin takes place near the root cap, and controlling this function is the efflux carrier PIN 3 (Friml *et al*., 2002a). PIN 3 coupled with PIN 2 (located in the epidermal regions) translocates auxin in a basipetal fashion, where PIN 2 drives auxin movement back towards the root elongation zone away from the root-cap (Friml *et al*., 2002a; Bennett *et al*., 1996; Swarup *et al*., 2001; Chen *et al*., 2002; Parry *et al*., 2001). AUX1 proteins are also aligned asymmetrically in the plasma membrane opposite of PIN 1 (Swarup *et al*., 2001), suggesting that these two carriers facilitate the movement of auxin towards the root tip.

Another protein family termed the Multidrug resistance (MDR)-type ATP-binding cassette (ABC) proteins has also been proposed to be involved in the transport of auxin. These proteins, specifically *Arabidopsis thaliana* MDR1 (AtMDR1) and *Arabidopsis thaliana* P-glycoprotein1 (AtPGP1), were initially identified as anion channels. The availability of double mutants of these two proteins confirmed that there was a reduced rate of auxin transport (Noh *et al*., 2000). Additionally, these proteins bind the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA), making for a stronger correlation that these MDR and PGP proteins participate in the transport of auxin in plants (Murphy *et al*., 2002).

Auxin, root growth and water deficit

Auxin plays a vital role in root growth and development (Ribaut and Pilet, 1994; Fu and Harberd, 2003; Blilou *et al*., 2005), and the studies presented in this section will focus on the promotive effects of these interactions. Root growth starts with cell wall extension, and per the acid growth hypothesis, apoplastic protons are wall-loosening agents that cause cell extension due to proton efflux facilitated by the plasma membrane ATPase (Rayle and Cleland, 1992; Fan and Neumann, 2004; Staal *et al*., 2011). Auxin has been shown to play a role in proton secretion by actively regulating the ATPase. Additionally, proton secretion facilitated by the ATPase has been shown to play a key role in primary root elongation and root hair development (Santi and Schmidt, 2009).

Auxin has also been shown to have important interactions in plants growing under water stress conditions. In water-stressed tissues, several studies have demonstrated that there is an accumulation of auxin and increased expression of auxin-inducible genes and proteins in the elongation zone of the maize and soybean primary root (Ribaut and Pilet, 1994; Poroyko *et al*., 2007, Spollen *et al*., 2008; Yamaguchi *et al*., 2010), suggesting that auxin accumulation may be one of the many mechanisms involved in primary root growth adaptation to plants growing under low ψ_w conditions. Also, studies by Nerya *et al*. (2004) and Kruk *et al*. (2005) identified that flavonoids and isoflavonoids accumulate in the apical region of water-stressed roots. While the accumulation of these compounds is likely to be involved in protection against oxidative damage (Nerya *et al*., 2004; Kruk *et al*., 2005), it was also hypothesized that these flavonoids can influence auxin transport by inhibiting PIN proteins, which could result in an accumulation of auxin in neighboring tissues (Mathesius *et al*., 1998; Subramanian *et al*., 2006).

Interactions of ABA and auxin

There are a number of studies describing interactions between ABA, auxin and shoot/root growth. In the youngest (upper) portions of hypocotyls in etiolated bean seedlings, Horemans *et al*. (1986) showed a positive correlation between ABA and auxin levels and cell expansion. Studies by Zhao *et al*. (2014) showed in rice seedlings that ABA plays an essential role in regulating primary root growth by interacting with auxin and mitogen–activated protein kinase (MAPK) signaling pathways and cell-cycle machinery. Additionally, ABA has been shown to interact with auxin in the formation of lateral root primordia (De Smet, 2006). More recently, Thole *et al*. (2014) presented evidence that ABA regulates primary root elongation through the activities of auxin and ethylene in *Arabidopsis*. However, the number of studies describing interactions of ABA, auxin, root growth and water stress are surprisingly limited. Importantly, a recent study provided compelling evidence of an interaction of ABA with auxin in the regulation of growth in rice and *Arabidopsis* primary roots (Xu *et al*., 2012). It was shown that ABA accumulation can modulate auxin transport in the root tip, which enhances proton secretion for maintaining primary root growth under moderate water stress conditions. This suggests not only does ABA signaling result in increased auxin transport, but shows that an interaction with IAA can be an important component of the role of ABA in maintaining primary root growth under water-stressed conditions.

Additional examples of ABA-auxin interactions include a study in *Arabidopsis*, where it was shown that the *abscisic acid insensitive 3* (*ABI3*) gene is modulated by farnesylation and involved in auxin signaling and lateral root development (Brady *et al*., 2003). A study by Deak and Malamy (2005) demonstrated that in the *lateral root*

development 2 (*lrd2*) mutant of *Arabidopsis*, auxin and ABA can facilitate lateral root initiation. This study revealed that lateral root initiation can be suppressed by ABA in plants growing at low ψ_w, but does not involve changes in primary root growth and development. Additionally, Wu *et al*. (1994) showed in maize that xyloglucan endotransglycosylase (XET) and expansin activity, two proteins that are necessary for cell wall loosening, increase within the apical region of the primary root elongation zone of plants growing under low ψ_w conditions, and that this is correlated with growth maintenance. Additionally, the increase in XET activity at low ψ_w was dependent on ABA buildup. Osato *et al*. (2006) also showed a similar response of XET activity and the dependency on ABA in *Arabidopsis*, and further concluded that transcript levels of XET were specifically regulated by auxin in the primary root tip. Taken together, the above studies establish some significant interactions that take place between ABA and auxin in the regulation of root growth under water-stressed conditions.

Objectives

The overall objective of this dissertation is to further understand the role of ABA accumulation in growth maintenance of water-stressed maize primary roots, by evaluating the inter-relationships of ethylene, ROS, auxin and growth. The specific objectives are as follows:

- Utilize a specific inhibitor of ethylene action to re-assess the conclusions of Spollen *et al*. (2000) that increased ethylene production is the cause of primary root growth inhibition in ABA-deficient roots growing at low ψ_w.

- Utilize a specific inhibitor of ethylene action to re-assess the conclusions of Smith (2011) that increased ethylene production is the cause of the increase in intracellular ROS in ABA-deficient plants growing at low ψ_w.

- Investigate whether the role of ABA in root growth maintenance at low ψ_w involves an interaction with IAA by utilizing inhibitors of auxin transport and direct addition of auxin to manipulate IAA levels in ABA-deficient roots growing at low ψ_w.

Figure 1. In maize seedlings, primary root growth is less sensitive than shoot growth to low water potentials (reproduced from Sharp, 1990).

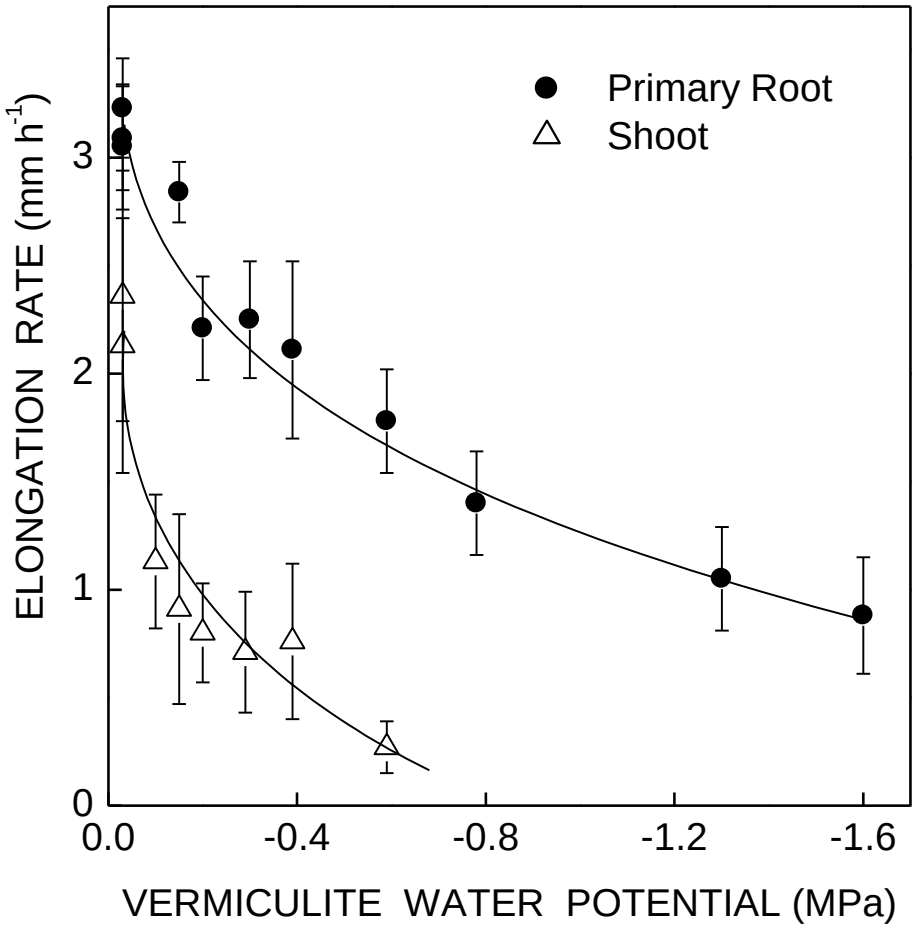


Figure 2. ABA biosynthetic pathway (modified from Taylor *et al*., 2000). The diagram illustrates the steps that are impaired by the chemical inhibitor of ABA synthesis fluridone (FLU), and in mutants of several species. The steps impaired in the *vp5* and *vp14* mutants of maize and by FLU as used in previous studies of primary root growth under water stress are designated in red.

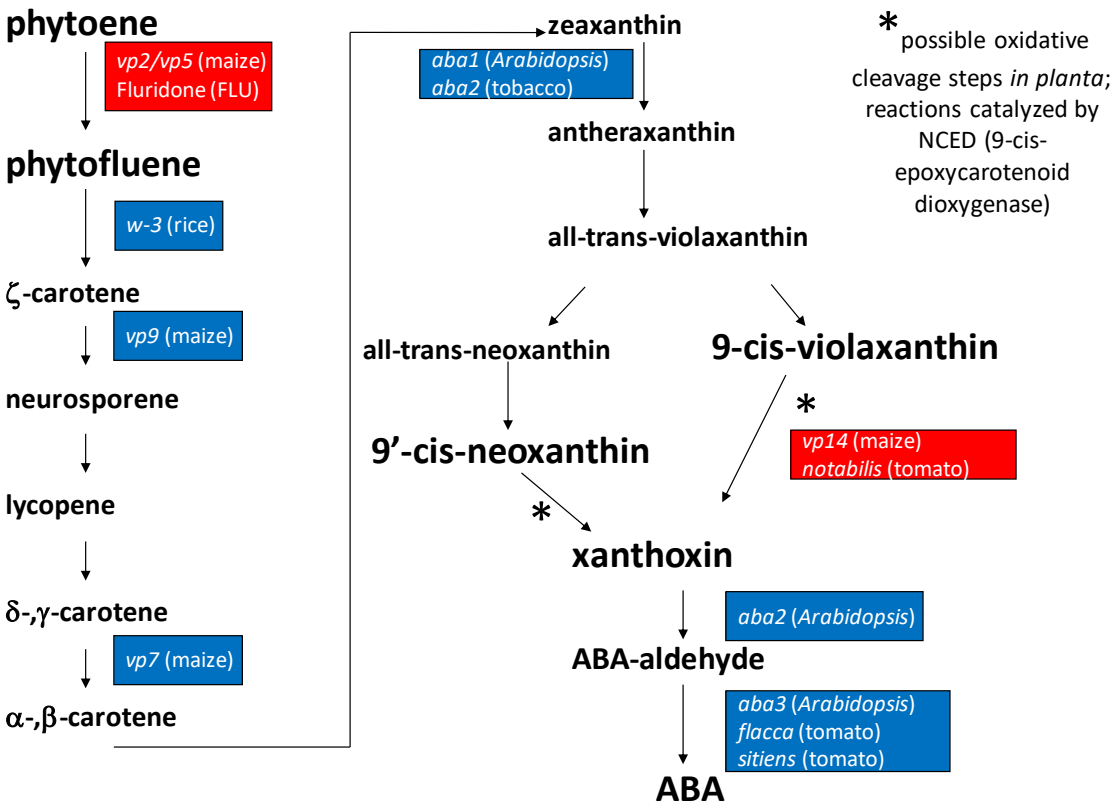


Figure 3. Primary root elongation and seedling ethylene evolution rate as a function of root tip (apical 10 mm) ABA content in *vp5*, *vp14* and fluridone-treated maize seedlings at low ψ_w (-1.6 MPa) in vermiculite. (reproduced from Sharp, 2002).

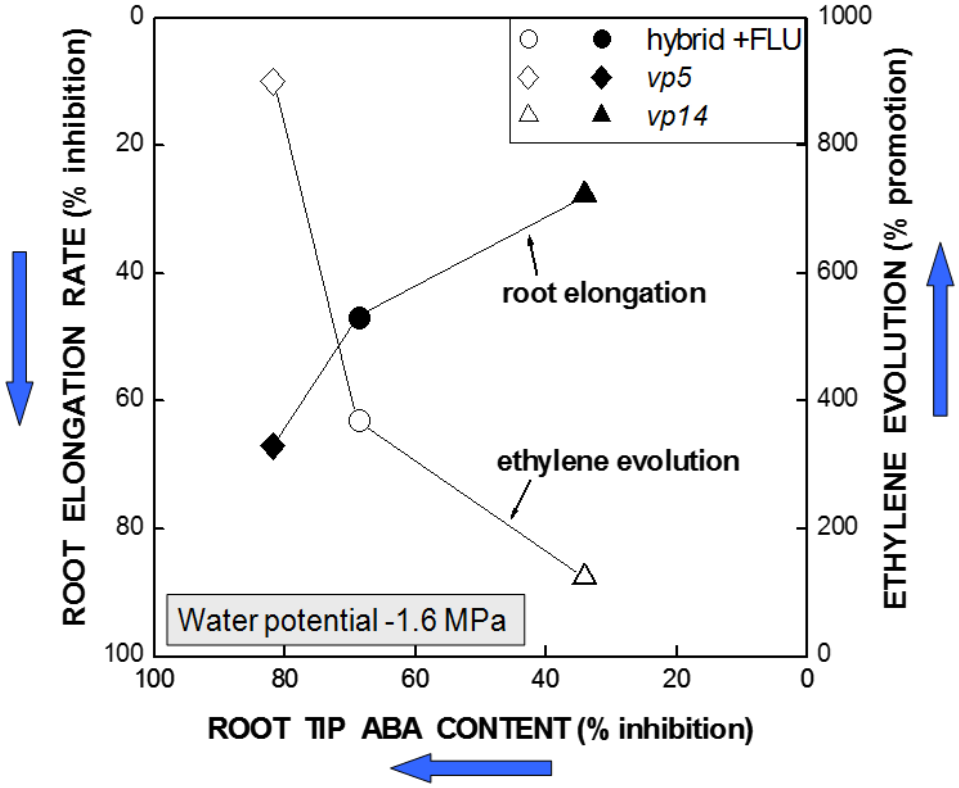


Figure 4. Apical 11 mm of maize primary roots of **(A)** FLU-treated and untreated and **(B)** wild type and *vp5* maize seedlings 48 h after transplanting to low ψ_w vermiculite (-1.6 MPa) (reproduced from Sharp *et al*., 1993).

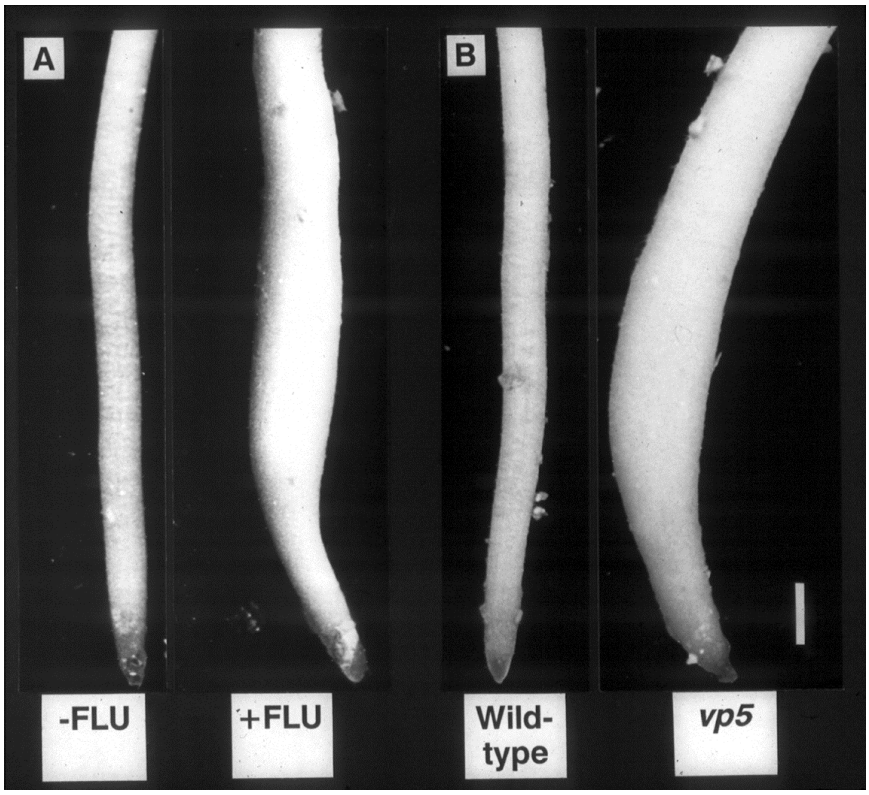
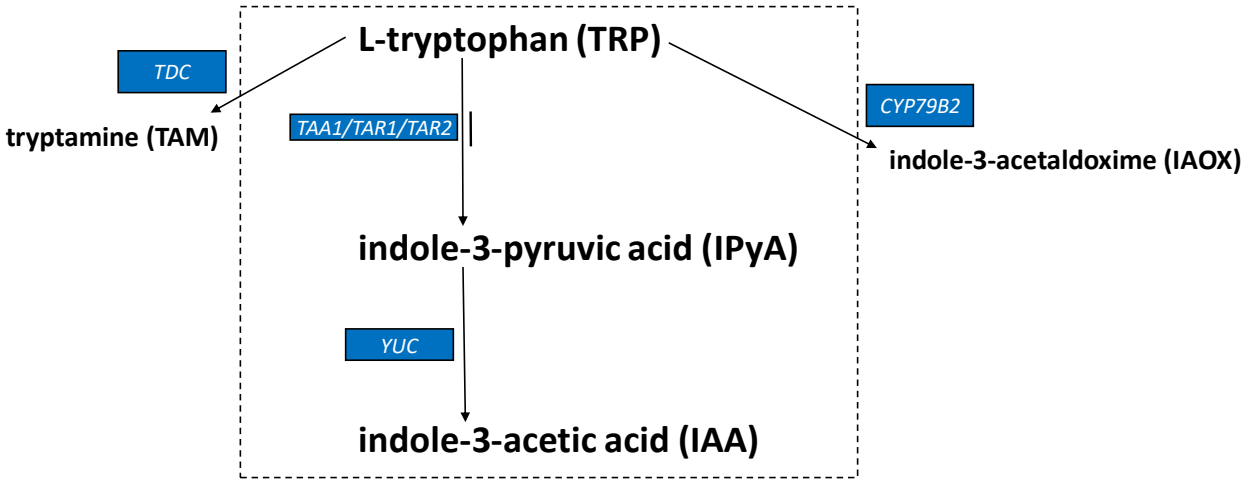


Figure 5. Auxin biosynthetic pathway (modified from Mano and Nemoto, 2012). The diagram illustrates the tryptophan-dependent portion of IAA biosynthesis (highlighted in the dashed-box) and associated enzymes.



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CHAPTER 1-SUPPLEMENT

RESTRICTION OF ETHYLENE SYNTHESIS PREVENTS THE INCREASE IN INTRACELLULAR ROS IN ABA-DEFICIENT ROOTS AT LOW WATER POTENTIALS AND RESTORES PRIMARY ROOT GROWTH

The studies presented in this section are supplemental to data presented in Smith (2011).

INTRODUCTION

As detailed in Chapter 1, ABA-deficient roots at low ψ_w exhibit both increased ethylene evolution rates (Spollen *et al*., 2000) and increased levels of intracellular ROS (Cho, 2006), and these effects together are associated with cellular damage and inhibition of primary root elongation. It was the principal goal of Smith (2011) to investigate the inter-relationship of ethylene and ROS in ABA-deficient roots by utilizing inhibitors of ethylene synthesis and action to determine whether increased ethylene production causes the increase in intracellular ROS in the root growth zone of water-stressed *vp14*. The results demonstrated that using the chemical inhibitor of ethylene synthesis AOA, one can successfully decrease endogenous ethylene levels, prevent the increase in intracellular ROS and restore primary root growth in ABA-deficient roots. These findings indicated that the interaction of ABA and ethylene in water-stressed roots is upstream of the production of ROS. Additionally, $AgNO_3$, an inhibitor of ethylene action, was also used to investigate interactions involving ABA, ethylene and ROS in water-stressed *vp14* roots (Smith, 2011). Results from that study demonstrated that $AgNO_3$ failed to restore primary root elongation, but decreased intracellular ROS levels in *vp14* seedlings. Failure of $AgNO_3$ to restore root elongation in *vp14* seedlings is of potential concern in interpreting the effects of that treatment.

To strengthen the conclusions of studies presented in Smith (2011), experiments reported in this section utilized an additional inhibitor of ethylene synthesis, AVG, to determine whether increased ethylene production causes or is the cause of the increase in intracellular ROS and primary root growth inhibition in ABA-deficient roots growing at

low $\psi_{w.}$ AVG was chosen because like AOA, it was effective in restoring elongation of ABA-deficient roots at low ψ_w in vermiculite-grown seedlings (Spollen *et al*., 2000).

MATERIALS AND METHODS

Plant material, growth conditions and root elongation measurements

Homozygous *vp14* and near isogenic wild-type (W22 genetic background) seed were derived by selfing the original seed from segregating ears that was supplied by Don McCarty, University of Florida, Gainesville. All experiments using AVG were conducted with homozygous *vp14* and wild-type seeds that were produced in the field in Puerto Rico in 2009. The use of an oxygenated PEG hydroponic system was used in which seedlings were transplanted into Plexiglas boxes containing a high ψ_w (-0.03 MPa) solution. Primary roots were allowed to grow for an initial 2 h period before water stress was imposed by gradual replacement with a solution of the same composition but with the addition of PEG 8000 (Sigma) to lower the ψ_w to -1.6 MPa. PEG solution was pumped into the bottom of the box at a flow rate of 1 mL min^{-1} over the course of 24 h, at which time the bulk solution ψ_w in the box had reached -1.6 MPa as described in Verslues *et al*. (1998) and Smith (2011) (Fig. 1). Primary root lengths were recorded at transplanting and at harvest at 24 h. The time course of root elongation rate for all experiments in which ethylene, ROS and ABA were measured was obtained by periodically marking the position of the primary root apices on the face of the Plexiglas boxes (using a green safe-light as described by Saab *et al*., 1990).

AVG treatment

AVG is a chemical inhibitor of pryidoxal phosphate, which is required for activity of ACC synthase (Amagasa *et al*., 1992). The hydroponic culture system was utilized to allow continuous application of AVG to inhibit ethylene synthesis. In each experiment, one box of 20 seedlings was grown for each of four treatments, as follows: wild type with or without inhibitor, and *vp14* with or without inhibitor.

Ethylene measurements

Ethylene evolution rates were measured from the 0-10 mm apical region of the primary root, as described by Smith (2011). Fifteen roots (five roots per sample) were harvested at 24 h after transplanting, and the excised segments were immediately transferred to gas-tight 10-mL syringes that were lined with moistened filter paper to prevent tissue dehydration. Preliminary experiments determined that ethylene evolution peaked between 18-24 h after imposition of the low ψ_w treatment. Ethylene was allowed to evolve for 20 min, since initial tests established that 20 min was the threshold before wound-induced ethylene commenced. The ethylene content of the head space was then measured by injecting a 9 mL sub-sample into the sample loop of a cold trap containing 100 mg absorbent (Parapak S, Supelco, Bellfonte, PA) that was cooled to -95°C with a mixture of acetone and liquid N_2 (DeGreef *et al*., 1976). The sample loop was then heated with boiling water to release the trapped ethylene into the carrier gas stream of a gas chromatograph (model 3400cx, Varian, Palo Alto, CA). Ethylene was identified by retention time compared to pure ethylene standards. Ethylene evolution rates were expressed as pmol ethylene kg^{-1} fresh weight s^{-1}.

Staining for intracellular ROS

Seedlings were removed from the growth boxes at 24 h after imposition of low ψ_w, and the apical region of the primary root of intact seedlings was placed in an iso-osmotic staining solution for approximately 30 min. As a precaution, aeration with air was used during the staining period so that roots would not be subjected to hypoxic conditions (as described in Cho, 2006). The staining solution consisted of 45 μM carboxy-H_2DCFDA (Molecular Probes, Eugene, OR) in 1 mM $CaSO_4$, with the addition of melibiose to lower the ψ_w to -1.6 MPa. Melibiose has been proposed as a suitable non-permeating osmoticum for imposing low ψ_w on plants in solution culture studies (Dracup *et al*., 1985). PEG was not used because preliminary tests showed that the high viscosity of PEG solutions interfered with the uptake of the dye. Carboxy-H_2DCFDA specifically stains for intracellular ROS. The dye readily crosses the plasma membrane due to the presence of diester moieties, which are then cleaved by intracellular esterases, exposing the oxidation site. Once oxidized, the dye produces a green fluorescence.

After the 30-min staining period, the apical 10 mm of the roots were excised and imaged using stereo-fluorescence microscopy (SMZ III, Leica, Germany) using a GFP filtration system, at an excitation of 400 nm and emission of 515/30 nm.

ABA measurements

In each of two separate experiments, all 20 seedlings were removed from each of the growth boxes at 24 h after imposition of the low ψ_w treatment, and the apical 10 mm of the primary roots were harvested for ABA quantification. After removing the apical 0.5 mm to remove the major portion of the root cap, root segments were immediately frozen

in liquid nitrogen and stored at -80°C. Removal of the apical 0.5 mm to remove the major portion of the root cap was necessary, as Saab *et al*. (1992) showed that ABA levels in the root cap were much higher compared to levels in the root growth zone. The segments were then freeze-dried, dry weights were measured, and ABA contents (five root tips per sample) were measured with a radio-immunoassay (Quarrie *et al*., 1988) as described by Saab *et al*. (1990) and Sharp *et al*. (1994), using a monoclonal antibody to ABA supplied by Babraham Bioscience Technologies (Cambridge, England). The radio-immunoassay (RIA) has a working range of 125-2000 pg ABA per vial. Vials were counted in a Beckman LS 6000IC scintillation counter.

RESULTS AND DISCUSSION

Determining the appropriate AVG concentration

A series of preliminary experiments was conducted to determine an appropriate concentration of AVG that would be effective in inhibiting ethylene synthesis, yet non-toxic due to long-term exposure. Since it was previously demonstrated that the inhibition of elongation in FLU-treated roots at low ψ_w could be almost fully prevented by AVG treatment (Spollen *et al*., 2000), recovery of root elongation in *vp14* was used as the primary indicator of AVG effectiveness in these experiments. The objective was to identify the lowest AVG concentration that resulted in root growth recovery in the mutant without significantly inhibiting root growth in the wild type. In addition, roots of *vp14* at low ψ_w exhibited a root-curling phenotype, which was also prevented by treatment with AVG and, therefore, was also presumably attributable to excess ethylene. This phenotype provided an additional indicator of the appropriate AVG concentration to be used.

A range of AVG concentrations from 0.1-5 µM was evaluated with wild-type and *vp14* seedlings growing at a ψ_w of -1.6 MPa. At 5 µM AVG, root elongation in wild-type plants was inhibited when compared to untreated wild-type controls. At the same concentration, root elongation rates of *vp14* plants were further inhibited when compared with untreated mutant controls, suggesting that the highest concentration of AVG tested was inhibitory due to toxicity. At 1 and 0.5 µM, AVG had no effect on wild-type or *vp14* root elongation when compared with untreated controls, suggesting that these concentrations were still toxic, therefore preventing root growth recovery in *vp14*. At 0.1 µM, AVG had no effect in wild-type plants but root elongation in *vp14* was restored to a rate that was not significantly different from that in AVG-treated or untreated wild-type seedlings (Fig. 2). Treatment with 0.1 µM AVG also fully prevented the root-curling phenotype in *vp14* roots.

Accordingly, an AVG concentration of 0.1 µM was chosen to evaluate the effects of AVG treatment on ethylene, ROS and ABA levels in *vp14* roots under water stress conditions. (It should be noted that in the studies of Spollen *et al*. (2000), AVG was only supplied as a pre-treatment during germination. Not unexpectedly, the AVG concentration used in that study [5 µM] proved to be much too high for the long-term treatments used in the present experiments.)

Intracellular ROS staining experiments

Figure 3 (D1-D10) shows that the increase in root tip ROS level in water-stressed *vp14* was completely prevented by treatment with 0.1 µM AVG. The AVG treatment had minimal effect on the basal levels of ROS in the wild-type roots (Fig. 3, B1-B10). For each treatment, all 10 images from two replicate experiments are presented.

Consistent with previous results using AOA (Smith, 2011), the complete prevention of the increase in ROS by suppression of ethylene synthesis suggests that the effect of ABA-deficiency on ethylene production in water-stressed roots is upstream of, and results in, the increase in ROS.

Ethylene measurements of AVG-treated and un-treated wild-type and vp14 roots

Figure 4 shows that the root tip ethylene evolution rate of *vp14* was significantly higher than in the wild-type at 24 h after water stress imposition. This finding is consistent with previous reports that ABA-deficiency causes increased ethylene evolution in water-stressed maize primary roots (Spollen *et al*., 2000; Sharp, 2002). Treatment with AVG completely prevented the increase in ethylene evolution in *vp14*, while having no effect on the wild type.

ABA measurements of AVG-treated and un-treated wild-type and vp14 roots

It was critical to measure ABA content in these experiments for two reasons. First, to confirm that the *vp14* mutant roots were indeed ABA deficient at low ψ_w, and second, to demonstrate that the effects of AVG treatment were not attributable to an unexpected recovery of ABA levels. (If ABA levels were restored in AVG-treated *vp14*, this would have confounded interpretation of the effect of ethylene suppression.) The root tip ABA contents of AVG-treated and untreated wild-type and *vp14* seedlings are shown in Figure 5. These results confirm that ABA levels were significantly reduced in *vp14* compared to the wild type, and were unaffected by treatment with AVG in both cases.

CONCLUSIONS

These results demonstrate that treatment with the chemical inhibitor of ethylene synthesis AVG can successfully decrease endogenous ethylene levels, prevent the increase in intracellular ROS and restore primary root growth in ABA-deficient seedlings growing under water stress conditions. As demonstrated in Smith (2011), results using AOA to inhibit ethylene synthesis are consistent with and strengthen this conclusion. Taken together, these findings indicate that the interaction of ABA and ethylene in water-stressed roots is upstream of the production of ROS.

Figure 1. Design of the Plexiglas growth boxes for hydroponic culture (original design by Verslues *et al*., 1998, with modifications as described in Smith, 2011). The kernels are suspended above perforated straw guides that help direct primary root growth. The solution is vigorously aerated with a mixture of air and oxygen via the tubing at the bottom of the box.

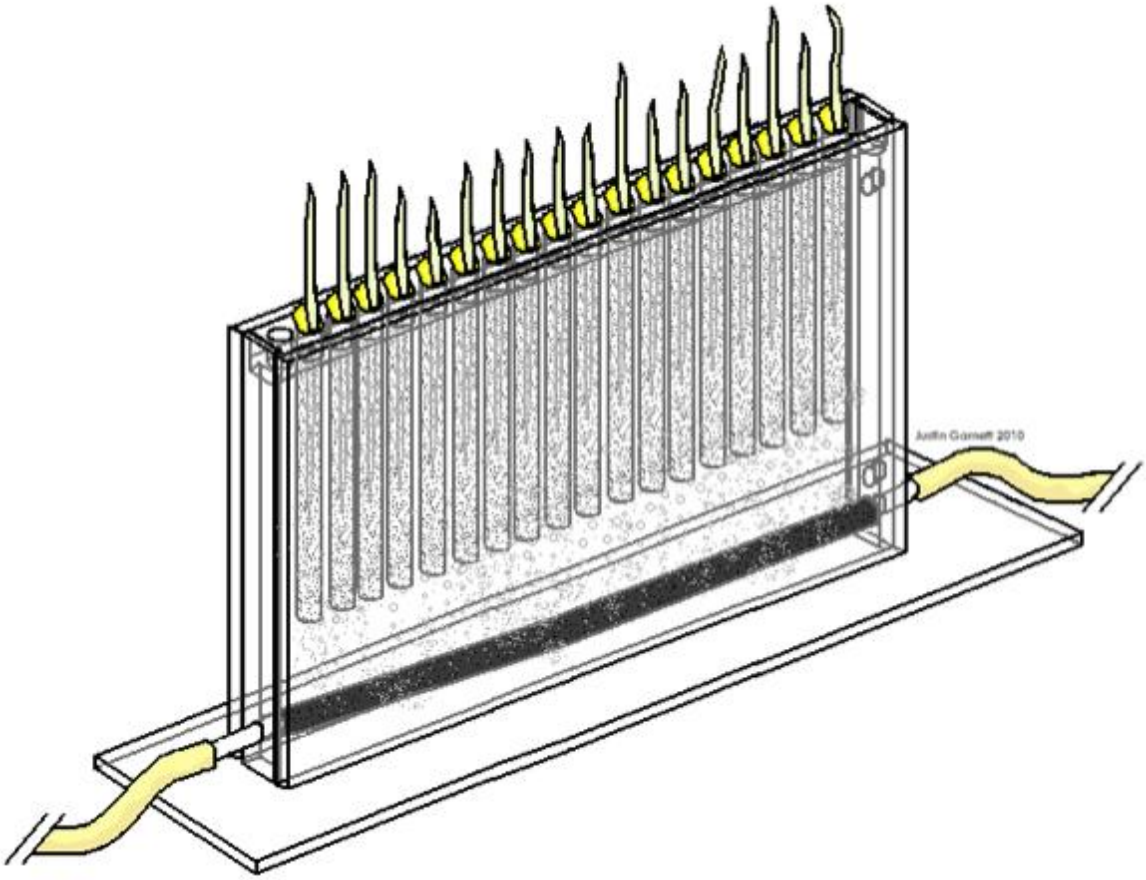


Figure 2. Primary root elongation rates of AVG-treated and untreated wild-type and *vp14* seedlings during 24 h after imposition of low ψ_w, during which time the PEG solution ψ_w in the growth box decreased to -1.6 MPa. Data are means ± SE (n = 80, combined from four experiments). A one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$).

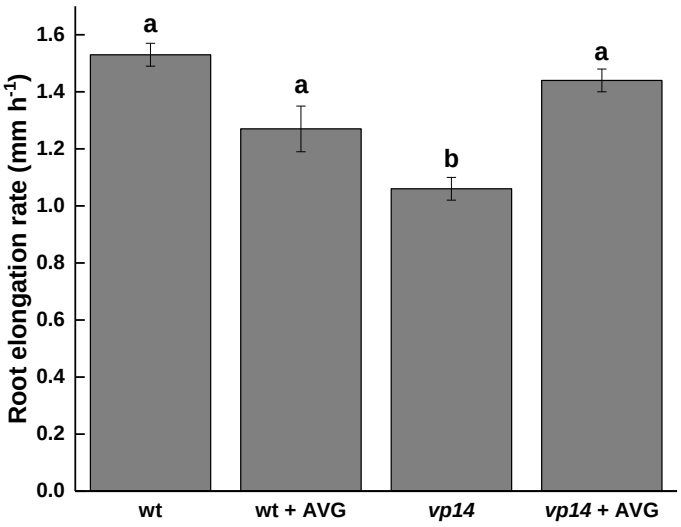
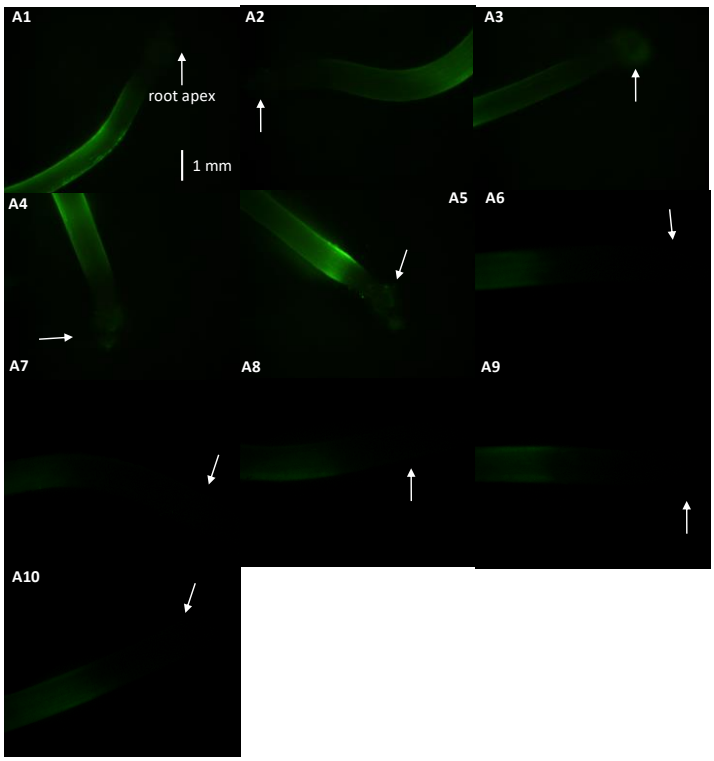
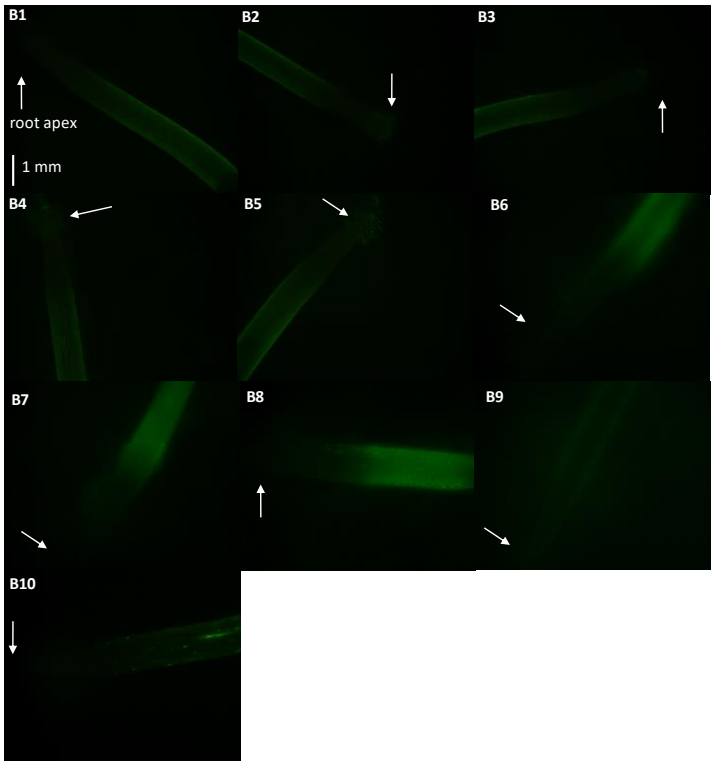


Figure 3. Fluorescence microscopy images of intracellular ROS levels in AVG-treated and untreated *vp14* and wild-type primary root tips. The measurements were made 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. For each treatment, all images from two replicate experiments are presented. **(A1-10)** wild-type; **(B1-10)** wild-type + AVG; **(C1-10)** *vp14*; **(D1-10)** *vp14* + AVG. The root apex is indicated by the white arrows. Similar results were obtained with AOA-treated and untreated wild-type and *vp14* plants (Smith, 2011).




B1
root apex
1 mm
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B3
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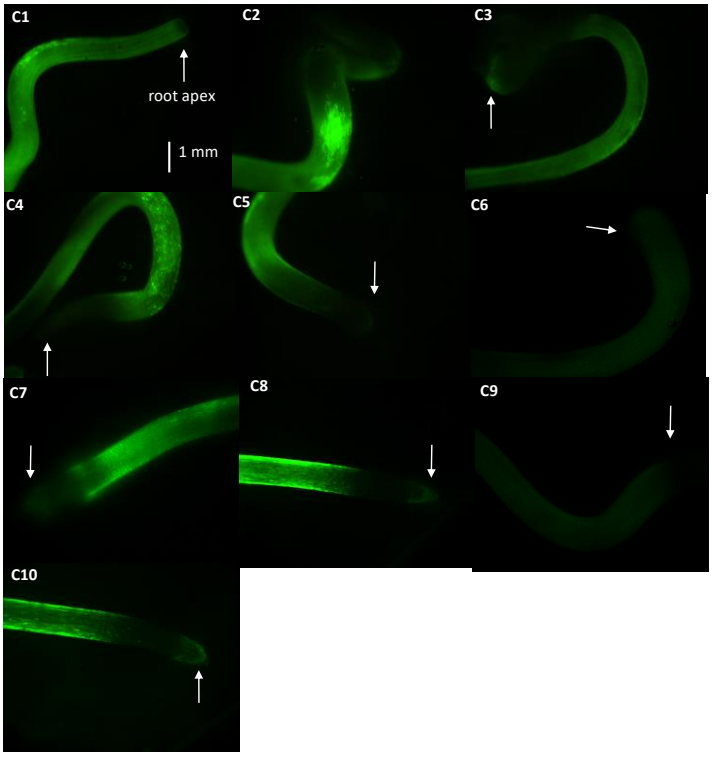
B9
B10


C1
root apex
1 mm
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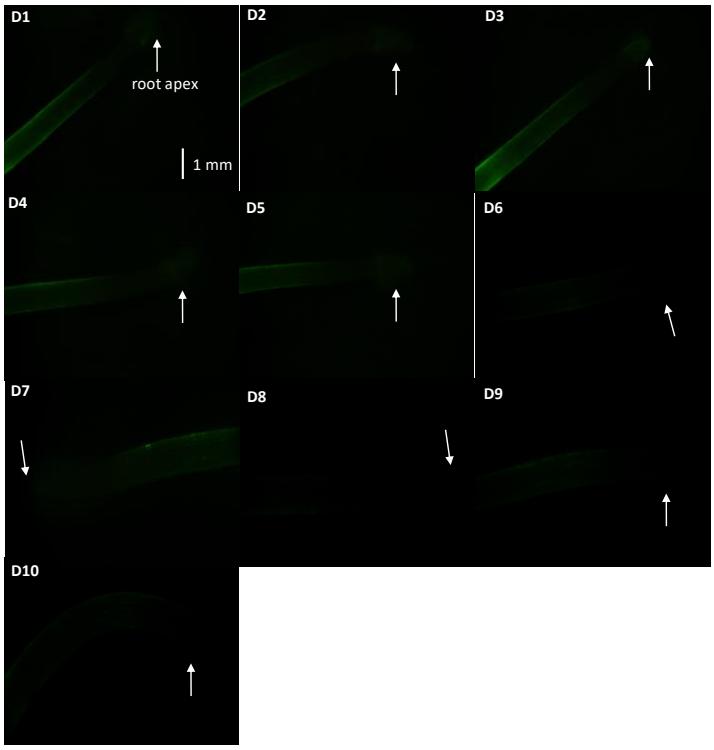
D1
root apex
1 mm
D2
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D7
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D10

Figure 4. Ethylene evolution rates from the apical 10 mm of the primary root of AVG-treated and untreated wild-type and *vp14* seedlings. Samples were taken 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. Data are means ± SE (n = 3). A one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$). The experiment was repeated with similar results. Similar results were obtained with AOA-treated and untreated wild-type and *vp14* plants (Smith, 2011).

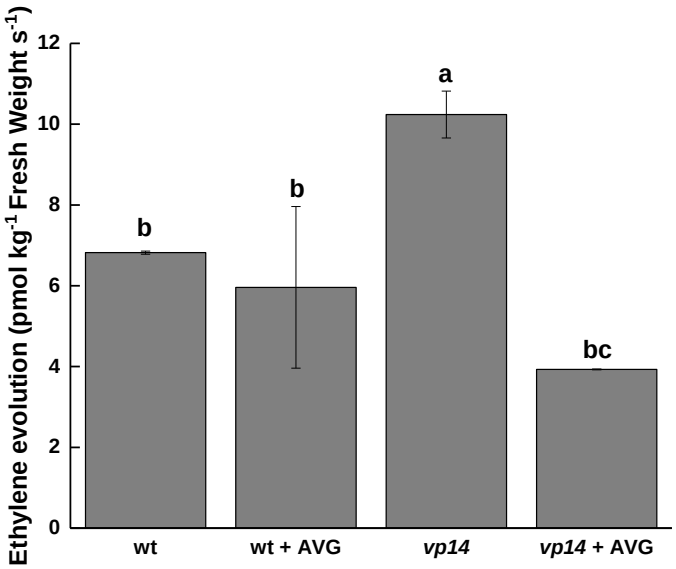
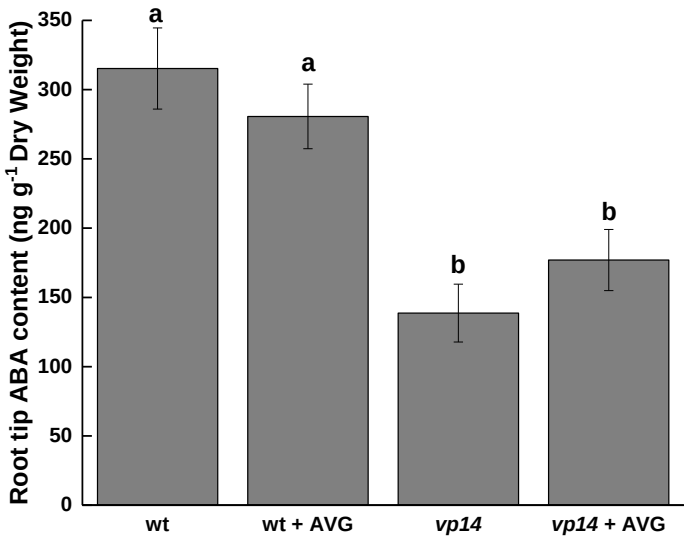


Figure 5. Primary root tip (apical 10 mm) ABA content of AVG-treated and untreated wild-type and *vp14* seedlings. The measurements were made 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. Data are means of ± SE (n =8, combined from two experiments). A one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$). Similar results were obtained with AOA-treated and untreated wild-type and *vp14* plants (Smith, 2011).



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CHAPTER 2

ROLE OF ABA IN ROOT GROWTH MAINTENANCE UNDER WATER STRESS: RE-EVALUATING THE INTERACTION WITH ETHYLENE

These studies were made possible in collaboration with The Dow Chemical Company

INTRODUCTION

As detailed in Chapter 1, previous work established that the ABA-deficient maize mutant *vp14* exhibits impaired primary root growth under water stress conditions (Saab *et al*., 1990; Sharp *et al*., 1994), which is associated with excess production of ethylene (Spollen *et al*., 2000) and intracellular ROS (Cho, 2006) in the root growth zone. In *vp14*, ABA synthesis is inhibited because of an impairment in one of the 9-cis-epoxydioxygenase (NCED) genes. NCED catalyzes the oxidative cleavage step of epoxy-carotenoids to xanthoxin, which represents the first committed step in ABA biosynthesis (Tan *et al*., 1997; Chapter 1, Fig. 2). Thus, the use of *vp14* for studies of the interaction between ethylene and ROS has the advantage that the inhibited step in ABA biosynthesis is downstream of the carotenoid pathway. Carotenoids act as ROS scavengers in plants and play a protective role in preventing oxidative damage (Armstrong and Hearst, 1996). Accordingly, inhibition of carotenoid synthesis could have been a confounding factor in earlier studies in which fluridone (FLU) or the *vp5* mutant were used to study effects of ABA deficiency (Saab *et al*., 1990; Sharp *et al*., 1994; Spollen *et al*., 2000).

To further understand the interrelationships between ABA, ethylene, ROS and growth in water-stressed roots, the *vp14* mutant was used to determine whether increased ethylene production causes or is the cause of the increase in intracellular ROS (Smith, 2011; Chapter 1 Supplement). A hydroponic culture system using oxygenated polyethylene glycol (PEG) solution to lower the ψ_w (Verslues *et al*., 1998) was used to allow controlled application of inhibitors of ethylene biosynthesis (AOA, AVG) and the ethylene action inhibitor silver nitrate ($AgNO_3$) during the growth of ABA-deficient roots, and the effects on primary root growth were analyzed. Results showed that inhibition of ethylene synthesis

using either AOA or AVG completely restored root growth and prevented the increase in intracellular ROS, demonstrating that in ABA-deficient water-stressed roots, the effect of ROS was downstream of ethylene. Treatment with $AgNO_3$ to inhibit ethylene action also prevented the increase in ROS, although the failure of this treatment to restore root elongation in *vp14* seedlings was of potential concern in interpreting the effects of the treatment.

However, recent discoveries have shown that both AOA and AVG can inhibit auxin biosynthesis independently on their effects of ethylene (Soeno *et al*., 2010). In addition, silver ions can increase auxin efflux, again independently of the effects on ethylene response (Strader *et al*., 2009). Because of these off-target effects of the inhibitors, it was critical to re-evaluate the role of ethylene, and its interaction with ROS production, in the growth inhibition of ABA-deficient roots at low Ψ_w, using an inhibitor that is specific to blocking ethylene synthesis or action. In this chapter, the effects of the inhibitor of ethylene perception 1-methylcyclopropane (1-MCP) were examined. 1-MCP is a gaseous compound that specifically blocks ethylene perception by competitively binding to ethylene receptors (Serek *et al*., 1994; Serek *et al*., 1995; Sisler *et al*., 1996; Sisler *et al*., 1999; Sisler *et al*., 2003). 1-MCP has been commonly used in the fruit ripening industry, and at an effective concentration, plants can remain ethylene insensitive for up to 12 days (Sisler and Serek, 1997). Therefore, 1-MCP was an ideal tool to re-evaluate the interactions between ABA, ethylene, ROS and root growth in ABA-deficient plants under water stress conditions. In addition, IAA measurements were made to provide an initial assessment of the effects of ABA deficiency on auxin levels in water-stressed roots.

MATERIALS AND METHODS

vp14 and wild-type plant material

Homozygous *vp14* and near-isogenic wild-type seeds (W22 genetic background) used for the experiments reported in this chapter were produced in 2009 from field-grown plants in Puerto Rico. Homozygosity of *vp14* material was confirmed by genotyping (Cho and Oliver, unpublished).

Growth experiments

Seeds of *vp14* and wild type were surface-sterilized in 5% NaClO solution for 45 min and rinsed with deionized water for 10 min. The seeds were then imbibed for 24 h in aerated 1 mM $CaSO_4$ and germinated for 48 h at 29 ± 1 ºC and near-saturation humidity in the dark on sterile germination paper (Anchor Paper, Hudson, WI, USA) that was saturated with the same solution (Spollen *et al*., 2000). Seedlings with primary roots that were 5-15 mm in length were then transplanted into Plexiglas boxes (30 seedlings per box) containing vermiculite (Therm-O-Rock East, Inc., PA, USA) at a mean ψ_w of -1.57 ± 0.01 MPa (data are means ± SE), as described in Sharp *et al*. (1988). The vermiculite was thoroughly mixed with a pre-calibrated volume of 1 mM $CaSO_4$ solution to obtain the desired ψ_w, which was measured in each experiment by isopiestic thermocouple psychrometry (Boyer and Knipling, 1965).

1-MCP treatment

Before transplanting, 200 mg of EthylBloc powder (1-MCP, formulated as a gas embedded in a cyclodextran polymer) (Dow Chemical Company, Midland Michigan,

USA; Lot #0004279123) was weighed in a 1 L glass beaker and placed into glass aquarium tanks (51.43 x 26.67 x 32.08 cm) that served as the treatment chambers. After transplanting, the Plexiglas growth boxes were placed in the tanks and 1 L of deionized water was added to the EthylBloc powder for a final concentration of 1-MCP of 200 mg L^{-1}. Since a significant amount of 1-MCP is released immediately after addition of liquid, a fitted Plexiglas lid was immediately placed on the tank and secured with heavy-duty vacuum grease (Dow Corning®) (Fig. 1). A total of four tanks were used, two containing untreated wild-type and mutant control plants, and two containing wild-type and mutant plants with the addition of 1-MCP. The final concentration of 1-MCP was determined during a series of preliminary dose-response experiments, as described below. Plants were then allowed to grow for 72 h, at which time the tanks were taken to a flow-hood where the remaining 1-MCP gas was expelled. Plants were then returned to the dark where primary root lengths were measured, and the apical 10 mm of the primary root was harvested for both ABA and IAA quantification.

To obtain overall root elongation rates, after 72 h tanks were taken to a flow-hood where the remaining 1-MCP gas was expelled, and then returned to the dark where plants were destructively harvested for final root length.

Exogenous ethylene application for sensitivity of root growth to ethylene

After germination, wild-type and *vp14* seedlings with primary roots that were 5-15 mm in length were transplanted to Plexiglas boxes containing vermiculite at a mean ψ_w of -1.63 MPa ± 0.02 (data are means ± SE). As shown in Table 1, the Plexiglas boxes were placed in six separate tanks (three per genotype) and the following treatments were

evaluated: four containing wild-type or mutant control plants, and two containing wild-type or mutant plants with the addition of 1-MCP (200 mg L^{-1}). Root growth was measured at 24 h as detailed below. Exogenous ethylene (99.5% purity) was then injected into tightly-sealed tanks containing one set of untreated wild-type and *vp14* plants and one set of 1-MCP-treated wild-type and *vp14*, and the plants were grown for a further 48 h. Ethylene was injected with a hypodermic syringe through a rubber septum giving a final ethylene concentration of 1 ppm in a volume of 37.85 L. After 72 h, tanks were taken to a flow-hood where the remaining 1-MCP and ethylene gas were expelled, and then returned to the dark where primary root length was measured and tissue harvested.

For measurements of root lengths at 24 h, tanks were taken to a flow-hood where the remaining 1-MCP was expelled and were then returned to the dark where primary root length was obtained by marking the position of the primary root apices on the face of the Plexiglas boxes under a green safe-light, as described in Saab *et al*. (1990). After marking, the growth boxes were returned to the respective treatment tanks, and 1-MCP was re-supplied and/or ethylene was injected according to the respective treatments.

Staining for intracellular ROS

Seedlings were removed from the growth boxes at 72 h, and the apical region of the primary root of intact seedlings was placed in an iso-osmotic staining solution for staining of intracellular ROS (as described in Chapter 1-Supplement). ROS intensity was analyzed from stereoscope images of wild-type and *vp14* roots using Metamorph Microscopy Image Analysis software (Molecular Devices Corp., Sunnyvale, CA). Total

ROS intensity levels shown in Table 2 represent the apical 10 mm regions of water-stressed wild-type and *vp14* primary roots.

ABA and IAA measurements

In each of two separate experiments, all seedlings (five root tips per sample) were harvested from the growth boxes at 72 h, and the apical 10 mm of the primary roots were harvested for ABA and IAA quantification. The fresh weights of the root segments were measured before immediately freezing in liquid nitrogen and storage at -80°C. Root segments were then freeze-dried (Labconco FreeZone 2.5 Lyophilizer), dry weights were measured, and plant material was sent to the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis. A 4000 QTRAP LC-MS/MS system (AB Sciex) was used for hormone analysis. The analysis system utilizes a hybrid triple quadrupole/linear ion trap mass spectrometer ideal for quantifying small molecules. A Shimadzu UHPLC using two high pressure pumps is coupled to the QTRAP instrument for separation of complex samples (danforthcenter.org/Targeted Metabolomics), and deuterium-labeled internal standards were prepared for each compound.

RESULTS AND DISCUSSION

Determining the appropriate 1-MCP concentration

A series of preliminary experiments was conducted to determine an appropriate concentration of 1-MCP that would be effective in inhibiting ethylene action, yet non-toxic due to long-term exposure. The objective was to identify a 1-MCP amount that resulted in root growth recovery in the mutant without significantly inhibiting root growth in wild-

type plants. 1-MCP concentrations ranging from 5-200 mg L^{-1} were studied. At all amounts tested, root elongation in wild-type and *vp14* seedlings was not significantly different from un-treated control plants, suggesting that the effects of 1-MCP are concentration independent once 1-MCP is bound to ethylene receptors. Or alternatively, this result could indicate that all tested 1-MCP concentrations were too low to be effective.

Because of the seemingly non-toxic effects, the highest 1-MCP concentration (200 mg L^{-1}) was chosen to evaluate the effects of 1-MCP treatment on ethylene-responsiveness, ROS, ABA, IAA and root growth in wild-type and *vp14* plants under water stress conditions.

Growth measurements of wild-type and vp14 roots using 1-MCP to inhibit ethylene action

The results demonstrate that in plants growing at low Ψ_w, 1-MCP was not effective in restoring primary root elongation of the *vp14* mutant (Fig. 2a). Additionally, 1-MCP treatment had no effect on the growth of wild-type primary roots. These results may suggest that the increase in ethylene in ABA-deficient plants growing at low Ψ_w does not explain primary root growth inhibition. Alternatively, 1-MCP may not be effective in this system. Because root elongation in 1-MCP-treated wild-type and *vp14* plants was not significantly different than in un-treated controls, it was critical to have an alternate way of assessing 1-MCP effectiveness in this system.

It is noteworthy that few studies have investigated the effects of 1-MCP on growing tissue. Therefore, because 1-MCP was unsuccessful in restoring primary root elongation in *vp14*, the effectiveness of the 1-MCP treatment itself was tested by addition of an inhibitory level of exogenous ethylene to both wild-type and *vp14* plants (Fig. 2b). Treatment with

ethylene at a concentration of 1 ppm resulted in inhibition of primary root growth in both wild-type and *vp14* seedlings. Pre-treatment with 1-MCP completely prevented the ethylene-induced root growth inhibition in both wild type and *vp14*, but did not restore root elongation of *vp14* plants back to the un-treated rate (Fig. 2b). Taken together, these results demonstrate that the 1-MCP treatment was effective in preventing ethylene-induced root growth inhibition in water-stressed roots. Accordingly, the inability of 1-MCP to restore root growth of *vp14* roots indicates that the increase in ethylene is not the cause of the inhibition of growth in ABA-deficient roots at low ψ_w, in contrast to previous conclusions (Spollen *et al*., 2000).

Consistent with studies presented here using the *vp14* mutant, similar results have been shown using the ABA-deficient and ethylene-insensitive double mutant of *Arabidopsis aba2-etr1* (Kobayashi and Sharp, unpublished). These studies demonstrated that ethylene insensitivity did not restore the inhibition of primary root elongation in *aba2* under water stress. For comparison, 1-MCP was used on *aba2 Arabidopsis* plants growing at low ψ_w, and the effects on primary root growth were assessed (Kobayashi and Sharp, unpublished). The results demonstrated that at several concentrations, 1-MCP did not restore the inhibition of primary root elongation in *aba2* under water stress, consistent with the present findings with the *vp14* mutant of maize.

Intracellular ROS staining experiments

At low ψ_w, intracellular ROS levels in 1-MCP-treated wild-type roots remained unaffected when compared to untreated plants (Fig. 3a; Fig. 3b). In *vp14* roots, plants exhibited a dramatic increase in intracellular ROS levels throughout most of the elongation

zone compared with wild-type plants (Fig. 3a; Fig. 3c), consistent with studies in Cho (2006), Smith (2011) and Chapter 1 Supplement. In 1-MCP-treated *vp14* plants, the increase in intracellular ROS was not significantly prevented (Fig. 3d), however the overall trend was similar to results shown in both the AOA (Smith, 2011) and AVG studies (Chapter 1 Supplement). The fluorescence microscopy images revealed that ROS levels were not uniform in either wild- type or *vp14* 1-MCP-treated or untreated roots under water stress. Therefore, quantification of ROS intensity from stereoscope images was necessary (Table 2). In the apical 10 mm region of roots at low ψ_w, the ROS intensity of untreated *vp14* plants increased to approximately 131% compared to 1-MCP treated and untreated wild-type levels. 1-MCP treated *vp14* roots showed a 69% lower intensity than untreated mutant roots and comparable levels to wild-type plants. The intensity data established that water-stressed *vp14* primary roots have higher ROS levels in the apical region compared to wild-type roots, however treatment with 1-MCP did not significantly reduce ROS levels in the growth zone of *vp14* roots at the $p<0.05$ or $p<0.1$ levels (Table 2). In 1-MCP-treated *vp14* roots, the ROS response is only significantly different than wild-type plants at the $p<0.27$ level (Table 2), which could suggest the sample size may be too limited to make a definitive conclusion on the role of ROS in these studies.

ABA measurements of 1-MCP treated and un-treated wild-type and vp14 roots

It was critical to measure ABA content in these experiments to confirm that the *vp14* mutant roots were indeed ABA deficient at low ψ_w. The root tip ABA contents of 1-MCP-treated and untreated wild-type and *vp14* seedlings are shown in Figure 4a. These results confirm that ABA levels were significantly reduced in *vp14* compared to the wild-type untreated plants. Additionally, there was an increase in ABA levels in 1-MCP treated

wild-type and *vp14* plants. ABA levels in 1-MCP-treated wild-type almost doubled when compared to untreated wild-type plants. ABA levels in 1-MCP-treated *vp14* also increased significantly when compared to untreated *vp14* plants. However, when compared to untreated and treated wild-type, 1-MCP-treated *vp14* roots were still significantly ABA deficient. These results suggest that decreased ethylene sensitivity can increase endogenous ABA concentrations. Similar results have been shown by Ghassemian *et al*. (2000) and Cheng (2009), who showed that mutants defective in their response to ethylene can have altered ABA sensitivity. These findings suggest that ethylene is a potential negative regulator of ABA synthesis.

IAA measurements of 1-MCP treated and un-treated wild-type and vp14 roots

The root tip IAA contents of 1-MCP-treated and untreated wild-type and *vp14* seedlings are shown in Figure 4b. IAA levels were significantly reduced in *vp14* compared to wild-type untreated plants, suggesting that ABA-deficient *vp14* plants may be auxin deficient. In 1-MCP treated wild-type and *vp14* plants, IAA content was significantly higher when compared to untreated plants, however the levels were not significantly different when compared to one another. Accordingly, 1-MCP treatment restored endogenous IAA levels in ABA-deficient roots growing at low ψ_w. Interestingly, however, root growth remained impaired. This result suggests that the action of auxin in primary root growth regulation requires an active ethylene pathway, as also suggested by Ruizicka *et al*. (2007) who demonstrated in *Arabidopsis* that ethylene responsiveness is required for auxin to affect primary root growth.

CONCLUSIONS

1-MCP proved to be an effective tool to re-assess the role of ABA, ethylene, ROS and root growth in *vp14* plants under water stress conditions. The results demonstrated that treatment with 1-MCP prevented the increase in intracellular ROS (although only at a significant level of $p<0.27$) but did not restore primary root growth in ABA-deficient roots. These results support the conclusion that the interaction of ABA and ethylene in water-stressed roots is upstream of the production of ROS in *vp14* plants growing at low Ψ_w, thus confirming studies in Smith (2011) and Chapter 1 Supplement. However, although studies by Spollen *et al*. (2000) concluded that an important role of ABA accumulation in the maintenance of maize primary root elongation at low Ψ_w is to restrict excess ethylene production, the studies using 1-MCP demonstrate that primary root growth inhibition in water-stressed ABA-deficient plants is not caused by increased ethylene production, nor by increased levels of ROS (Fig. 5).

Additionally, the studies presented in this chapter revealed that ABA-deficient *vp14* plants are auxin deficient (Fig. 4b), and suggest that interactions of ABA with auxin could potentially play a key role in regulating maize primary root growth under water deficit conditions. The interactions between ABA and auxin in *vp14* plants under water stress are the focus of the studies in the following chapter.

Table 1. Treatments and times for exogenous ethylene (C_2H_4) application for sensitivity of root growth to ethylene experiments for wild-type (wt) and *vp14* plants. Treatments and times represented here correspond to the data presented in Figure 2b.

wild-type		***vp14***	
0-24 h	24-72 h	0-24 h	24-72 h
wt	wt	*vp14*	*vp14*
wt + 1-MCP	wt + 1-MCP + C_2H_4	*vp14* + 1-MCP	*vp14* + 1-MCP + C_2H_4
wt	wt + C_2H_4	*vp14*	*vp14* + C_2H_4

Table 2. Average intensity of H_2DCFDA staining in the apical 10 mm region of 1-MCP-treated and untreated primary root tips. The measurements were made 72 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Nine individual roots per treatment (combined from two experiments) were quantified by using Metamorph program and the ROS intensity means represented here correspond to the ROS images presented in Figure 3. Different letters indicate significant differences between treatments ($p < 0.05$; $p < 0.1$; $p < 0.27$) and data are means ± SE.

	ROS Intensity		
	$p<0.05$	$p<0.1$	$p<0.27$
wild type	75.8 ± 16.3^{ab}	75.8 ± 16.3^{ab}	75.8 ± 16.3^{a}
wild type + 1-MCP	71.1 ± 17.4^{ab}	71.1 ± 17.4^{ab}	71.1 ± 17.4^{a}
vp14	99.4 ± 23.9^{a}	99.4 ± 23.9^{a}	99.4 ± 23.9^{b}
vp14 + 1-MCP	73.5 ± 19.2^{ab}	73.5 ± 19.2^{ab}	73.5 ± 19.2^{a}

Figure 1. Design of the 1-MCP treatment chamber. Seedlings were transplanted into Plexiglas boxes containing low ψ_w vermiculite (-1.6 MPa) and placed in glass aquarium tanks along with a glass beaker containing the chemical EthyBloc. 1-MCP was released immediately after addition of water to the compound. A Plexiglas lid secured with heavy-duty vacuum grease was attached to the tank to prevent 1-MCP gas escape.

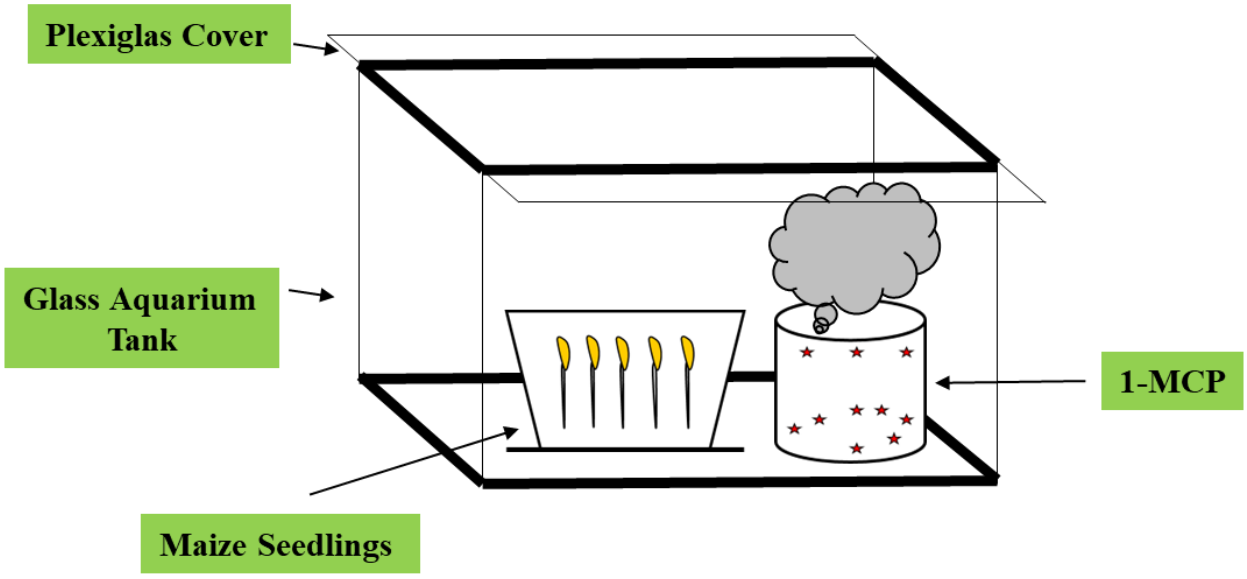
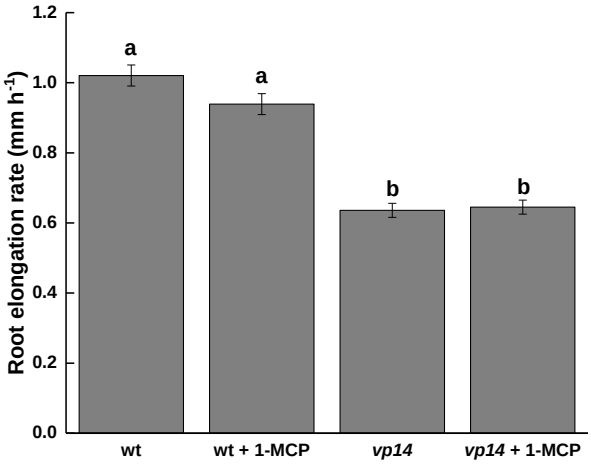


Figure 2. (A) Average primary root elongation rates of 1-MCP-treated or untreated wild-type (wt) and *vp14* seedlings during 72 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n = 80, combined from two experiments). A one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$). **(B)** Average primary root elongation rates of wild-type and *vp14* primary roots during the 24-72 h time-period after transplanting to vermiculite at a ψ_w of -1.6 MPa. Seedlings were treated or untreated with 1-MCP (200 mg L^{-1}) from 0-24 h, followed by treatment with exogenous ethylene (1 ppm) from 24-72 h. Primary root lengths were measured at 24 h (by marking the position of the root apices on the Plexiglas face) and at 72 h (by destructive harvesting). Data are means ± SE (n = 60, combined from two experiments). A one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$).

A


Root elongation rate (mm h-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
wt
wt + 1-MCP
vp14
vp14 + 1-MCP

B

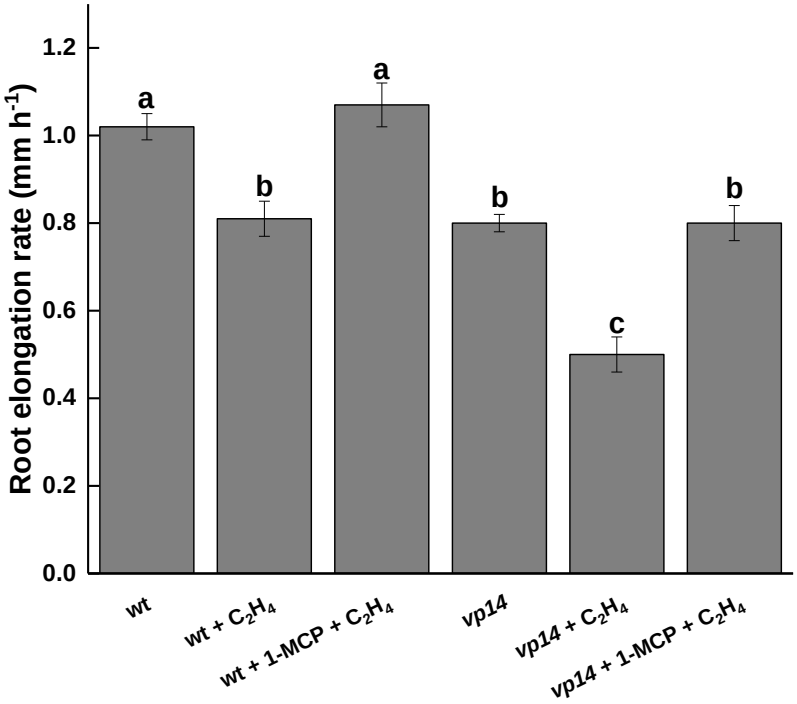
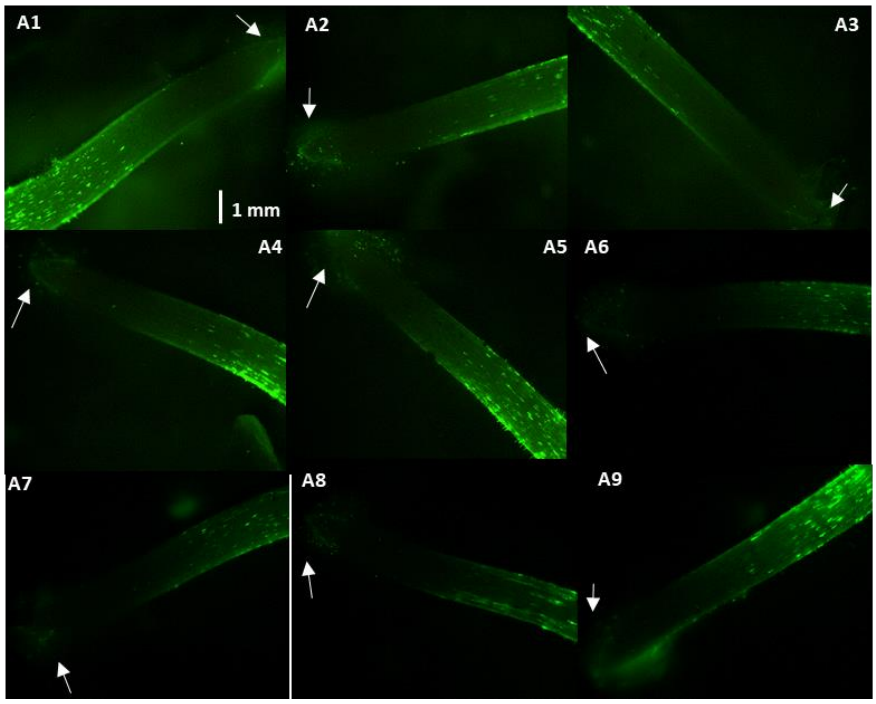
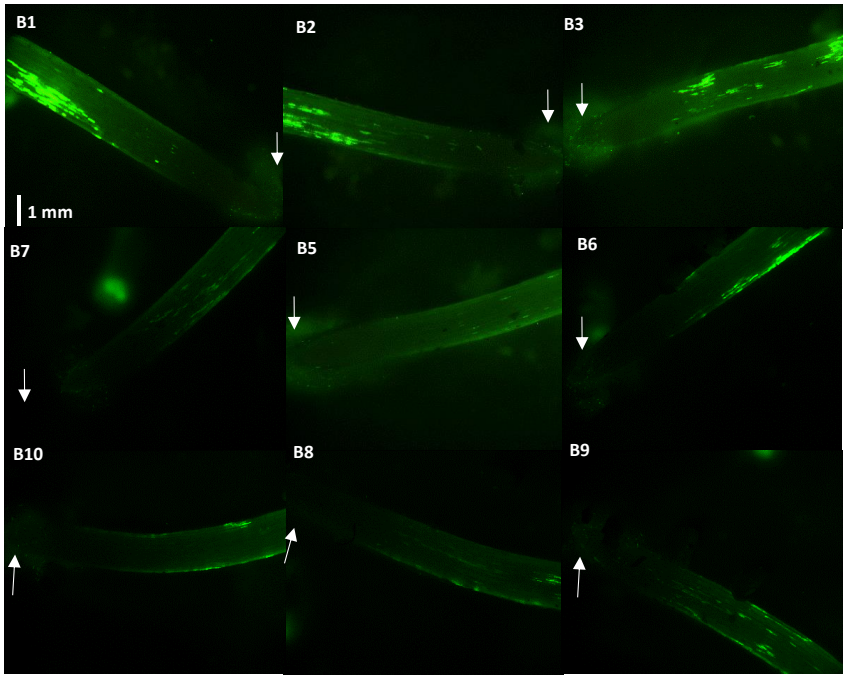

Root elongation rate (mm h-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
b
c
b
wt
wt + C2H4
wt + 1-MCP + C2H4
vp14
vp14 + C2H4
vp14 + 1-MCP + C2H4

Figure 3. Fluorescence microscopy images of intracellular ROS levels in 1-MCP-treated and untreated *vp14* and wild-type primary root tips. The measurements were made 72 h after transplanting to low ψ_w vermiculite (-1.6 MPa). For each treatment, all images from two replicate experiments are presented. **(A1-9)** wild-type; **(B1-9)** wild-type + 1-MCP; **(C1-9)** *vp14*; **(D1-9)** *vp14* + 1-MCP. The root apex is indicated by the white arrows.

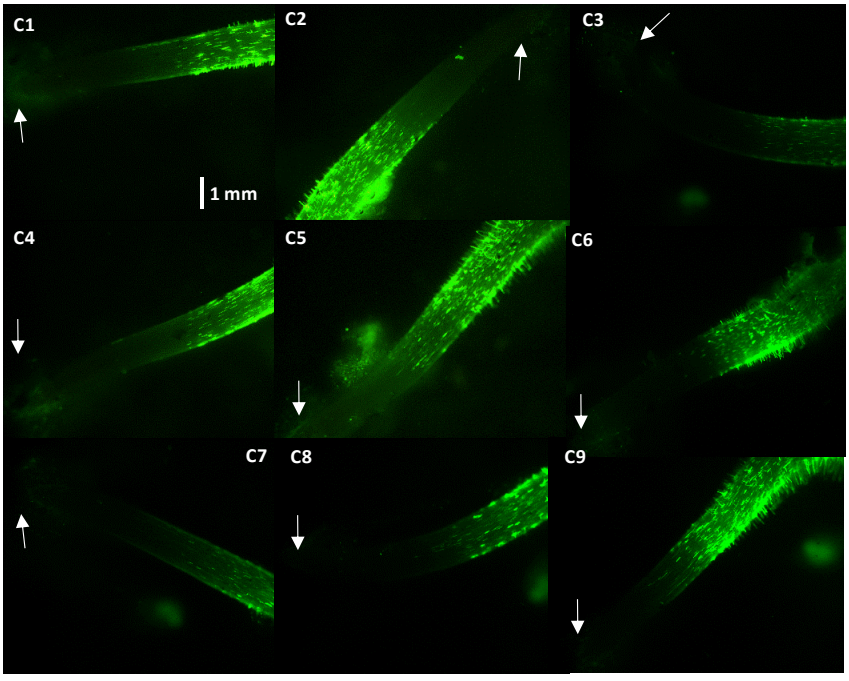
A



B



C



D

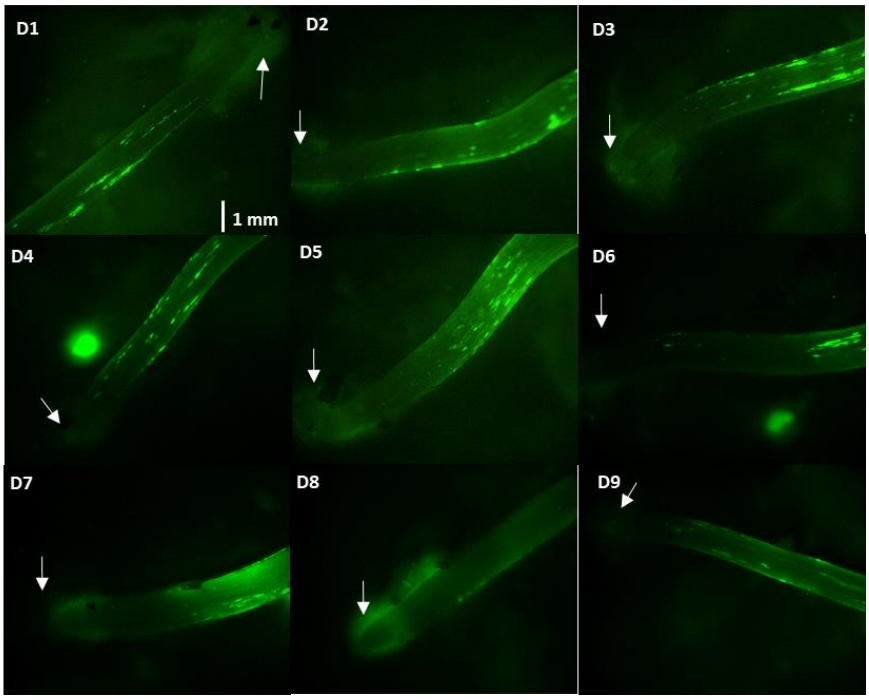
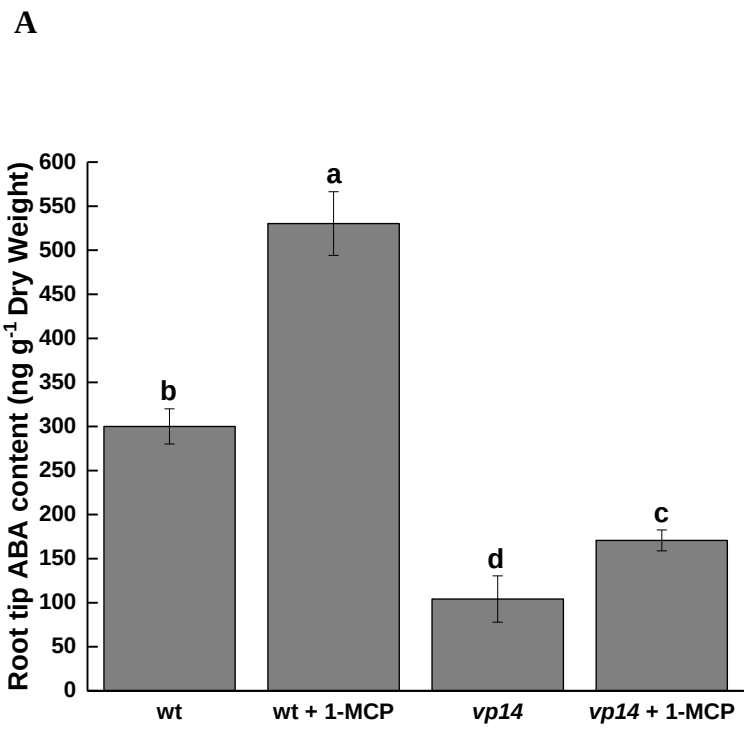


Figure 4. Primary root tip (apical 10 mm) **(A)** ABA and **(B)** IAA contents of 1-MCP-treated and untreated wild-type and *vp14* seedlings. The measurements were made 72 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means of ± SE (n = 6 combined from two experiments). In each set of data, a one-way ANOVA was used to compare data and different letters indicate significant differences between treatments ($p < 0.05$).



B

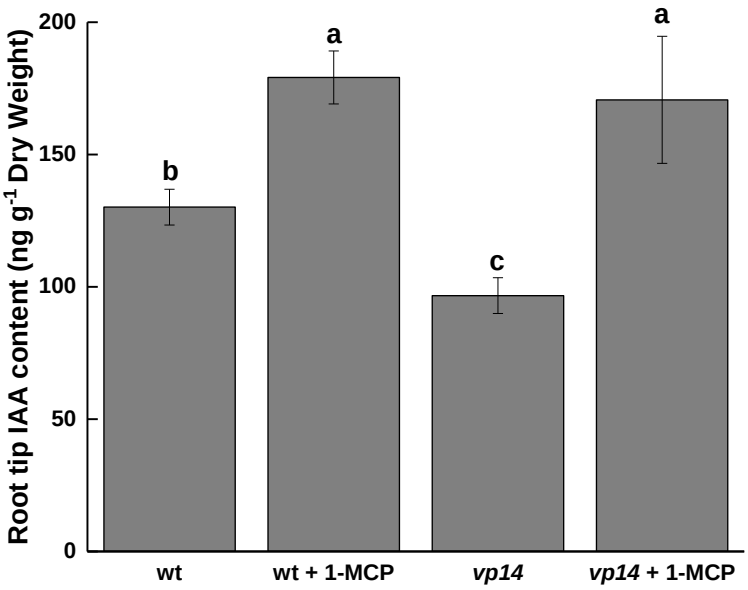
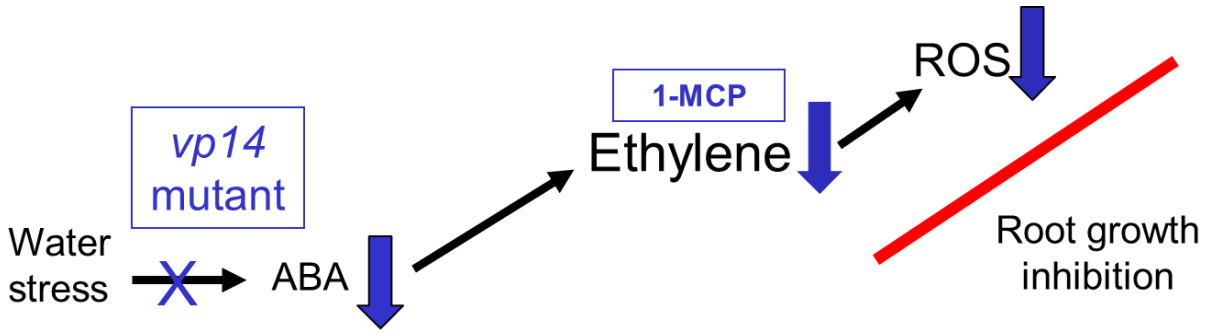

Root tip IAA content (ng g-1 Dry Weight)
200
150
100
50
0
a
b
c
a
wt
wt + 1-MCP
vp14
vp14 + 1-MCP

Figure 5. Illustration depicting that inhibition of ethylene (and ROS) via 1-MCP in ABA-deficient maize plants at low ψ_w does not restore (as indicated by the red line) primary root growth.



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CHAPTER 3

ABA REGULATES AUXIN LEVELS IN THE PRIMARY ROOT GROWTH ZONE AT LOW WATER POTENTIALS

INTRODUCTION

ABA-deficient maize primary roots growing at low ψ_w exhibit both increased ethylene production (Spollen *et al*., 2000) and increased levels of intracellular ROS (Cho, 2006), both of which are correlated with primary root growth inhibition. It was the goal of Smith (2011) and Chapter 1 Supplement to determine whether the increased ethylene production causes or is caused by the increase in intracellular ROS using the *vp14* mutant to impose ABA deficiency. Using inhibitors of ethylene synthesis (AOA, AVG) or action ($AgNO_3$), those studies concluded that the effects of intracellular ROS are downstream of ethylene signaling, and that the increase in ethylene production and/or ROS was the cause of root growth inhibition. However, as detailed in Chapter 2, the ethylene synthesis inhibitors AOA and AVG can also inhibit auxin biosynthesis (Soeno *et al*., 2010), while silver ions can promote auxin efflux (Strader *et al*., 2009). Therefore, it is possible that the restoration of growth in ABA-deficient seedlings growing at low ψ_w resulting from the action of these inhibitors could be due to an interaction with auxin. Because of this possibility, it was critical to re-evaluate the causal interrelationships between ABA, ethylene, ROS and root growth in ABA-deficient plants under water stress. Studies using the specific inhibitor of ethylene action 1-MCP confirmed that the effects of intracellular ROS are downstream of ethylene, but also demonstrated that the increase in ethylene and/or ROS was not the cause of primary root growth inhibition in *vp14* plants growing under low Ψ_w conditions (Chapter 2).

Like the relationship between ABA and ethylene, ABA and auxin levels are often closely inter-related during plant growth and development (Horemans *et al*., 1986; Suzuki *et al*., 2001; Brady *et al*., 2003; Monroe-Augustus *et al*., 2003; Thole *et al*., 2014).

Additionally, studies by Xu *et al*. (2012) demonstrated that under moderate water stress in rice and *Arabidopsis*, ABA accumulation can modulate auxin transport in the primary root tip, which enhances proton secretion for maintaining root growth. These studies suggest that a certain level of ABA is necessary to maintain the appropriate amount of auxin in water-stressed roots. Consistent with this possibility, IAA measurements in the studies presented in Chapter 2 indicated that IAA levels in the root growth zone of *vp14* roots were significantly lower than in the wild type under low Ψ_w conditions.

In the experiments reported in this chapter, two approaches were taken to examine the effects of altered IAA levels in the root growth zone of ABA-deficient roots at low ψ_w. First, two inhibitors of auxin transport, N-1-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), were utilized to modify rates of IAA transport in the root apical region. Second, IAA levels were directly increased by addition of IAA to the root growth zone. The results using both approaches support the hypothesis that the growth inhibition of ABA-deficient roots under water stress is causally related to decreased IAA levels in the root growth zone.

MATERIALS AND METHODS

vp14 and wild-type plant material

Studies presented in this chapter were conducted with homozygous *vp14* and near-isogenic wild-type seeds (W22 genetic background) that were produced in the field in Puerto Rico in 2009.

Growth experiments

All experiments utilized the following growth conditions. As described in Chapter 2, seeds were surface-sterilized in 5% NaClO for 45 min and rinsed with deionized water for 10 min. The seeds were then imbibed at 25 °C for 24 h in aerated 1 mM $CaSO_4$ solution. After 24 h, seeds were germinated between sterile sheets of germination paper moistened with 1 mM $CaSO_4$ at 29 °C in the dark. Seedlings with primary roots that were 5-15 mm in length were then transplanted into Plexiglas boxes containing vermiculite corresponding to a ψ_w of -1.57 MPa (mean ψ_w of all experiments in this chapter). The vermiculite was thoroughly mixed with a pre-determined volume of 1 mM $CaSO_4$ to obtain the desired ψ_w, which was measured by isopiestic thermocouple psychrometry in each experiment (Boyer and Knipling, 1965). The boxes were modified such that the middle portion of the box can be removed to gain access to the primary roots (Fig. 1). The seedlings were then grown at 29 ± 1 ºC and near-saturation humidity in the dark.

Experimental approach of the auxin manipulation studies

In preliminary studies, the IAA transport inhibitors NPA and TIBA were applied using the hydroponic culture system (Verslues *et al*., 1998; Smith, 2011). However, the

results demonstrated that continuous application of the inhibitors to the entire primary root of ABA-deficient and wild-type plants growing at low Ψ_w had a strongly negative effect on root elongation in both wild-type and *vp14* seedlings (data not shown). Therefore, an experimental approach was developed that allowed application of the IAA transport inhibitors, as well as IAA itself, via agarose blocks that were applied just above the growth zone of roots growing at low Ψ_w in the vermiculite seedling system. This method was based on a study of hydrotropism by Takano *et al*. (1995), which demonstrated that hydrotropism can be induced by applying small agarose blocks with and without the addition of sorbitol unilaterally to the tip of a primary root.

It was decided to apply the agarose blocks above the growth zone, rather than directly to the growth zone, for several reasons. First, auxin is transported towards the root tip in the inner cortex (acropetal transport) and is re-distributed away from the apex near the epidermal layers (basipetal transport) (Friml *et al*., 2002a). With the agarose blocks placed strategically above the growth zone, it was anticipated that one could potentially increase auxin levels within the apical region of the root by preferentially restricting basipetal transport. Second, direct manipulation of auxin levels in the growth zone is complex because concentration may vary depending on the region of the growth zone being investigated (Petersson *et al*., 2009). Third, in the growing region, there are areas of growth acceleration and deceleration that are associated with Ψ_w gradients, where lower Ψ_w occur in faster growing regions (Nonami and Boyer, 1989). This situation greatly complicates the determination of the appropriate Ψ_w of the agarose blocks (see below). In contrast, in the basal region of the root axis, cells have ceased elongation and tissues typically have a Ψ_w that is in equilibrium with the surrounding media. Lastly, as the apical region continues

to grow and the tissues elongate, agarose blocks placed on the growing region would become displaced toward the basal region of the root, thus making the manipulation of auxin levels in the growth zone increasingly complex. For these reasons, it was decided to bilaterally apply agarose blocks to the basal region immediately above the growth zone of wild-type and *vp14* primary roots.

Determining the Ψ_w of the agarose blocks

To ensure that the agarose blocks did not change the Ψ_w of the root, which could cause artefactual effects on root elongation, it was essential to adjust the Ψ_w of the blocks to be iso-osmotic with the root Ψ_w. Because the Ψ_w of the basal region determined the Ψ_w of the agarose block, it was critical to measure the tissue water status of this region accurately. Furthermore, there could be differences in the tissue Ψ_w between genotypes as well as changes over time. Therefore, the time-course of basal tissue Ψ_w of wild-type and *vp14* roots was examined. It is important to note that although the Ψ_w of the basal region determined the appropriate Ψ_w of the agarose block, the apical region Ψ_w could also influence the experimental design. As stated previously, the apical region of primary roots has a lower Ψ_w than the surrounding media and the mature region behind the growth zone, in order to drive water transport into the growing cells. If the Ψ_w of the apical region is lower than that of the agarose block added to mature tissues, water from the block could flow into the growing region. If the magnitude of this gradient differed between the wild-type and *vp14* roots, this could again confound results. Because of this potential complication, the apical region Ψ_w was also measured in both wild-type and *vp14* roots.

The length of the elongation zone in wild-type and *vp14* roots growing at a Ψ_w of -1.6 MPa was determined to be 0-6 and 0-4 mm from the root apex, respectively (Cho, 2006). When measuring the Ψ_w of growing tissue by excised tissue psychrometry, it is necessary to have some attached mature tissue to provide a water source and prevent cell wall relaxation during the measurement, which would otherwise erroneously lower the tissue Ψ_w (Cavalieri and Boyer, 1982). For this reason, an additional 6 and 4 mm of mature tissue, respectively, were included with the growth zones in wild-type and *vp14* plants. Accordingly, to accurately determine the Ψ_w of the apical and basal regions in wild-type and *vp14* plants, the Ψ_w of the apical 0-12/0-8 mm regions and the basal 13-20/11-20 mm regions were measured, respectively.

At 24, 36, 48 and 72 h after transplanting, the apical and basal regions from fifteen primary roots of each genotype were rapidly harvested and transferred to a Styrofoam ice chest that was lined with moist filter paper to maintain close to saturation humidity. The roots were accessed through sleeved-hand access ports. All subsequent tissue manipulations were performed within the box to minimize tissue water loss after excision. Five segments were placed inside each of three psychrometer cups, and the Ψ_w was then determined by isopiestic thermocouple psychrometry. Additionally, average primary root elongation at 24, 36, 48 and 72 h after transplanting was determined by marking the position of the primary root apices on the face of the Plexiglas boxes (using a green safe-light as described by Saab *et al.*, 1990).

Application of agarose blocks to wild-type and *vp14* primary roots

A pair of agarose blocks (2 mm^3) of known ψ_w was applied bilaterally to the 11-13 mm region of wild-type and *vp14* primary roots, as described in Takano *et al*. (1995) and Miyamoto *et al*. (2002) (Fig. 2). Agar (Sigma-Aldrich) at a concentration of 1% (w/v) was prepared in 50 mL deionized water. The agarose solution was then distributed between two polystyrene square petri plates (Fisher Scientific) at 25 mL per plate and left to solidify. Twenty-five mL of melibiose solution (0.22 g ml^{-1}/.0258 M) was poured on top of the solidified agarose in each plate to decrease the agarose ψ_w. After 24 h, the remaining solution on top of the plates was poured off and the plates were used for experiments. During the 24 h period, the melibiose diffused into the agar, thus lowering the ψ_w to -1.6 MPa (the same as the water potential of the vermiculite and basal region of wild-type and *vp14* primary roots-see Results). Preliminary tests showed that the 24-h period was sufficient for the ψ_w of the plates to reach equilibrium, which was verified by measurements of ψ_w of the top and bottom layers of the agarose (method developed by van der Weele *et al*., 2000 and modified by D. Smith). The reasoning for using melibiose as an osmoticum in these studies is described in Chapter 1-Supplement.

Application of NPA/TIBA and IAA to wild-type and *vp14* primary roots

A 1 mM stock of NPA solution was prepared by dissolving 29.13 mg of NPA (Sigma-Aldrich) in 100 mL of deionized water. From the 1 mM stock, 5 µL was added to 49.995 mL of agarose solution before solidification to give a final concentration of 0.1 µM NPA. A 1 mM stock of TIBA was prepared by dissolving 49.98 mg of TIBA (Sigma-Aldrich) in 50 mL of deionized water. From the 1 mM stock, 5 µL was added to 49.995

mL of agarose media before solidification to give a final concentration of 0.1 μM TIBA. In experiments where IAA was administered to the roots, a 1 mM stock solution was prepared by dissolving 17.51 mg of IAA (Sigma-Aldrich) in 5 mL of 1 N NaOH and bringing to 100 mL volume with deionized water. From the 1 mM stock, 5 μL (0.1 μM IAA), 25 μL (0.5 μM IAA) and 50 μL (1 μM IAA), was added to 49.995, 49.975, 49.95 mL of agarose solution before solidification.

In each of two separate auxin transport inhibitor experiments, one box of 30 seedlings was grown for each of the following six treatments; wild type; wild type + NPA; wild type + TIBA; *vp14*; *vp14* + NPA; *vp14* + TIBA. After 36 h of growth, agarose blocks (control plants) or agarose blocks containing 0.1 μM NPA or TIBA were placed at the basal 11-13 mm regions of wild-type and *vp14* roots and plants were left to grow until 60 h. In addition, each experiment included one box each of 30 wild-type and *vp14* seedlings that were grown for 36 h.

In each of two separate IAA addition experiments, one box of 30 seedlings was grown for each of the following eight treatments; wild type; wild type + 0.1μM IAA; wild type + 0.5μM IAA; wild type + 1μM IAA; *vp14*; *vp14* + 0.1μM IAA; *vp14* + 0.5μM IAA; *vp14* + 1μM IAA. After 36 h of growth, agarose blocks (control plants) or agarose blocks containing 0.1 μM IAA, 0.5 μM IAA or 1 μM IAA were placed at the basal 11-13 mm regions of wild-type and *vp14* roots and plants were left to grow until 60 h. In addition, each experiment included one box each of 30 wild-type and *vp14* seedlings that were grown for 36 h.

For all experiments, primary root lengths were recorded at transplant and until harvest at either 36 or 60 h by periodically marking the position of the root apices on the

face of the Plexiglas boxes. In each experiment, all 30 seedlings were removed from the growth boxes at 36 or 60 h and the apical 10 mm of the primary roots were harvested for ABA and IAA quantification (10 root tips per sample). The fresh weights of the root segments were measured, and the segments were then immediately frozen in liquid nitrogen and stored at -80°C. The segments were then freeze-dried, dry weights measured, and the samples were sent to the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center for hormone analysis, as described in Chapter 2.

Application of 1-MCP and IAA to wild-type and *vp14* primary roots

Additional experiments were conducted in which both 1-MCP and IAA were administered to wild-type and *vp14* roots growing at low ψ_w. In these experiments, one box of 30 seedlings was grown for each of the following six treatments; wild type; wild type + 1-MCP; wild type + 1-MCP + IAA; *vp14*; *vp14* + 1-MCP; *vp14* + 1-MCP + IAA. Plexiglas boxes were placed in six separate tanks (per genotype) as described in Chapter 2. Two boxes contained wild-type or *vp14* control plants, and the remaining four boxes contained wild-type or *vp14* plants with the addition of 1-MCP (200 mg L^{-1}). A fitted Plexiglas lid was immediately placed on the tanks and secured with heavy-duty vacuum grease and growth was monitored for 36 h. After 36 h, agarose blocks (control plants) or agarose blocks containing 1 μM IAA were placed bilaterally at the 11-13 mm region of one set of 1-MCP–treated wild-type and *vp14* primary roots and plants were left to grow for another 24 h. The remaining set of 1-MCP treated plants were left to grow for another 24 h without the addition of agarose blocks or agarose blocks containing IAA.

For all 1-MCP and IAA addition experiments, primary root lengths were recorded at transplant and until harvest at 60 h by periodically marking the position of the root apices on the face of the Plexiglas boxes (see Chapter 2 for details of 1-MCP replenishment after marking). After 60 h, tanks were taken to a flow-hood where the remaining 1-MCP gas was expelled and were then returned to the dark where the roots were harvested for final length measurements. The apical 10 mm of the roots of all seedlings were then harvested for ABA and IAA quantification as described above.

Application of ABA to *vp14* primary roots

A 1 mM ±ABA stock was prepared by dissolving 26.43 mg of ±ABA (Sigma-Aldrich) in 5 mL of absolute ethanol and bringing the solution to 100 mL with deionized water. After germination, wild-type and *vp14* seedlings with primary roots that were 5-15 mm in length were transplanted to Plexiglas boxes containing vermiculite at a mean ψ_w of -1.58 MPa (measured by isopiestic thermocouple psychrometry) that had been mixed with 1 mM $CaSO_4$ and ±ABA at a final concentration of 0.5 mM. The concentration of ABA was determined in previous studies as described in Sharp *et al*. (1994), Spollen *et al*. (2000) and Cho (2006).

In each of two separate experiments, one box of 30 seedlings was grown for each of the following three treatments; wild type; *vp14*; *vp14* + ABA. Primary root lengths were recorded at transplant and until harvest at 60 h by periodically marking the position of the root apices on the face of the Plexiglas boxes. In each experiment, all 30 seedlings were removed from the growth boxes at 60 h and the apical 10 mm of primary roots were harvested for ABA and IAA quantification as described above (10 root tips per sample).

RESULTS AND DISCUSSION

Root apical and basal region ψ_w analysis

Water potential measurements of the apical (encompassing the growth zone) and basal (adjacent to the growth zone) regions of the primary root of wild-type and *vp14* seedlings were made at 24, 36, 48 and 72 h after transplanting to low vermiculite at a ψ_w of -1.6 MPa (Fig. 3). In both regions, the results demonstrate that at 24 h, tissue ψ_w had not yet declined to the ψ_w of the vermiculite. However, by 36 h, the ψ_w of both regions in both genotypes had further decreased and reached a ψ_w of approximately -1.6 MPa, and remained at similar values at 48 and 72 h. There were no significant differences between the genotypes in either region at any time point.

It was anticipated that the ψ_w of the apical region would be lower than that of the basal region, due to the existence of growth-induced ψ_w gradients (Nonami and Boyer, 1989). However, results showed that this was not the case. This finding may have been due to the relatively large amount of mature tissue that was included with the growth zone for the apical region ψ_w measurements (to avoid cell wall relaxation errors), combined with the slow growth rates and hence diminished ψ_w gradients in water-stressed roots. Collectively, these effects may have obscured the ψ_w gradients between the apical and basal regions.

Corresponding to the root tip ψ_w data, primary root elongation rates of wild-type and *vp14* seedlings at 24, 36, 48 and 72 h are presented in Figure 4. These data were included as a baseline comparison to the subsequent experiments in which control agar blocks are added. Results show that wild-type roots grew at a significantly higher rate compared to *vp14* over the course of 72 h, and that the growth rate of *vp14* roots was stable.

However, the growth rate of wild-type plants decreased over time, potentially because of depletion of seed reserves by the 72 h time-point.

From the above data, it was concluded that the agarose blocks could be added to wild-type and *vp14* roots at the same Ψ_w and at the same time. Therefore, agarose blocks prepared to a water potential of -1.6 MPa and applied at 36 h were used for the auxin transport inhibitor and IAA addition studies.

Objective of auxin transport inhibitor and IAA addition studies

As described in Chapter 2, ABA-deficient primary roots growing under low ψ_w conditions exhibited reduced IAA levels in the root growth zone (Chapter 2, Fig. 4b). The goal of the following studies was to determine if restoring auxin levels could restore primary root elongation. To investigate this hypothesis, two methods of auxin manipulation were chosen. First, inhibitors of auxin transport (NPA, TIBA) were utilized in an attempt to disrupt the basipetal direction of auxin flow above the root growth zone, thereby increasing endogenous auxin levels within the growth zone, of ABA-deficient roots growing at low ψ_w (Fig. 5a). The second method of auxin manipulation involved the addition of IAA directly to ABA-deficient roots to restore normal IAA levels within the root growth of ABA-deficient roots growing under low ψ_w conditions (Fig. 5b).

Determining the appropriate NPA or TIBA concentrations.

A series of preliminary experiments was conducted to determine the appropriate concentrations of NPA and TIBA that would be effective in inhibiting auxin transport, yet non-toxic due to excessive concentration and/or long-term exposure. Since it was

previously demonstrated that the inhibition of elongation in *vp14* roots at low ψ_w could be almost fully prevented by treatment with AOA or AVG (Smith, 2011; Chapter 1-Supplement), and these inhibitors have also been implicated in affecting auxin levels (Soneo *et al*., 2010), recovery of root elongation in *vp14* was used as the primary indicator of NPA and TIBA effectiveness in these preliminary experiments. The objective was to identify the lowest NPA and TIBA concentrations that resulted in root growth recovery in the mutant without significantly inhibiting root growth in the wild type.

A range of both NPA and TIBA concentrations from 0.1-0.5 µM was studied. At concentrations from 0.2-0.5 µM, root elongation was strongly inhibited in wild-type and *vp14* roots, suggesting that these concentrations were potentially toxic (data not shown). In contrast, NPA and TIBA concentrations of 0.1 µM fully restored root elongation in *vp14* to a rate that was not significantly different from that in NPA or TIBA-treated or untreated wild-type seedlings (Fig. 6). In wild-type plants, neither NPA or TIBA had any significant effect on root elongation. Accordingly, NPA and TIBA concentrations of 0.1 µM were chosen for detailed evaluation of effects on root growth, auxin and ABA levels in *vp14* and wild-type roots under water stress conditions.

Growth measurements of wild-type and vp14 roots using NPA or TIBA to inhibit auxin transport.

Growth measurements were assessed during the 36 h prior to the application of NPA and TIBA and during the following 24 h period (Fig. 6). The root elongation rates of untreated wild-type and *vp14* seedlings to which agarose blocks without the inhibitors were applied were consistent with those in Figure 4, demonstrating that addition of the blocks did not have a negative effect on root elongation in either genotype. After the addition of

either NPA and TIBA, root growth rates of *vp14* increased to the same level as untreated wild-type roots (Fig. 6). Neither NPA or TIBA treatment had any effect on the growth of wild-type roots (Fig. 6). The detailed time-course of growth from the same experiment (Fig. 7) shows that addition of NPA or TIBA increased growth of *vp14* roots rapidly and to above wild-type rates by the 48 h time-point. This effect was transient, however, as the growth rates declined back to wild-type levels by 60 h.

IAA and ABA measurements of NPA- and TIBA-treated and untreated wild-type and vp14 roots.

Taken together, the results in Figures 6-7 suggest that the NPA and TIBA treatments could effectively be restoring IAA levels in the growth zone of ABA-deficient roots at low ψ_w, thereby resulting in recovery of root elongation to the wild-type rate. To test this hypothesis, the IAA content of the root apical 10 mm region was measured at both 36 and 60 h to determine respective levels before and after NPA and TIBA application (Fig. 8a). The results demonstrate that IAA levels were significantly reduced in untreated *vp14* compared to the wild-type at both 36 and 60 h, consistent with the results presented in Chapter 2. However, in both NPA- and TIBA-treated *vp14* roots, IAA levels were completely restored to wild-type levels by 60 h. In NPA- and TIBA-treated wild-type plants, in contrast, addition of the inhibitors had no effect on root tip IAA levels, suggesting that ABA sufficiency or alternative mechanisms may result in a greater ability to degrade the excess auxin in the growth zone. An alternate explanation could be that auxin transport in water-stressed wild-type roots was already blocked. Interestingly, this is not the first result observed of an apparent lack of result in wild-type plants in response to chemical inhibitors (Spollen *et al*., 2000; Smith, 2011; Chapter 1 Supplement; Chapter 2), which

could suggest that water-stressed wild-type roots exhibit a varied-range of insensitivity to applied ethylene and auxin inhibitors.

These results confirm that ABA-deficient roots under water stress are auxin deficient in the growth zone. Importantly, the results also demonstrate that primary root growth restoration in NPA- and TIBA-treated *vp14* plants was not the result of a restoration of ABA levels, which were significantly lower in *vp14* than in the wild-type at both 36 and 60 h after transplanting and were unaffected by the inhibitor treatments in both genotypes (Fig. 8b). Accordingly, this result indicates that inhibition of auxin transport using NPA or TIBA can effectively restore primary root elongation by restoring endogenous IAA levels in the root growth zone of ABA-deficient plants under water stress conditions.

Direct application of IAA restores root growth of ABA-deficient seedlings at low ψ_w

A series of experiments was conducted to determine an appropriate IAA concentration that would be effective in restoring auxin levels in the root growth zone of ABA-deficient roots at low ψ_w, yet non-toxic due to excessive concentration and/or long-term exposure. It has been established that water-stressed *vp14* roots are auxin deficient, and that inhibition of auxin transport using NPA and TIBA fully restored root elongation (Fig. 6; Fig. 7) and IAA levels (Fig. 8a) in the root growth zone. Accordingly, recovery of root elongation in *vp14* was used as the primary indicator of IAA effectiveness in these preliminary experiments. The objective was to identify the lowest IAA concentration that resulted in root growth recovery in the mutant without significantly inhibiting root growth in the wild type.

A range of IAA concentrations, 0.1, 0.5 and 1 µM, was studied. Growth measurements were assessed during the 36 h prior to the addition of IAA, and during the

following 24 h period (Fig. 9). The root elongation rates of untreated wild-type and *vp14* seedlings were consistent with those in Figures 6 and 7. After the addition of IAA, at all concentrations tested root elongation rates of *vp14* increased to the same level as untreated wild-type roots (Fig. 9). In contrast, none of the IAA treatments had a significant effect on the elongation of wild-type roots. The detailed time-course of root elongation in the same experiments (Fig. 10) shows that the addition of IAA at all concentrations tested rapidly promoted root elongation in *vp14*, such that at 48 h, root elongation rates were greater than in treated and untreated wild-type roots (Fig. 10).

Similar to the auxin transport inhibitor studies, this dramatic increase in *vp14* root elongation was a transient phenomenon. By 60 h, the growth rate of *vp14* roots treated with 0.1 µM IAA had returned to the wild-type rate and was also declining in the 0.5 µM treatment. However, roots treated with 1 µM IAA were still increasing in their elongation rate at 60 h (Fig. 10). While the goal of this experiment was to not to achieve an elongation rate in *vp14* that was higher than in the wild-type, these results are very interesting, suggesting that at least in ABA-deficient roots, supplemental IAA can stimulate root growth under water-stressed conditions. However, as already noted, this was not the case in wild-type roots.

IAA and ABA measurements of IAA-treated and un-treated wild-type and vp14 roots.

To further investigate the different responses to applied IAA between water-stressed *vp14* and wild-type roots, IAA levels were measured in the apical region of the roots with and without the applied IAA treatments (Fig. 11a). In addition, root tip IAA levels were measured in *vp14* and wild-type roots growing under well-watered conditions.

Consistent with previous results (Fig. 8a), IAA levels were significantly reduced in untreated *vp14* compared with wild-type roots at both 36 and 60 h after transplanting to low ψ_w conditions (Fig. 11a). In the 0.1 µM IAA treatment, at 60 h, IAA levels in the apical region of *vp14* roots was restored correlating with the recovery of root elongation to the wild-type rate (Fig. 9; Fig. 10). The 0.1 µM IAA treatment did not significantly increase IAA levels in the wild-type roots, consistent with the lack of effect on root elongation in this treatment. Interestingly, however, IAA levels in the 0.5 and 1.0 µM IAA treatments were significantly higher in both wild-type and *vp14* roots than in the untreated and 0.1 µM IAA treatments. These increases in IAA content were an anticipated result for the *vp14* roots based upon the increase in root elongation to above wild-type rates seen at 60 h after transplanting during the time-course of growth (Fig. 10). However, the increases in IAA content in the 0.5 and 1 µM IAA treatments of wild-type roots were not expected, since there was no effect on root elongation in these treatments. These data suggest that while the applied IAA was taken up similarly by the wild-type and *vp14* roots, this translated to an increase in root elongation only in the *vp14* mutant and not in the wild-type roots. Accordingly, these findings suggest that wild-type roots could be insensitive to variations in IAA content in this range as a result of their normal levels of ABA accumulation. Importantly, ABA measurements confirmed that the restoration of root elongation by the applied IAA treatments in *vp14* roots was not due to restoration of ABA content in the root apical region was significantly reduced in untreated *vp14* compared with wild-type roots at both 36 and 60 h and was unaffected in either genotype in any of the applied IAA treatments (Fig. 11b).

The results of the applied IAA experiments confirm that the reduced elongation of ABA-deficient roots at low ψ_w is casually related to reduced levels of IAA in the root growth zone. Further, the results demonstrate the increasing IAA to above the normal wild-type levels can enhance root elongation to above wild-type rates, but only in an ABA-deficient background.

Interestingly, while the results show that ABA-deficient roots at low ψ_w are IAA-deficient compared with the wild-type, both genotypes had reduced IAA contents compared with their well-watered controls (Fig. 11a). Accordingly, while the findings of this study indicate that IAA plays a growth-maintaining role in water-stressed roots, consistent with the conclusions of Xu *et al*. (2012), this effect occurs despite a stress-induced reduction in IAA levels in the root growth zone. This latter result is not consistent with previous findings by Ribaut and Pilet (1994), who showed that in maize primary roots under water stress, IAA levels in the apical region were significantly higher when compared to well-watered roots. The explanation for this difference is not clear, but could be due to the differences in methods, genetic background and severity of stress imposition. Ribaut and Pilet (1994) imposed low ψ_w by addition of mannitol in a hydroponic medium, resulting in rapid stress development to -1.66 MPa over a 1, 2 or 3 h period.

Objective of 1-MCP and IAA addition studies

As described in Chapter 2, treatment with the inhibitor of ethylene binding 1-MCP completely restored the auxin level in the root growth zone of ABA-deficient plants growing at low ψ_w (Chapter 2, Fig. 4b). However, the restoration of auxin content did not translate to a recovery of root growth, in contrast to the results of the auxin transport

inhibitor and IAA addition studies (Fig. 6; Fig. 9). These contrasting results suggest the hypothesis that an active ethylene response is required for the growth-promotive effect of IAA in water-stressed roots. To test this hypothesis, the purpose of the following studies was to determine if 1-MCP pre-treatment prevents the recovery of root growth following application of IAA to *vp14* roots under low ψ_w conditions. As described in Chapter 2, seedlings were exposed to a 1-MCP concentration of 200 mg L^{-1} from 0-36 h after transplanting. At 36 h, agarose blocks containing 1 µM IAA (the highest concentration tested in the previous experiments) were applied and root lengths were then measured at 48 and 60 h.

Growth measurements of wild-type and vp14 seedlings using 1-MCP and IAA to restore primary root elongation at low ψ_w

During the first 36 h, 1-MCP treatment had no effect on the root growth of either wild-type or *vp14* roots (Fig 12; Fig. 13), similar to results previously shown in Chapter 2. Following the addition of IAA at 36 h, there was no significant effect on root growth in 1-MCP-treated or untreated wild-type plants, consistent with the lack of applied IAA in the wild-type in previous experiments (Fig. 9; Fig. 10). However, in contrast to the previous finding that IAA could restore root growth in *vp14* seedlings, the addition of IAA to 1-MCP-treated *vp14* roots had no effect on root elongation (Fig. 12; Fig. 13). These results support the hypothesis that the ability of auxin to restore primary root elongation in ABA-deficient plants growing at low ψ_w is dependent on a functional ethylene pathway, and are consistent with several previous studies. For example, Ruizikia *et al*. (2007) demonstrated that ethylene can regulate *Arabidopsis* root growth through effects on auxin biosynthesis and transport-dependent auxin distribution, and studies by Strader *et al*. (2010) showed that

ethylene can direct auxin to control *Arabidopsis* root epidermal cell expansion by utilizing the *eto1* ethylene overproducer mutant. Results demonstrated that overproduction of ethylene could partially restore auxin responsiveness in these mutants, suggesting that both auxin and ethylene are tightly regulated. Also, Stepanova *et al*. (2007) showed that there is early activation of auxin and ethylene responses at the root-cell level as well as changes in gene expression in wild-type versus auxin and ethylene *Arabidopsis* mutants, suggesting that both auxin and ethylene can regulate each other's biosynthesis and response pathways. Additionally, the tightly-regulated interactions between auxin and ethylene can be seen quite clearly in auxin mutants, as they exhibit potent ethylene response defects (Swarup *et al*., 2002)

Objective of ABA addition studies

The results of the IAA transport inhibitor and IAA application studies indicate that IAA deficiency in the growth zone is a major cause of the inhibition of root growth in ABA-deficient seedlings under low ψ_w conditions. To confirm that the reduced IAA levels in *vp14* roots were indeed the result of ABA-deficiency and not a pleiotropic effect of the mutation, the following studies were conducted to examine whether restoration of ABA levels could restore endogenous IAA levels in the root growth zone.

As demonstrated in Cho (2006) and Smith (2011), addition of ABA at a concentration of 0.5 mM to *vp14* roots growing at low ψ_w (-1.6 MPa) can completely restore primary root growth in association with restoration of root tip ABA content. This protocol was based on the findings of Sharp *et al*. (1994), who demonstrated that the requirement for such a high applied ABA concentration to restore the internal level in the

root tip of fluridone-treated and *vp5* mutant seedlings was because of very limited ABA uptake from the dry media. Similar results are shown in Figures 14 and 15.

Growth measurements of wild-type and vp14 seedlings using ABA to restore primary root elongation at low ψ_w

Root length measurements of *vp14* seedlings were assessed during 60 h after transplanting to low ψ_w vermiculite with and without the addition of 0.5 mM ABA (Fig. 14). The results demonstrate that root elongation was restored almost to the rate of wild-type roots by as early as 12 h after transplanting and was maintained at this rate throughout the remaining 48 h (Fig. 14). These results are comparable to the data presented in Spollen *et al*. (2000), where ABA was added back to FLU-treated and *vp5* roots growing at the same low ψ_w conditions.

IAA and ABA measurements of wild type, vp14 and ABA-treated vp14 plants.

The IAA content of the root apical 10 mm region was measured at 60 h to determine levels without and with ABA addition (Fig. 15a). The results again show that IAA levels were significantly reduced in untreated *vp14* compared to wild-type roots, and demonstrated that in ABA-treated *vp14* plants, root-tip IAA content was completely restored to wild-type levels. The ABA content of the root apical 10 mm region was measured at 60 h to determine levels after experiment completion (Fig. 15b). Consistent with previous results, these results again show that ABA levels were significantly reduced in *vp14* compared to wild-type in un-treated roots. Additionally, in ABA-treated *vp14* roots, root-tip ABA content was completely restored to wild-type levels. These results using the ABA-deficient mutant *vp14* confirm previous studies of Cho (2006),

demonstrating that ABA is required for root growth maintenance under water stress conditions.

Taken together, the results of the IAA transport inhibitor, IAA addition and ABA addition studies confirm that an important role of ABA in sustaining primary root growth under water stress conditions is to maintain auxin levels in the root growth zone at an appropriate level.

CONCLUSIONS

The findings presented in this chapter describe the interactions of ABA, auxin and ethylene in the regulation of maize primary root growth under low ψ_w conditions. First, the results using 1-MCP to specifically block ethylene action demonstrated that increased ethylene (and ROS) are not the cause of the inhibition of growth in ABA-deficient roots under water stress, in contrast to previous conclusions (Spollen *et al*., 2000). Instead, the results revealed that at low Ψ_w, ABA-deficiency causes inhibition of primary root elongation in association with decreased auxin content in the root growth zone. It was demonstrated that root growth could be restored by restoring the IAA content of the root growth zone using each of three independent methods: manipulation of auxin transport using the IAA transport inhibitors NPA and TIBA, direct application of IAA, and application of ABA to restore ABA content. The hypothesized mechanism of the auxin transport inhibitors in restoring the IAA levels in the root growth zone is that by applying the compounds directly above the growth zone, they successfully disrupted basipetal auxin flow and thereby increased the endogenous IAA levels within the growth zone of ABA-deficient roots (Fig. 16a). Restoration of auxin content and root growth was also achieved

by direct application of IAA to ABA-deficient roots (Fig. 16b). Finally, the IAA levels in the root growth zone were also restored by restoration of ABA levels, confirming that the reduced levels of IAA in *vp14* roots under water stress are a direct result of ABA deficiency. Taken together, the results suggest that the ratio of auxin and ABA is a critical component in the regulation of primary root growth under water-stressed conditions. These findings are in agreement with those of Horemans *et al*. (1986), who showed that the concentration gradients of ABA and IAA within the growing region of bean hypocotyl were increased compared to the mature region with lower levels correlating with decreased cell expansion, suggesting that ABA and IAA are tightly-linked in the regulation of growth.

Supporting the above findings are studies by Rowe *et al*. (2016) who demonstrated in *Arabidopsis* that auxin transport via PIN1 is limited under osmotic stress conditions, that enhanced PIN2 activity can lead to a lead to a reduction in auxin content, meristem size and primary root growth, and that these processes are regulated by ABA. A study by Wang *et al*. (2017) showed in rice that the addition of ABA can modulate mRNA expression levels of *OsPIN* genes involved in auxin transport. Additionally, this group tested if ABA levels could influence local auxin concentrations, using a *DR5-GUS* reporter, in rice roots. The results showed that increased concentrations of ABA could increase *DR5* expression levels in the root tip and outer dermal layers of the primary root, which resulted in increased root elongation. The present findings are also consistent with those of Xu *et al*. (2012), who showed that ABA can modulate auxin transport in the root tip of *Arabidopsis* and rice to maintain root growth under moderate water stress conditions. Collectively, these findings suggest that an interaction of ABA with auxin influx and efflux proteins is

important for regulating IAA levels and, therefore, primary root growth under water-stressed conditions.

The present results also suggest that the role of IAA in maintaining root growth under water stress in *vp14* roots requires a functional ethylene pathway (Fig. 16c). Consistent with these findings is a study by Strader *et al*. (2010), who demonstrated in Arabidopsis roots that ethylene directs auxin to control cell expansion. Inconsistent with the above findings is a study by Li *et al*. (2017), who demonstrated in *Arabidposis* that at low ABA concentrations, root growth operates via an ethylene-independent pathway and requires auxin signaling and transport.

Taken together, the results presented in this chapter involving the interactions of ABA and auxin will lead to a greater understanding of the signal transduction pathways and other primary mechanisms involved in the regulation of root growth maintenance by ABA under water deficit conditions.

GENERAL DISCUSSION

The findings presented in this dissertation described the complex interactions of multiple hormones including ABA, auxin and ethylene in the regulation of maize primary root growth under water-stressed conditions. These studies demonstrated that ABA, auxin and their interactions are responsible for growth maintenance of roots growing under water stress conditions. Several studies have provided evidence that ABA and auxin-related genes and proteins are altered under abiotic stress conditions including salinity, high-light and flooding. However, there are very few water-stress specific studies in the literature to support a direct interaction of ABA and auxin in the maintenance of root elongation.

Among the few is a study by Xu *et al*. (2012), who provided some definitive evidence that a function of ABA in *Arabidopsis* and rice is to modulate auxin transport in the root growth zone for maintenance of growth by enhancing proton secretion. The studies reported in Chapters 2 and 3 of this study provide conclusive evidence that ABA-deficient water-stressed maize primary roots are auxin deficient, and that the restoration of normal IAA levels can completely restore root elongation. Consistent with the studies of Xu *et al*. (2012), it was demonstrated that ABA is directly regulating auxin levels in the growth zone resulting in an overall adaptation of roots growing under water stress. The significance of the findings presented here make a clear indication of the importance of hormone balance, ratios and sensitivity, and how a shift in any of these components can completely alter the growth response.

The detailed studies presented in this dissertation establish a physiological foundation of how root growth responds to low ψ_w conditions. What has not been speculated on are the proposed mechanisms by which auxin is able to completely restore root elongation in ABA-deficient water-stressed plants. It is plausible that auxin itself has a direct effect on aquaporin activity independent of ABA directly correlating to increased water uptake which is one aspect of plant development that could account for the increase in growth rate achieved in auxin manipulation studies. To support this speculation, a study by Peret *et al*. (2012) demonstrated in *Arabidopsis* that auxin regulates aquaporin function to facilitate lateral root emergence. Additionally, a complete restoration of growth would require increases in either cell division, elongation or both. Increased cell elongation would require modifications of cell wall extension, which is facilitated by proton efflux by way of the plasma membrane ATPase. Auxin is known to play a vital role in this tightly-

regulated process. What is not understood from the studies presented here and which requires more intense investigation is determining the ABA and auxin-inducible genes and proteins involved in the maintenance of root growth of plants growing under water-limited conditions. Moving forward, further understanding of gene and protein regulation and the signal transduction pathways activated or deactivated would provide some key elements to further understand the primary mechanisms involved in the interaction of ABA and IAA in the maintenance of root growth under low ψ_w conditions.

Figure 1. Design of the Plexiglas growth boxes used to apply agarose blocks to the primary roots of seedlings growing in vermiculite at low ψ_w. The middle portion of the box can be removed to gain access to the apical region of the primary roots.

Figure 2. Illustration depicting a maize seedling transplanted to low ψ_w vermiculite (-1.6 MPa) with the addition of agarose blocks at the region 11-13 mm from the apex of the primary root.

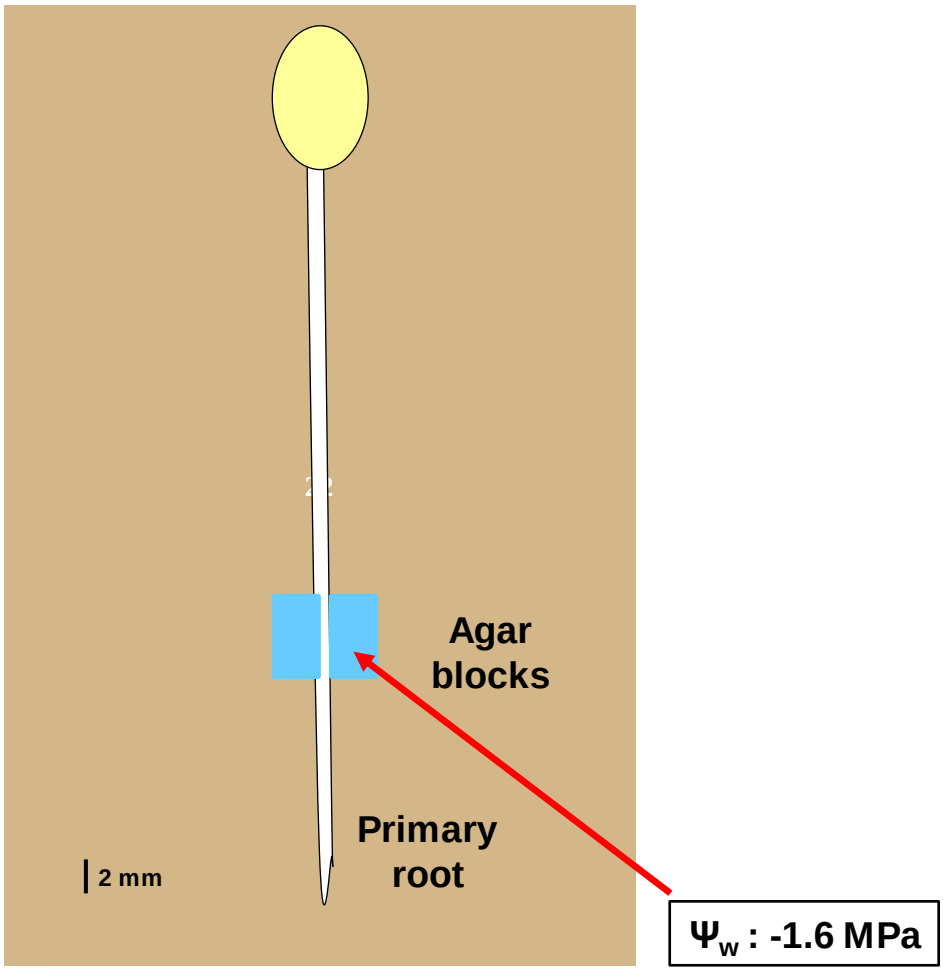


Figure 3. Time course of tissue ψ_w in the growth zone and adjacent mature region of the primary root of wild-type and *vp14* seedlings after transplanting to vermiculite at a ψ_w of -1.6 MPa. **(A)** The apical 0-12/0-8 mm (wild type/*vp14*) and **(B)** basal 13-20/11-20 mm (wild type/*vp14*) regions of the roots were measured. A two-way ANOVA for each set of data was used to compare wild-type and *vp14* over different times. Data are means ± SE (n=3) and different letters indicate significant differences ($p < 0.05$).

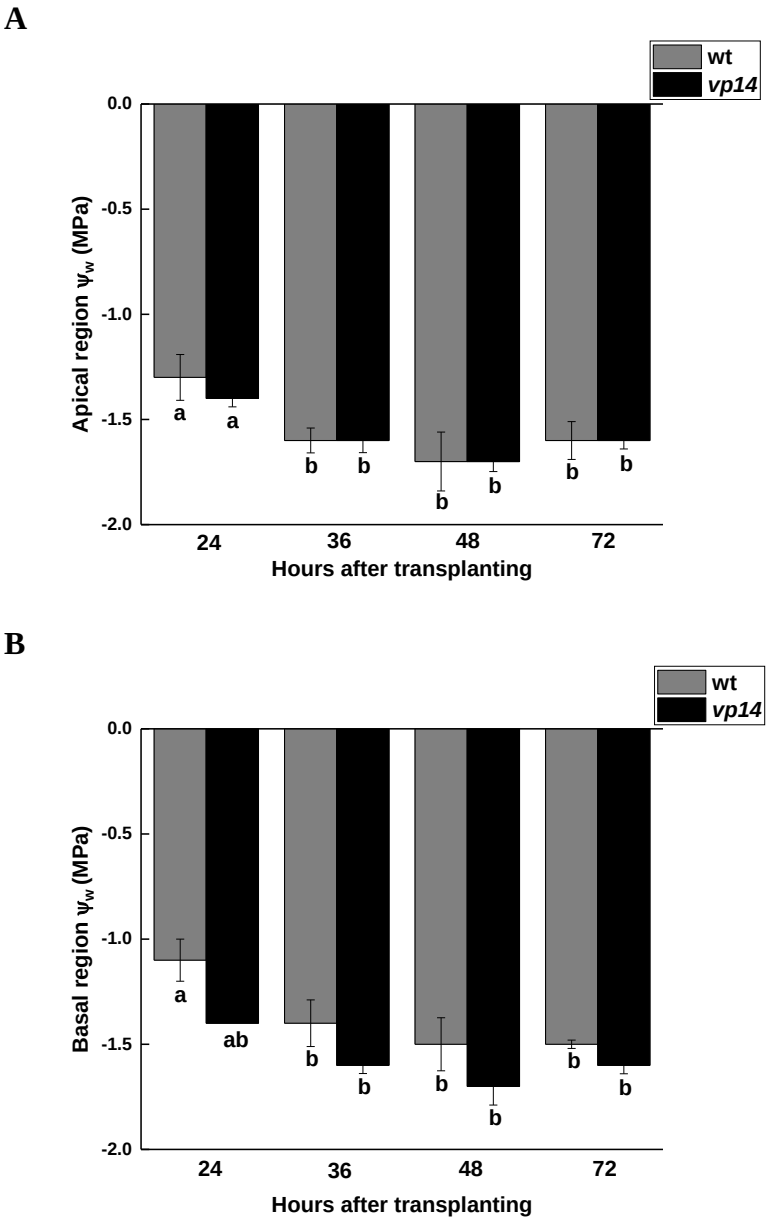


Figure 4. Average primary root elongation rates of wild-type and *vp14* seedlings during 72 h after transplanting to vermiculite at a ψ_w of -1.6 MPa. Data are means ± SE (n=30/time-period, combined from two experiments). A two-way ANOVA was used to compare wild-type and *vp14* roots over different times after transplanting and different letters indicate significant differences ($p < 0.05$).

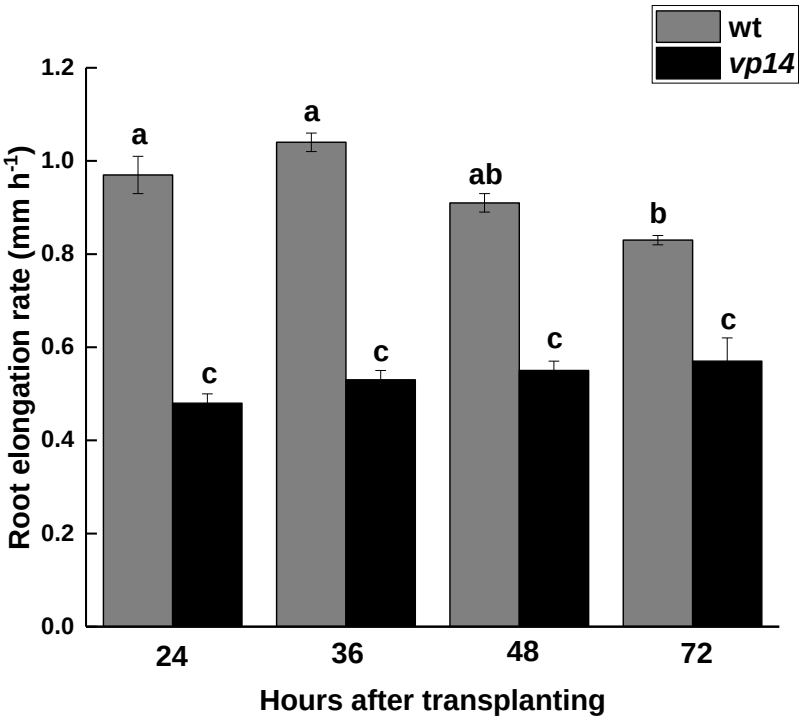


Figure 5. (A) Illustration depicting how blocking auxin transport via application of the IAA transport inhibitors NPA and TIBA above the root growth zone could preferentially disrupt the basipetal flow of IAA in the outer tissues, thereby increasing IAA levels within the growth zone and resulting in root growth recovery of ABA-deficient seedlings growing at low ψ_w. **(B)** Illustration depicting how application of IAA above the root growth zone could increase IAA levels within the root growth zone, thereby resulting in root growth recovery of ABA-deficient seedlings growing at ψ_w.

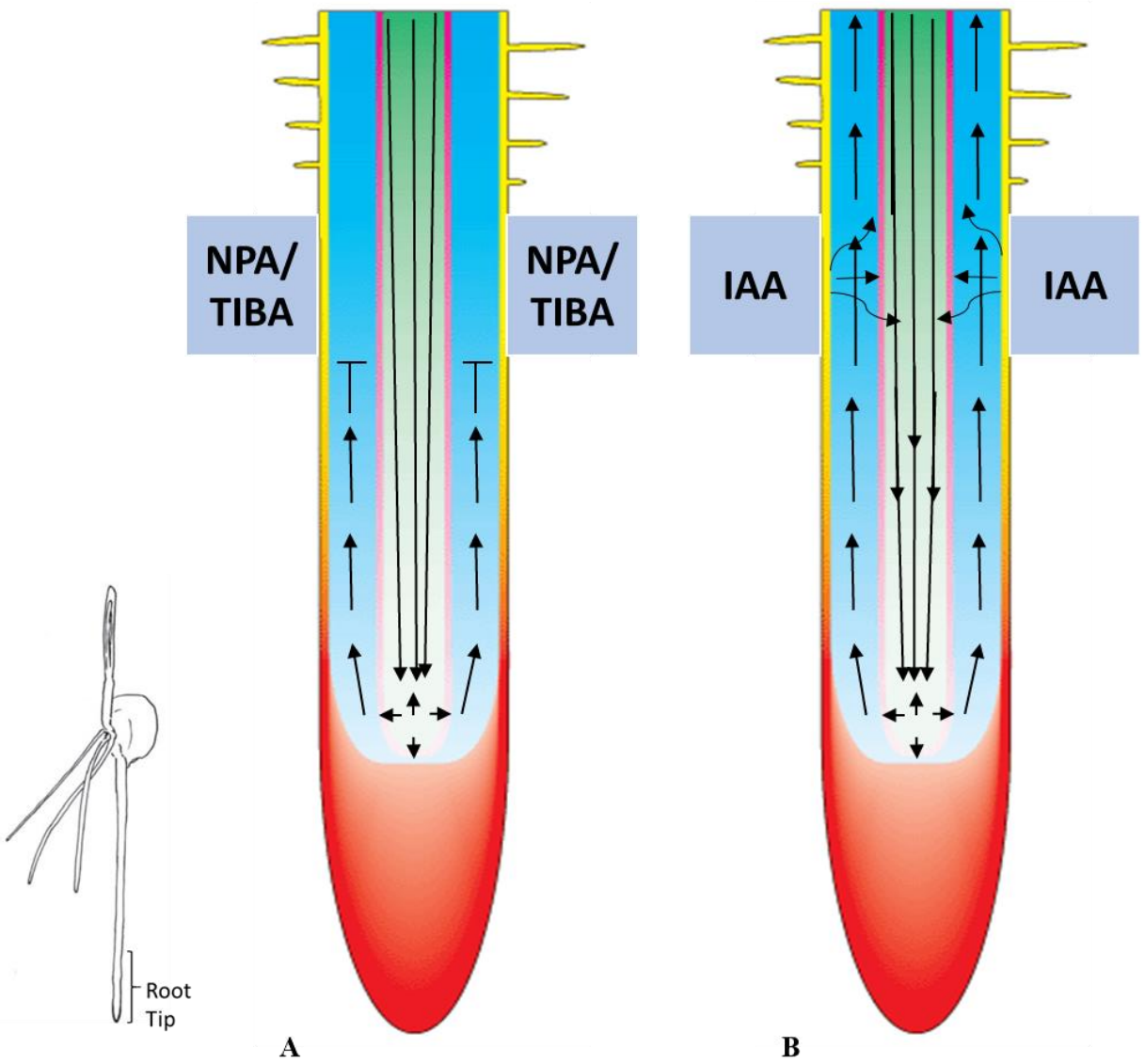

NPA/
TIBA
NPA/
TIBA
IAA
IAA
Root
Tip
A
B

Figure 6. Primary root elongation rates of untreated wild-type and *vp14* seedlings during 0-36 h and 36-60 h after transplanting to low ψ_w vermiculite (-1.6 MPa), and of wild-type and *vp14* seedlings during the 36-60 h period following application of NPA and TIBA via agarose blocks applied at 36 h. Agarose blocks without inhibitors were applied to the un-treated control roots. Data are means ± SE (n = 60, combined from two experiments). A one-way ANOVA was used to compare data, and different letters indicate significant differences ($p < 0.05$).

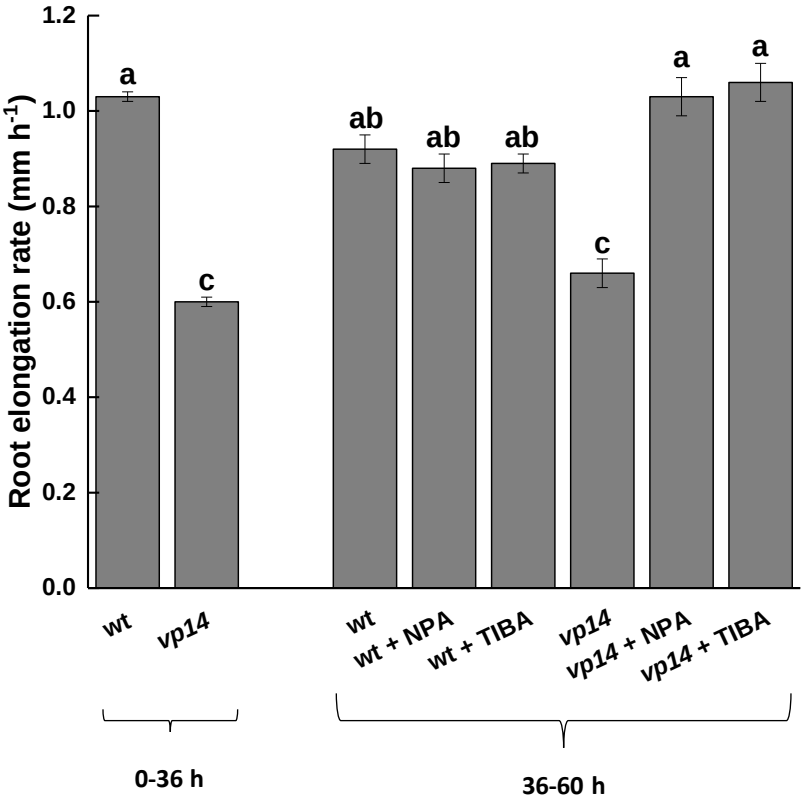


Figure 7. Primary root elongation rates during consecutive 12 h periods of NPA and TIBA-treated (applied at 36 h) and untreated wild-type and *vp14* seedlings during 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n = 60, combined from two experiments). See Figure 6 legend for additional details of treatment application.

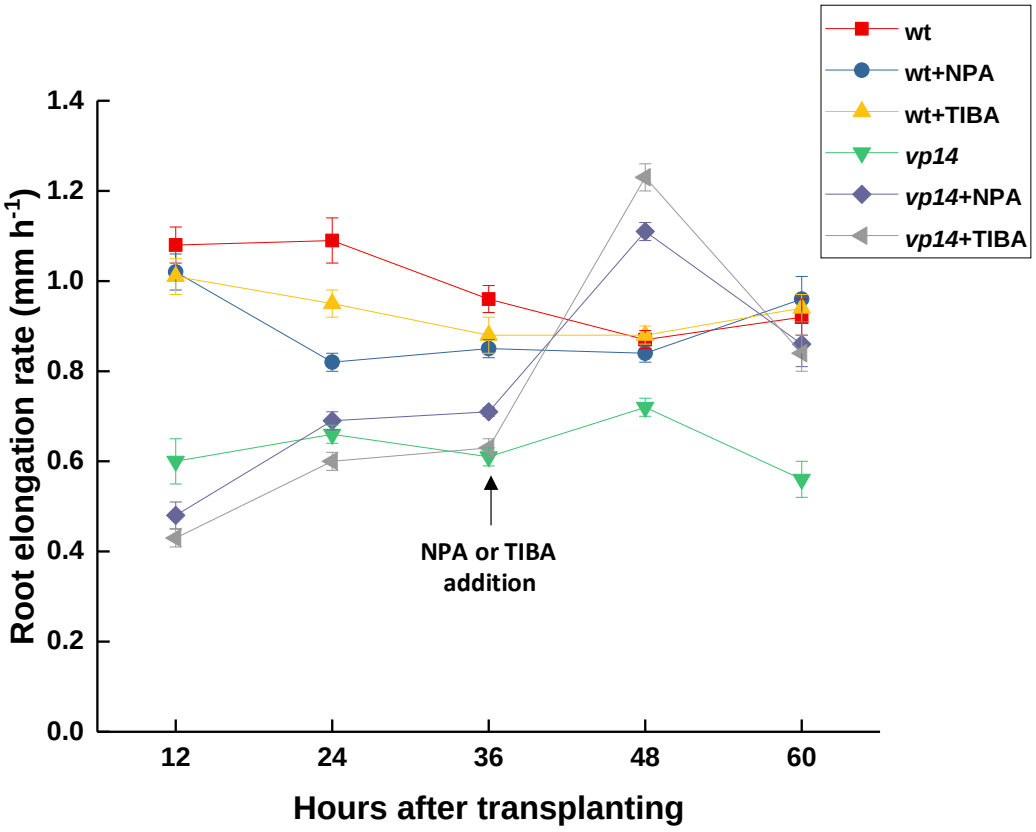
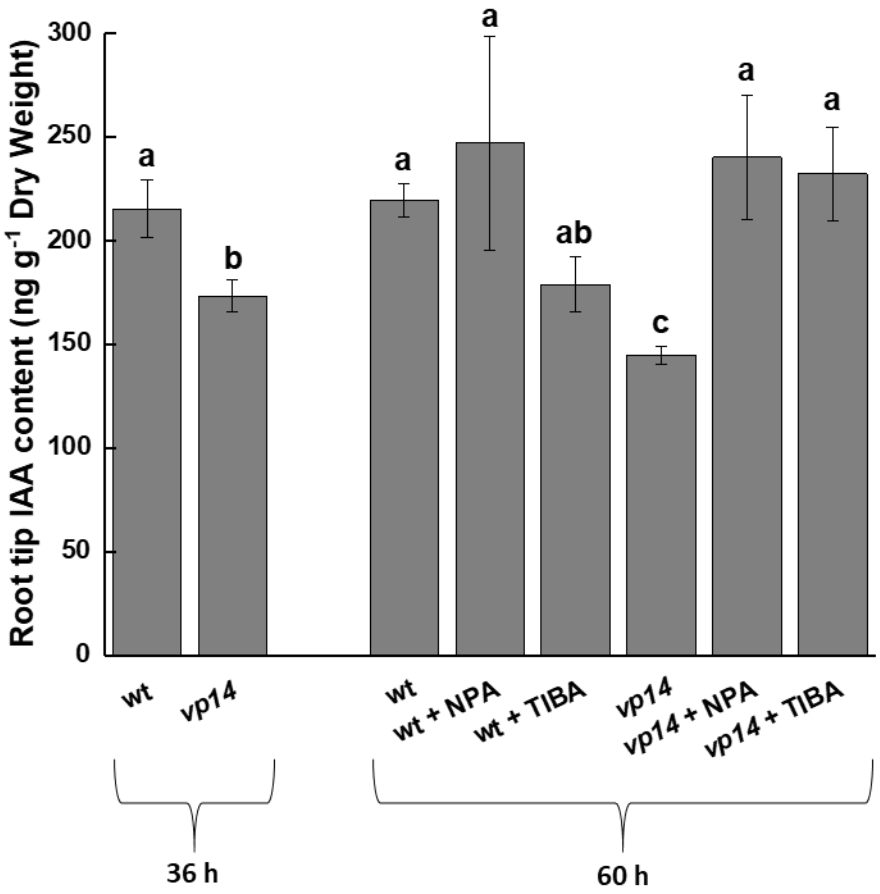


Figure 8. (A) IAA content and **(B)** ABA content of the primary root tip (apical 10 mm) of un-treated wild-type and *vp14* seedlings at 36 and 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa), and of wild-type and *vp14* seedlings treated with NPA or TIBA at 60 h following application of the inhibitors at 36 h. Data are means ± SE (n=6, combined from two experiments). In each set of data, a one-way ANOVA was used to compare data and different letters indicate significant differences ($p < 0.05$). See Figure 6 legend for additional details of treatment application.

A



B

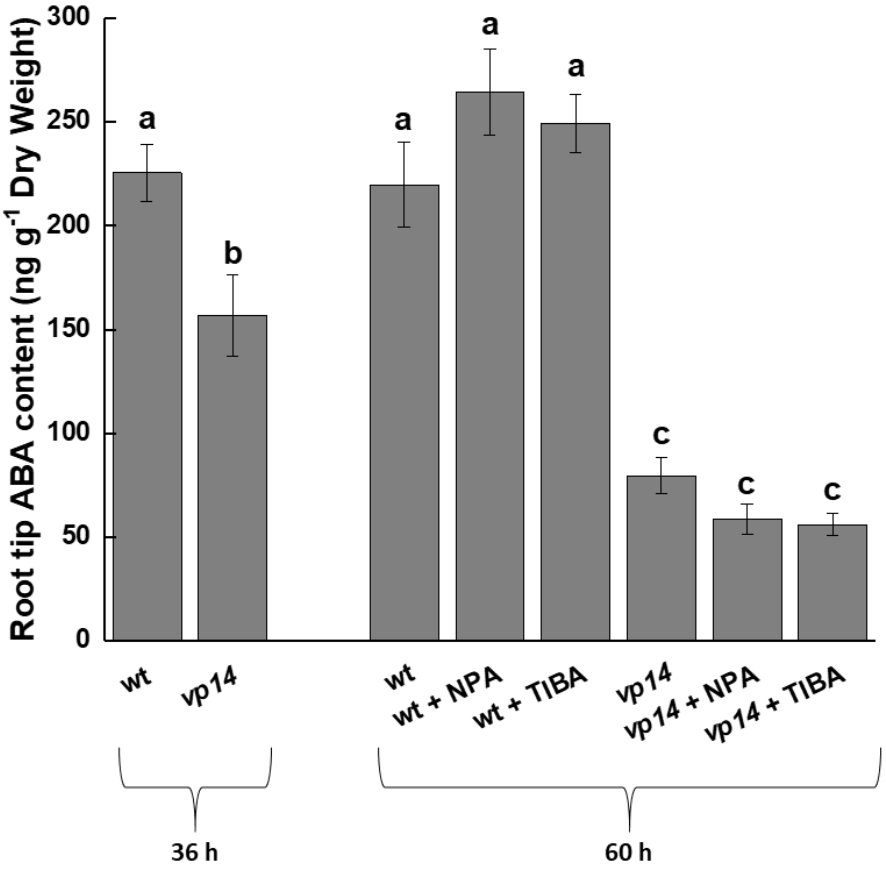

Root tip ABA content (ng g-1 Dry Weight)
300
250
200
150
100
50
0
a
b
a
a
a
c
c
c
wt
vp14
wt
wt + NPA
wt + TIBA
vp14
vp14 + NPA
vp14 + TIBA
36 h
60 h

Figure 9. Primary root elongation rates of un-treated wild-type and *vp14* seedlings during 0-36 and 36-60 h after transplanting to low ψ_w vermiculite (-1.6 MPa), and of wild-type and *vp14* seedlings during the 36-60 h period following application of various concentrations of IAA via agarose blocks applied at 36 h. Agarose blocks without IAA were applied to the untreated control roots. Data are means ± SE (n = 60, combined from two experiments). A one-way ANOVA was used to compare data, and different letters indicate significant differences ($p < 0.05$).

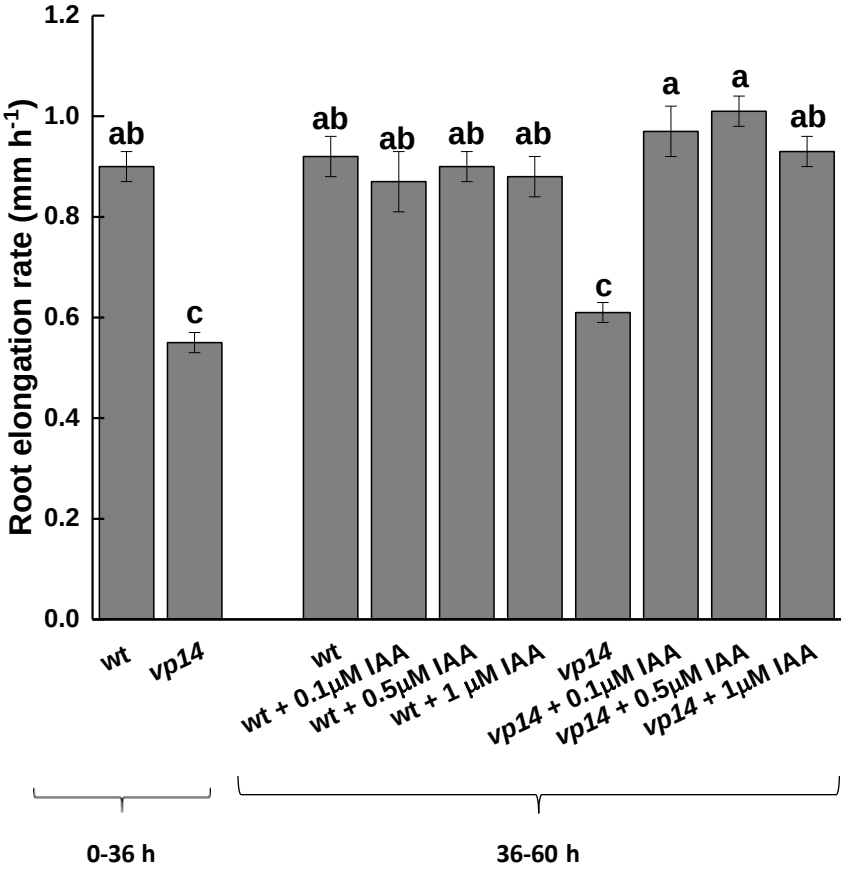


Figure 10. Primary root elongation rates during consecutive 12 h periods of IAA-treated (applied at 36 h) and untreated wild-type and *vp14* seedlings during 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n = 60, combined from two experiments). See Figure 9 legend for additional details of treatment application.

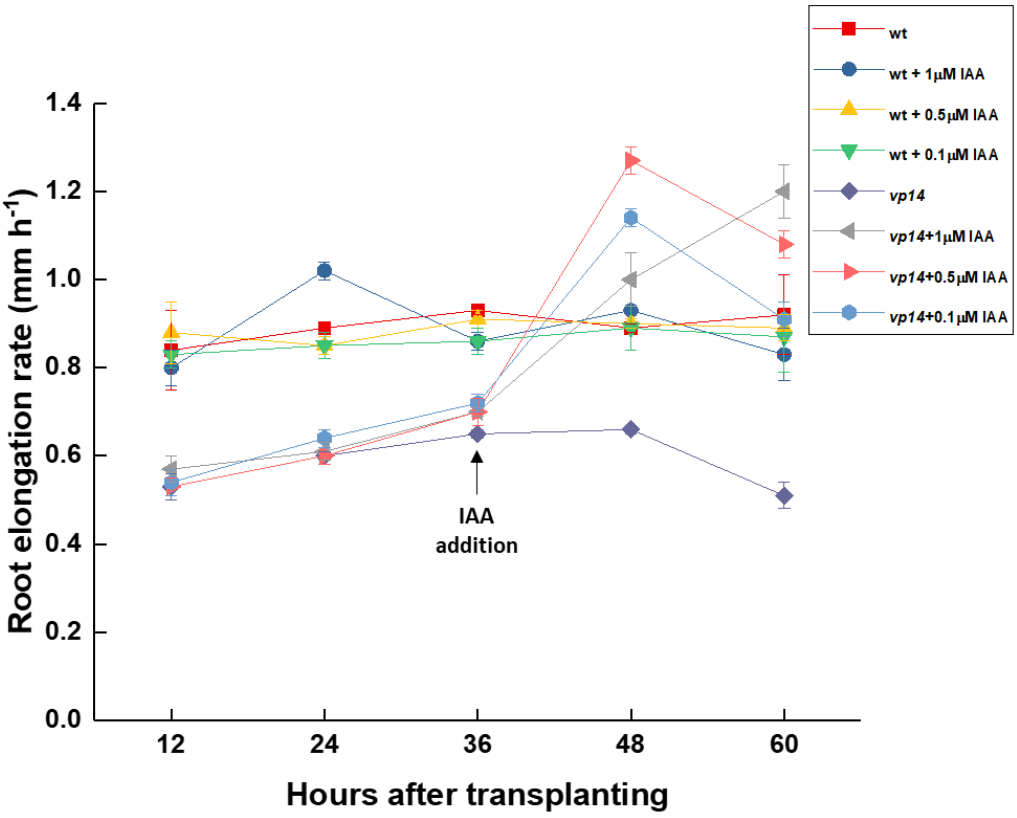
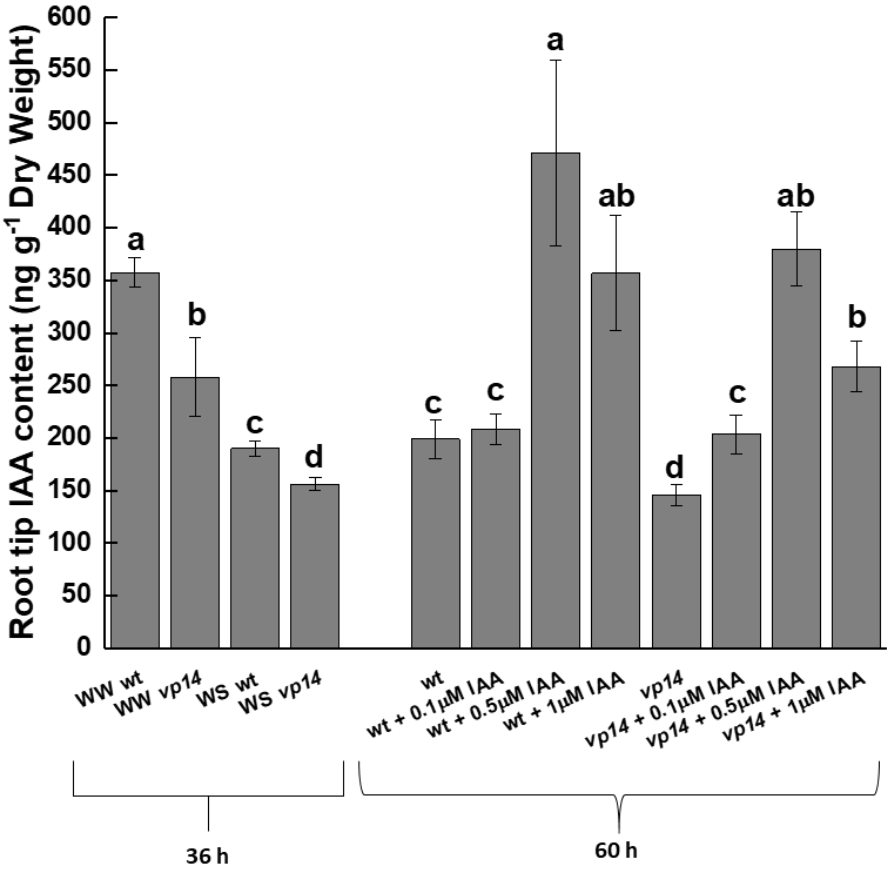


Figure 11. (A) IAA content and **(B)** ABA content of well-watered (WW, -0.03 MPa) and water-stressed (WS, -1.6 MPa) untreated roots of the primary root tip (apical 10 mm) of wild-type and *vp14* seedlings at 36 h after transplanting, and of untreated or IAA-treated roots of water-stressed seedlings at 60 h following application of IAA at 36 h. In each figure, data are means ± SE (n=6, combined from two experiments) and a one-way ANOVA was used to compare data; different letters indicate significant differences ($p < 0.05$). See Figure 9 legend for additional details of treatment application.

A



B

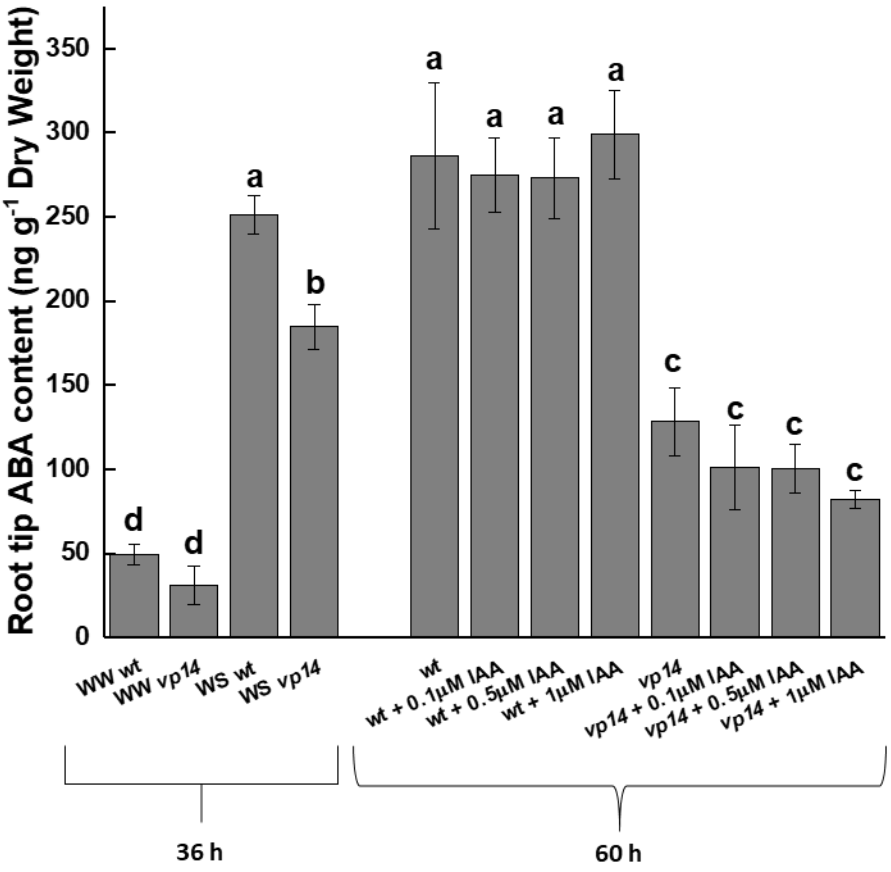

Root tip ABA content (ng g-1 Dry Weight)
0
50
100
150
200
250
300
350
d
d
a
b
a
a
a
a
c
c
c
c
WW wt
WW vp14
WS wt
WS vp14
wt
wt + 0.1µM IAA
wt + 0.5µM IAA
wt + 1µM IAA
vp14
vp14 + 0.1µM IAA
vp14 + 0.5µM IAA
vp14 + 1µM IAA
36 h
60 h

Figure 12. Primary root elongation rates of un-treated and 1-MCP-treated wild-type and *vp14* seedlings during 0-36 and 36-60 h after transplanting to low ψ_w vermiculite (-1.6 MPa), and of 1-MCP-treated wild-type and *vp14* seedlings during the 36-60 h period following application of 1µM IAA via agarose blocks applied at 36 h. Agarose blocks without IAA were applied to the untreated and 1-MCP control roots. Data are means ± SE (n = 30). A one-way ANOVA was used to compare data and different letters indicate significant differences ($p < 0.05$).

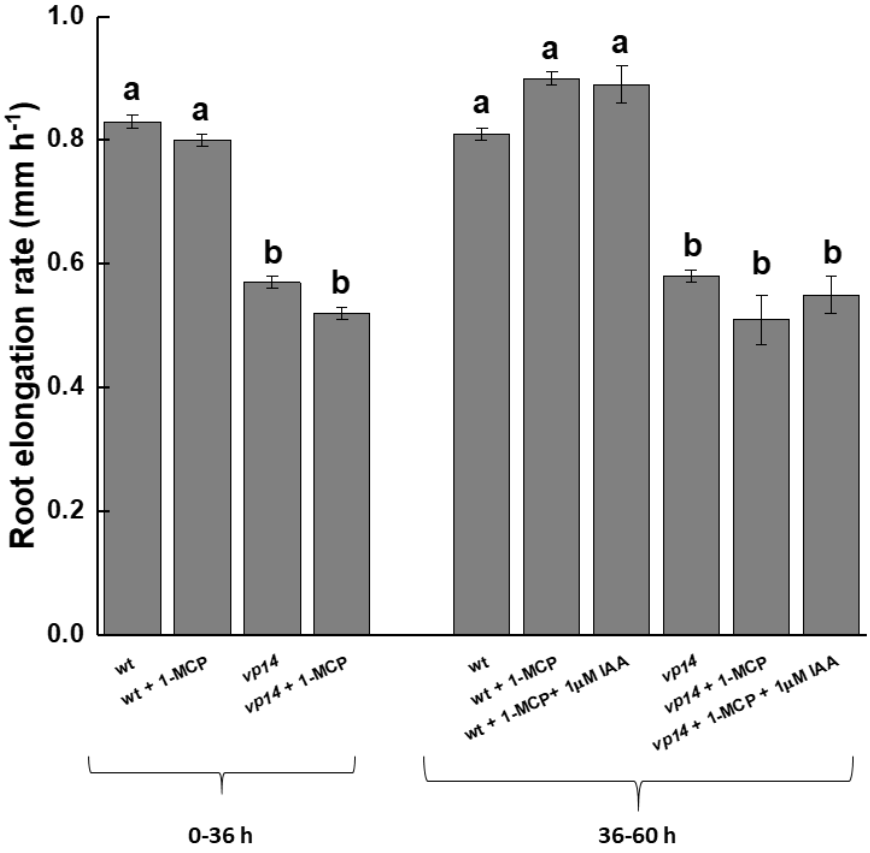


Figure 13. Primary root elongation rates during consecutive 12 h periods of 1-MCP (added at time 0), IAA-treated (added at 36 h) and untreated wild-type and *vp14* seedlings during 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n =30). See Figure 12 legend for additional details of treatment application.

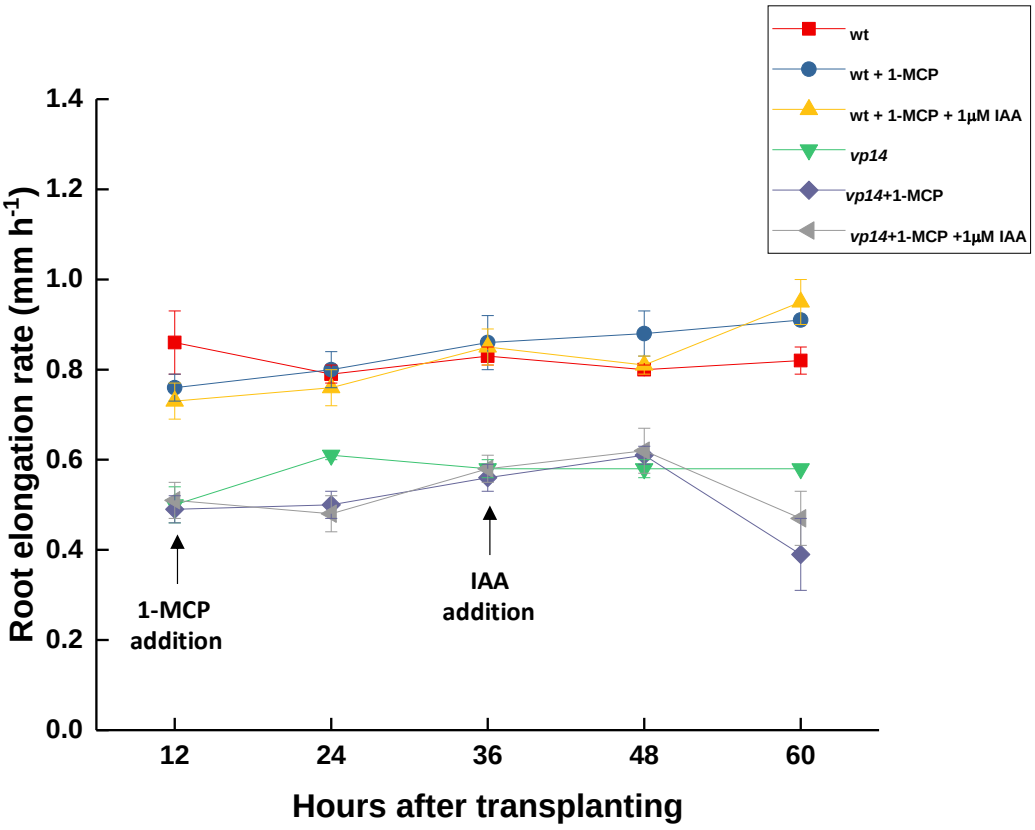


Figure 14. Root length increase of wild-type, *vp14* and ABA-treated *vp14* seedlings during 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n =60), combined from two experiments.

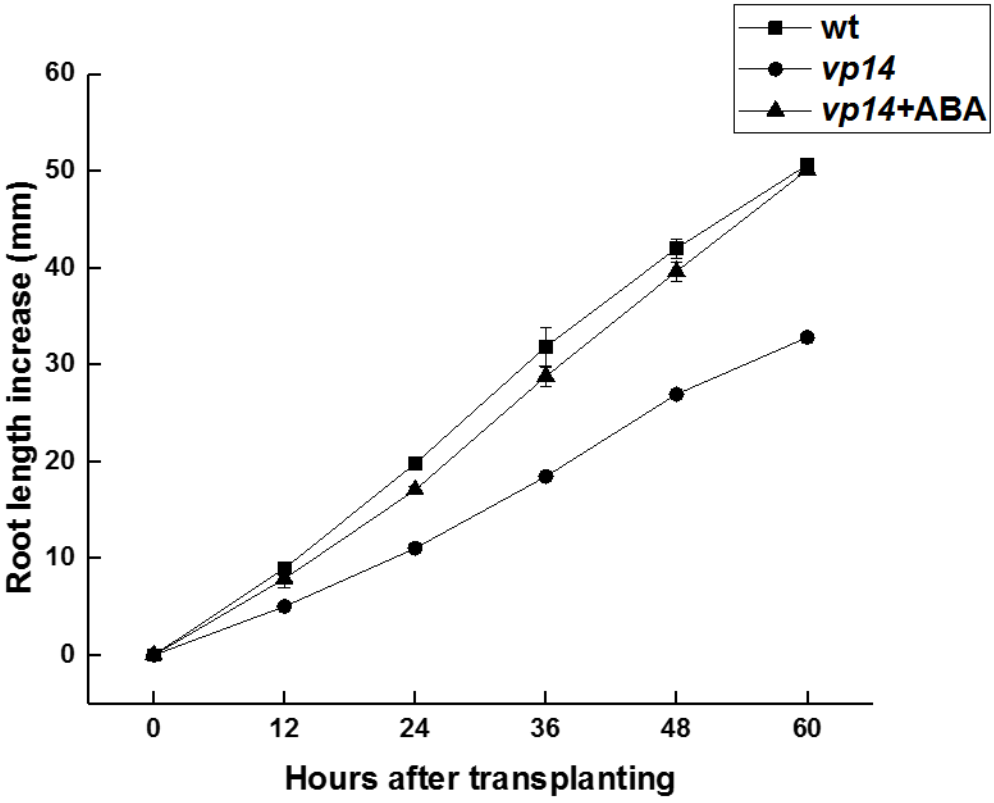
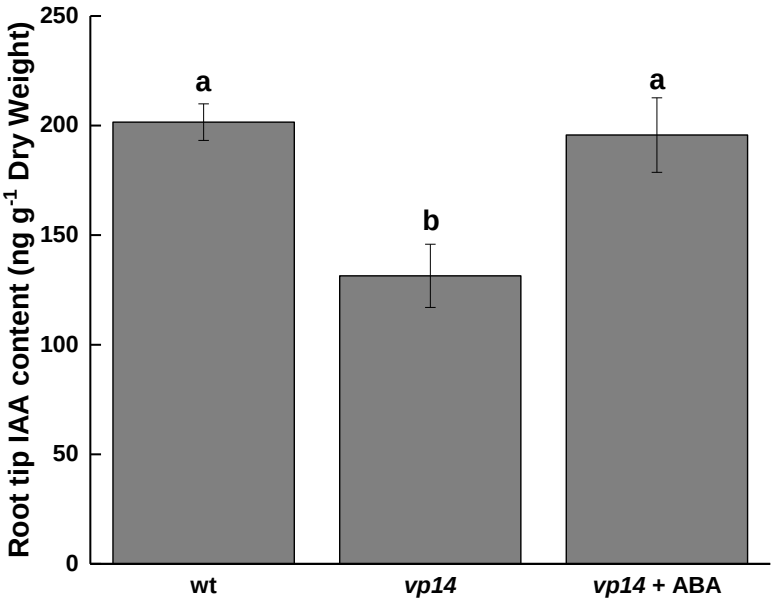


Figure 15. (A) IAA content and **(B)** ABA content of the primary root tip (apical 10 mm) of wild-type, *vp14* and ABA-treated *vp14* seedlings at 60 h after transplanting to low ψ_w vermiculite (-1.6 MPa). Data are means ± SE (n=6, combined from two experiments). In each set of data, different letters indicate significant differences between treatments at the 0.05 level (Fisher's LSD test).

A



B

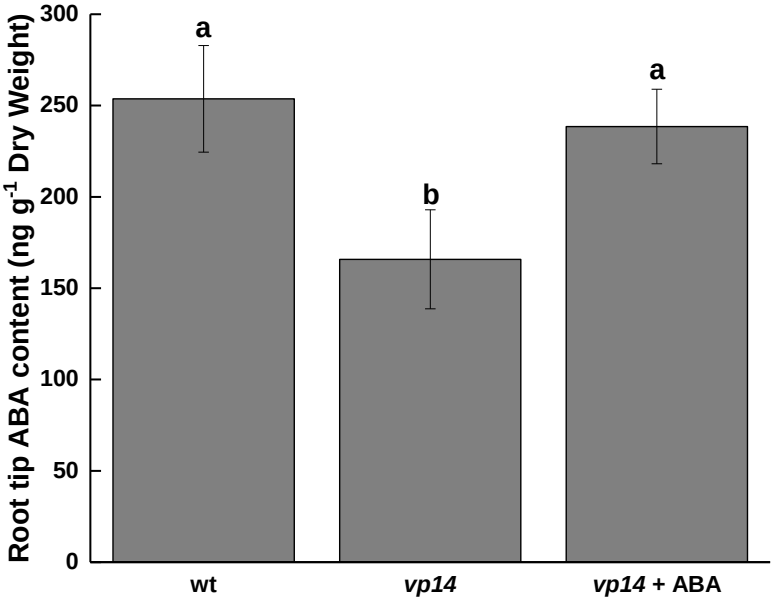
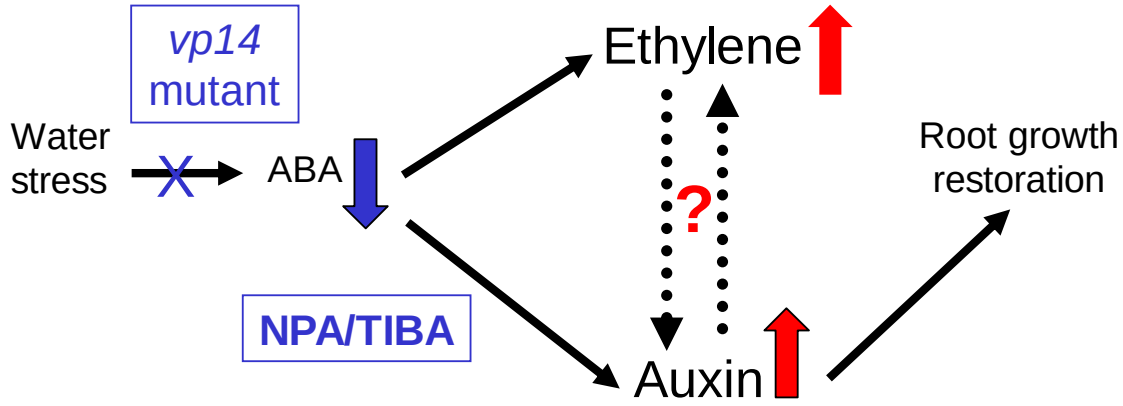
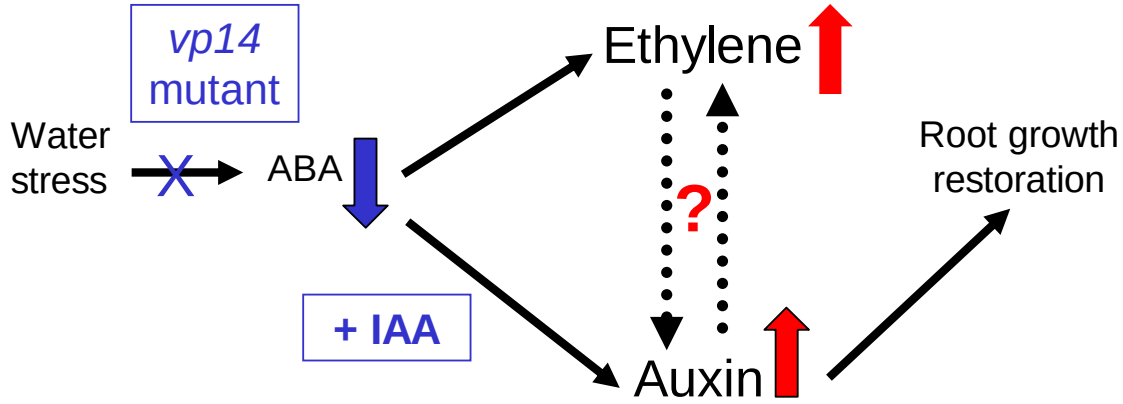

Root tip ABA content (ng g-1 Dry Weight)
300
250
200
150
100
50
0
a
b
a
wt
vp14
vp14 + ABA

Figure 16. Illustrations depicting that **(A)** blocking auxin transport using the inhibitors NPA and TIBA and **(B)** addition of IAA to ABA-deficient maize plants at low ψ_w can completely restore primary root elongation by restoring endogenous IAA levels in the root growth zone. **(C)** Illustration depicting that addition of 1-MCP and IAA to the root growth zone of ABA-deficient maize plants at low ψ_w does not restore primary root elongation.

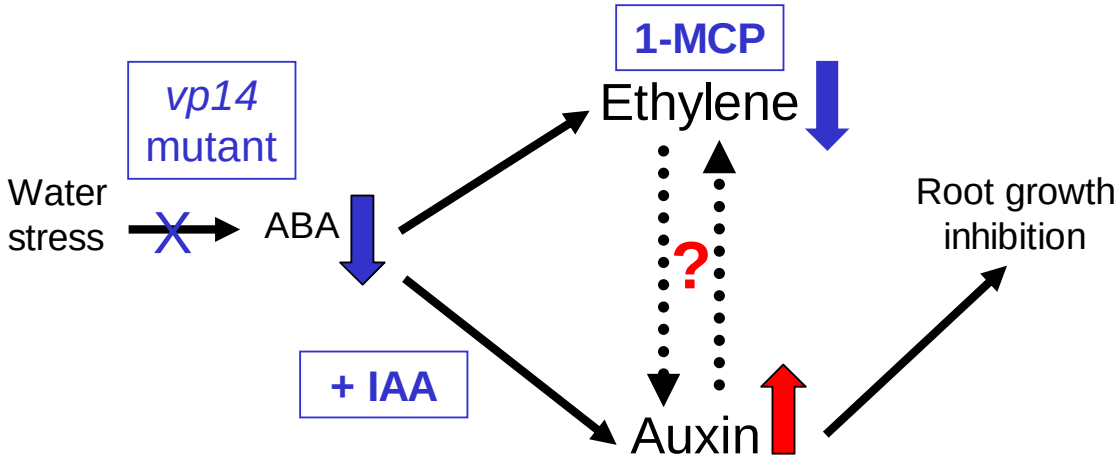
A



B



C


1-MCP
vp14
mutant
Ethylene
Water
stress
ABA
?
Root growth
inhibition
+ IAA
Auxin

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APPENDIX I

COMPARSION OF ABA MEASUREMENTS BY RADIOIMMUNOASSAY (RIA) AND LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

INTRODUCTION

In previous studies of the role of ABA in root growth regulation under waters stress by Sharp and colleagues, ABA was measured with a radioimmunoassay (RIA) (Quarrie *et al*. (1988), as described by Saab *et al*. (1990) and Sharp *et al*. (1994). The RIA method was also used in previous studies of ABA/ethylene/ROS interactions in roots of the *vp14* mutant by Cho (2006), and Smith (2011), and as described in Chapter 1-Supplement of this dissertation. The RIA method of ABA quantification uses the monoclonal antibody MAC252 (Babraham Bioscience Technologie, Cambridge, England) and has a high selectivity for (+)- *cis*, *trans*-ABA. Plant tissues such as maize (leaves and roots), wheat and lupin can be successfully analyzed using the RIA without interference from alternate immunoreactivity (Quarrie *et al*., 1988). Additionally, the RIA has a working range of 100-4000 pg (0.4-15 pmol) of ABA per tube and vials are counted in a scintillation counter (Beckman LS 6000IC), which yields results in either counts (cpm) or disintegrations per minute (dpm). However, a drawback to using the RIA is the inability to obtain multiple hormone measurements in one sample, which is advantageous when investigating hormonal interactions.

The Proteomics and Mass Spectrometry facility located at the Donald Danforth Plant Science Center in St. Louis uses a LC-MS technique in which ten acidic plant hormones including ABA and IAA can be analyzed in one biological sample. Quantification of these compounds can be successfully accomplished in extracts from vegetative tissues including roots, leaves, fruits and seeds. Therefore, this method of analysis was chosen for measurements of ABA and IAA in the studies presented in Chapters 2 and 3 of this dissertation. In order to compare the ABA results with those of

previous studies that utilized the RIA for ABA quantification, a comparison of ABA measurements using the two methods is presented below.

MATERIALS AND METHODS

vp14 and wild-type plant material and growth conditions

All experiments in this section were conducted with homozygous *vp14* and wild-type seeds (W22 genetic background) that were produced in the field in Puerto Rico in 2009. Seeds were surface-sterilized in 5% NaClO for 45 min and rinsed with deionized water for 10 min. The seeds were then imbibed for 24 h in aerated 1 mM $CaSO_4$ solution at 25° C and then germinated for 48 h on sterile germination paper that was saturated with the same solution at 29 ± 1 ºC in the dark. Seedlings with primary roots that were 5-15 mm in length were then transplanted into Plexiglas boxes containing vermiculite corresponding to a high (-0.03 MPa) or low (-1.6 MPa) ψ_w. The vermiculite was mixed with 1 mM $CaSO_4$ solution to obtain the desired ψ_w, which was measured by isopiestic thermocouple psychrometry. Transplanted seedlings were grown at 29 ± 1 ºC and near-saturation humidity in the dark.

RIA and LC-MS ABA measurements

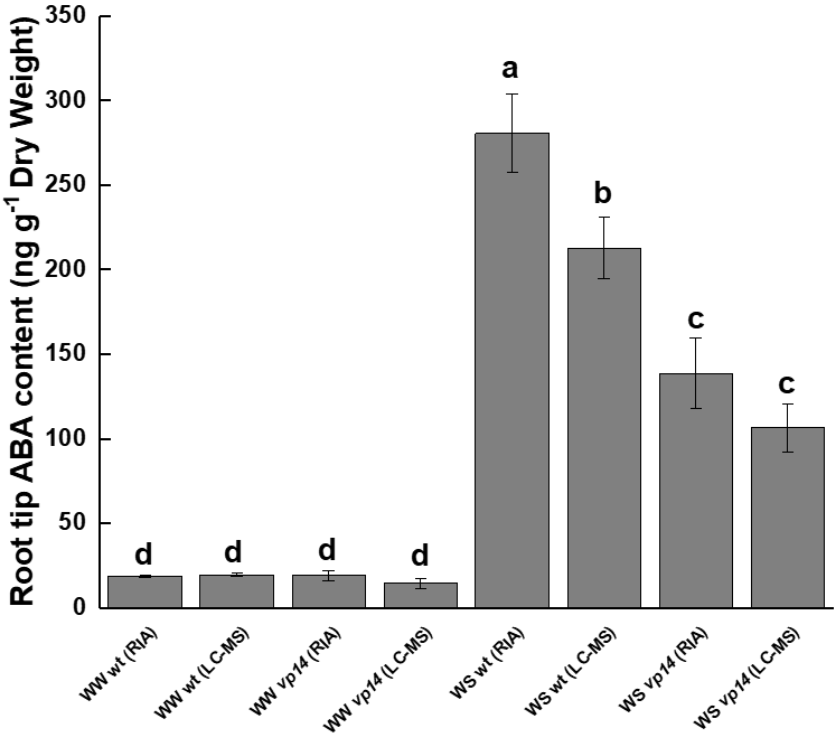
Separate experiments were conducted to collect samples for ABA analysis by RIA or by LC-MS. The seedlings were removed from the growth boxes at 48 h after imposition of the low ψ_w treatment, and the apical 10 mm of the primary roots were harvested for ABA quantification. Root segments were immediately frozen in liquid nitrogen, fresh weights were recorded, and the samples were stored at -80°C. Before analysis, root segments were

freeze-dried, dry weights measured, and ABA contents (five root tips per sample for RIA analysis; 10 root tips per sample for LC-MS analysis) were then measured using the RIA as described in Chapter 1-Supplement, or by LC-MS as described in Chapter 2.

RESULTS AND DISCUSSION

The results showed that under both well-watered and water-stressed conditions, the RIA and LC-MS techniques yielded very comparable values of ABA in both wild-type and *vp14* roots (Fig. 1a; Fig. 1b). At high ψ_w, ABA levels were below 20 ng g^{-1} DW in both wild-type and *vp14* mutant roots. These values are consistent with studies showing that the ABA contents of wild-type and *vp14* root tips are not significantly different under well-watered conditions (Cho, 2006). In the low ψ_w treatment, ABA levels increased to 200-300 ng g^{-1} DW in wild-type roots and were decreased by approximately 50% in *vp14* roots when measured using either technique. Taken together, these results confirm previous ABA measurements obtained using the RIA of *vp14* and wild-type plants growing under both well-watered and water-stressed conditions, and indicate the LC-MS method of quantification can be used in studies involving ABA and its interactions with other hormones as described in Chapters 2 and 3.

Figure 1. Primary root tip (apical 10 mm) ABA content of wild-type and *vp14* seedlings measured using the RIA or LC-MS method. The measurements were made 48 h after transplanting to high (-0.03) or low ψ_w vermiculite (-1.6 MPa). Data are means of ± SE (n=3). A one-way ANOVA was used to compare data, and different letters indicate significant differences between treatments ($p < 0.05$).



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APPENDIX II

IS THE ETHYLENE PRECURSOR 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) INVOLVED IN MAIZE PRIMARY ROOT GROWTH REGULATION UNDER WATER STRESS?

INTRODUCTION

ACC, the immediate precursor to ethylene (Fig. 1), is a simple small-chain molecule. ACC is produced from S-adenosyl-L-methionine (SAM) in a reaction catalyzed by ACC-synthase (ACS), which belongs to the pyridoxal phosphate (PLP)-dependent group of enzymes. ACC is converted to ethylene by ACC-oxidase (ACO) in the presence of oxygen. Under abiotic stress, both ethylene and ACC can accumulate in plant tissues, and ACC can be transported from roots to shoots (McManus, 2012). One of the classic examples of these molecules accumulating under stress was demonstrated in shoots of tomato when roots were exposed to hypoxia under flooding conditions. Under these conditions, an increased amount of ACC is loaded into the xylem and transported to the shoot, where it is converted to ethylene (Bradford and Yang, 1980). Additionally, increases in long-distance transport of ACC have been observed in some studies under drought conditions (Tudela *et al*., 1992; Davies *et al*., 2000; Sobeih *et al*., 2004; Skirycz *et al*., 2011).

It was previously reported that ABA-deficient mutants under water stress exhibited increased production of ethylene (Spollen *et al*., 2000; Cho, 2006; Smith, 2011). To test the hypothesis that the increase in ethylene was a cause of the inhibition of primary root elongation, the effects of two different ethylene synthesis inhibitors, AOA and AVG, and an inhibitor of ethylene binding, 1-MCP, were examined (Spollen *et al*., 2000; Smith, 2011; Chapter 1-Supplement; Chapter 2). The results demonstrated that using either AOA or AVG to inhibit ethylene synthesis completely restored primary root elongation in ABA-deficient roots growing at low ψ_w (Spollen *et al*., 2000; Smith, 2011; Chapter 1-Supplement). However, root growth was not restored in studies using 1-MCP, as described in Chapter 2.

AOA and AVG prevent ethylene synthesis by inhibiting the activity of ACC-synthase (Yu and Yang, 1979; Yang and Hoffmann, 1984). Therefore, a conceivable hypothesis could be that accumulation of ACC, rather than ethylene itself, may be the cause of growth inhibition in ABA-deficient roots under water stress. Consistent with this hypothesis, Van de Poel and Van Der Straeten (2014) discussed a possible role of ACC acting as a signaling molecule independently of the effects of ethylene. This hypothesis was tested by examining the effects of treatment with 2-aminoisobutyric acid (AIBA), a structural analog and therefore competitive inhibitor of ACC (Fig. 1), on the growth of *vp14* and wild-type roots growing under well-watered and water-stressed conditions.

MATERIALS AND METHODS

vp14 and wild-type plant material, growth conditions and root elongation measurements

All experiments were conducted with homozygous *vp14* and near isogenic wild-type seeds (W22 genetic background) that were produced in the field in Puerto Rico in 2009. Seedlings were grown at either high (-0.03 MPa) or low (-1.6 MPa) ψ_w and with and without the addition of AIBA to the solution using the oxygenated PEG hydroponic culture system developed by Verslues *et al*. (1998), with modifications as described in Smith (2011) and Chapter 1-Supplement. Primary root lengths were recorded at transplanting and after 24 h (using a green safe-light as described by Saab *et al*., 1990) during which time the solution ψ_w of the PEG-treatment had decreased to -1.6 MPa.

RESULTS

Determining the appropriate AIBA concentration.

A series of preliminary experiments was conducted to determine an appropriate concentration of AIBA. Since it was previously demonstrated that the inhibition of elongation in *vp14* roots at low ψ_w could be fully prevented by AOA and AVG treatment (Smith, 2011; Chapter 1-Supplement), which block ACC-synthase, it was anticipated that recovery of root elongation in water-stressed *vp14* could be used as the primary indicator of AIBA effectiveness in these experiments. Initially, it was the objective to identify an AIBA concentration that resulted in root growth recovery in the mutant without significantly inhibiting root growth in wild-type plants, and a range of AIBA concentrations ranging from 1-100 µM was investigated. Surprisingly, however, at all AIBA concentrations tested, the elongation rates of AIBA-treated *vp14* roots were not significantly different from untreated roots (data not shown). However, elongation of AIBA-treated wild-type roots was substantially enhanced. These results suggest that the effects of AIBA are consistent over a wide-range of concentrations where the chemical is effective yet non-toxic.

Effect of AIBA treatment on root elongation of wild-type and vp14 roots under well-watered and water-stressed conditions.

Because of the seemingly non-toxic effects, AIBA concentrations of 1 and 100 µM were chosen to further evaluate the effects of AIBA treatment on root elongation in wild-type and *vp14* roots under well-watered and water-stressed conditions (Fig. 2). At both concentrations, AIBA treatment had no effect on the elongation of water-stressed *vp14* roots. In contrast, both treatments resulted in a dramatic increase in the elongation of wild-

type roots under water stress, such that their elongation rates were not significantly different from untreated wild-type roots growing under well-watered conditions. Treatment of well-watered roots with 1 µM AIBA resulted in severe root growth inhibition, however.

DISCUSSION

Previously**,** the hydroponic culture system has proven to be a useful tool in performing ethylene inhibitor studies and investigating their interactions (Smith, 2011). The results presented in this section demonstrate that using AIBA, a structural analog of ACC, greatly enhances primary root elongation in wild-type but not ABA-deficient primary roots under water stress conditions (Fig. 2). The fact that root elongation is increased in the wild-type to well-watered levels suggests that ACC may be a potent negative regulator of primary root growth under water-stressed conditions. In contrast, AIBA treatment inhibited the growth of well-watered wild-type roots. These results suggest that ACC plays a positive role in the growth of well-watered roots, but could also reflect greater toxicity of the inhibitor under well-watered conditions. Further adding to the complexity, AIBA treatment had no effect on the growth of water-stressed *vp14* roots, potentially suggesting that the positive action of ACC on root growth under water stress involves an interaction with ABA. Further clarification of these interesting hypotheses requires more detailed investigation.

A study presented by Habben *et al*. (2014) utilized an ACC synthase (ACS) mutant of maize, *Zm-ACS6* (a maize ubiquitin promoter fused to the mutant *Zm-ACS6* to silence ACS) to demonstrate that restriction of ethylene biosynthesis at the step of ACS can protect

maize reproductive growth and increase grain yield in the field under drought conditions. However, in this study, the effects of ACC and ethylene on growth could not be separated.

In addition to ACC, there are 3 conjugated forms of ACC that have been identified. These forms include malonyl-ACC (MACC), γ-glutamyl-ACC (GACC) and jasmonyl-ACC (JA-ACC). MACC was identified in seedlings of buckwheat and wheat leaves (Amrhein *et al*., 1981; Hoffman et al., 1982) and is synthesized by way of the enzyme N-malonyl transferase (AMT), which was purified from tomato (Martin and Saftner, 1995). GACC was identified in tomato extracts of ACC-N-malonyl-transferase (Martin *et al*., 1995), and is synthesized by way of the enzyme γ-glutamyl-transferase (GGT) (Martin *et al*., 1995; Martin and Slovin, 2000). The third form of ACC is jasmonyl-ACC (JA-ACC), which was identified during an amino acid conjugate screening of JA, quantified using gas chromatography-mass spectrometry (GC-MS) (Staswick and Tiryaki, 2004). In *Arabidopsis*, JA-ACC was shown to inhibit root growth independently of JA signaling utilizing the JA-signaling mutant *coi1-35* (Staswick and Tiryaki, 2004). These are the only ACC forms to have been identified in plants, and their functions have not been fully investigated. However, it is speculated that these ACC molecules can actively contribute to the available ACC pool in planta, which could conceivably affect endogenous ethylene content.

Figure 1. Ethylene biosynthetic pathway illustrating associated enzymes (right) and the site of action inhibitors of ethylene synthesis (left).

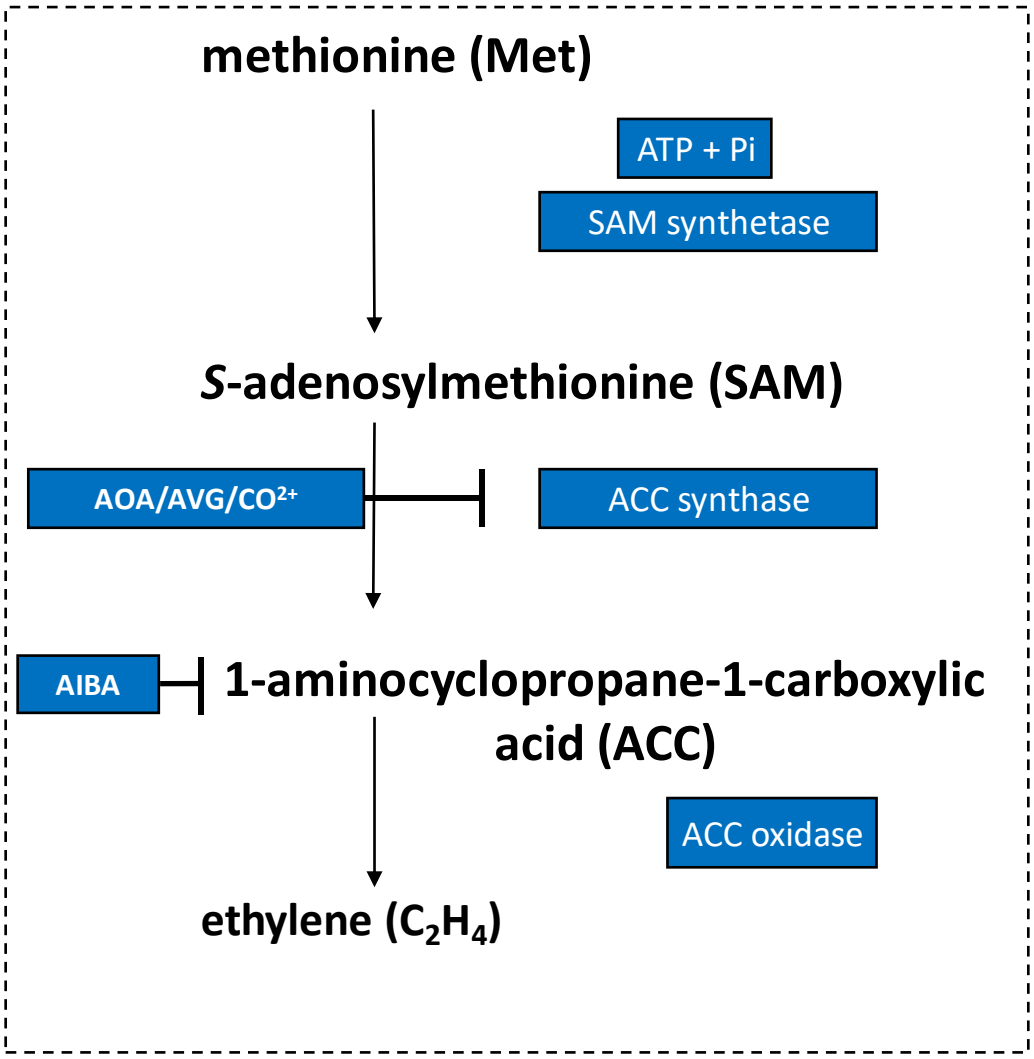
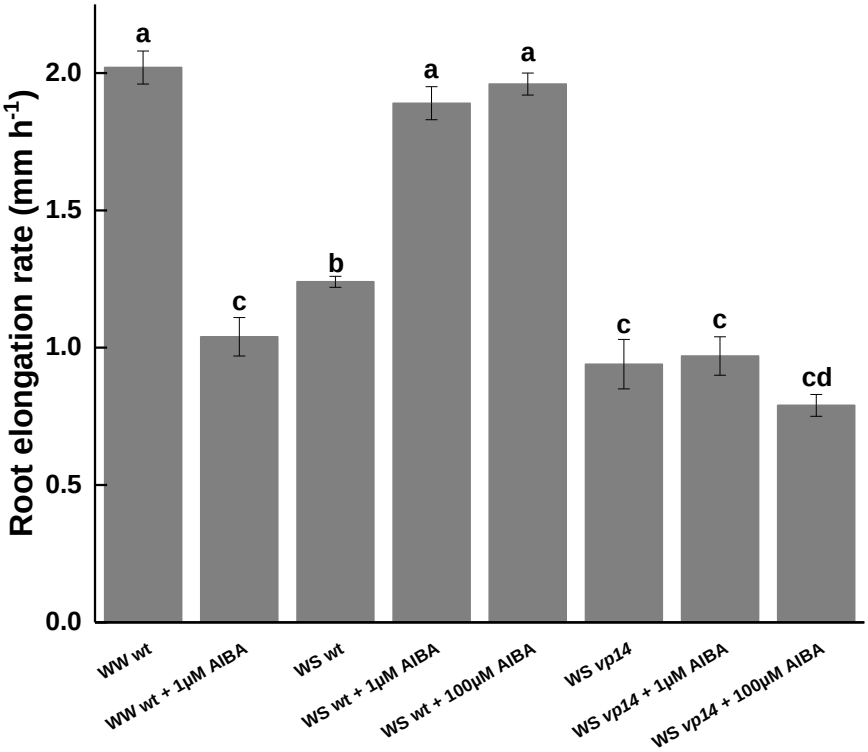


Figure 2. Average primary root elongation rates of AIBA-treated and untreated wild-type and *vp14* seedlings during 24 h under well-watered (WW) conditions and after imposition of water stress (WS), during which time the solution ψ_w in the growth box decreased to -1.6 MPa. Data are means ± SE (n = 40, combined from two experiments). A one-way ANOVA was used to compare data, and different letters indicate significant differences between treatments ($p < 0.05$).



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VITA

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Upon receiving his Ph.D. degree, he plans to pursue a career focused on crop improvement with a focus on drought, plant hormones and root development using skills acquired from studies of both whole plant physiology and molecular biology.