ABSCISIC ACID: INTERACTIONS WITH ETHYLENE AND REACTIVE OXYGEN SPECIES IN THE REGULATION OF ROOT GROWTH UNDER WATER DEFICIT

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DANTÉ O. SMITH

Dr. Robert E. Sharp, Thesis Supervisor

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The undersigned, appointment by the Dean of the Graduate School, have examined the thesis entitled

ABSCISIC ACID: INTERACTIONS WITH ETHYLENE AND REACTIVE OXYGEN SPECIES IN THE REGULATION OF ROOT GROWTH UNDER WATER DEFICIT

Presented by Danté O. Smith

A candidate for the degree of Master of Science

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

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Robert E. Sharp

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Melvin J. Oliver

_____
Stephen G. Pallardy

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ABSTRACT

The phytohormone abscisic acid (ABA) is involved in several responses to plant water deficits, including an important role in root growth maintenance. Previous studies of the ABA-deficient maize mutant *viviparous 14* (*vp14*) showed that ABA-deficiency under water deficit conditions causes impaired primary root growth, which was associated with excess production of ethylene and also a dramatic increase in intracellular reactive oxygen species (ROS) in the root growth zone, resulting in loss in membrane integrity and, ultimately, cell death. Studies in other systems have shown that stress-induced ROS production can trigger ethylene production and, conversely, that ethylene can lead to excess ROS production. The objective of this research was to further understand the inter-relationships between ethylene, ROS and ABA in water-stressed roots, by using the *vp14* mutant to determine whether the increase in ethylene is the cause or result of the increase in ROS. The suitability of a hydroponic culture system, in which low water potentials are imposed using oxygenated solutions of polyethylene glycol, for studies of the *vp14* mutant was evaluated. This system was then used to allow continuous application of aminooxyacetic acid (AOA), an inhibitor of ethylene synthesis, during the growth of ABA-deficient roots at low water potentials. This treatment completely

prevented the increase in ethylene evolution from the root growth zone, allowing definitive assessment of the effect of inhibiting ethylene synthesis on ROS levels. Intracellular ROS levels were evaluated using a fluorescent indicator dye combined with fluorescence microscopy. The results showed that inhibition of ethylene synthesis completely prevented the increase in intracellular ROS levels and restored root growth in ABA-deficient water-stressed roots. These findings indicate that the interaction of ABA and ethylene is upstream of, and results in, the production of ROS. Further analysis with this system will lead to a greater understanding of the signal transduction pathway and primary mechanisms involved in the regulation of root growth by ABA under water deficit conditions.

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

Water deficit is one of the most limiting factors affecting plant growth and development. Among environmental factors, water deficit is the leading cause of crop yield loss in the US and globally (Boyer, 1982). Under water deficit conditions, plants suffer from cellular damage and this is typically accompanied by an increase in plant-body temperature (Henckel, 1964). Many land plants typically grow in unfavorable conditions and are termed "stressed" (Boyer, 1982). As a result of being "stressed", plants evolutionarily 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 is a very complex trait. 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 stress perception and signaling (chemical and hydraulic) of plants under water deficit, and also the utilization of altered gene expression patterns through transgenic manipulation (Chaves *et al.*, 2003). However, the overall complexity of drought adaptation is still poorly understood and requires additional research.

Root growth and water deficit

Root growth arises from new cell production and expansion in the root apical meristem. The maize (*Zea mays* L.) root system forms a network of embryonic and postembryonic roots. Specifically, the primary root is a part of the embryonic system. The primary root is formed within the maize embryo; this region becomes noticeable 10-

15 days after pollination (Yamashita and Uneo, 1992). After germination, cell expansion in the maize primary root covers a 12 mm region beginning from the apex (Burstrom, 1953; Erikson and Sax, 1956). The tissue that is wounded when the primary root emerges through the seed coat is termed the coleorhiza, which encases the end of the newly emerged primary root. The primary root of maize has the ability to remain active throughout the entire life cycle of the plant.

Water uptake is essential for plant growth and development. Under drought conditions, plants can experience a decrease in water potential (ψ_w), resulting in cellular damage and loss of turgor that adversely affect growth. In plants growing in drying soil, the growth of the root system is typically less inhibited than shoot growth (Fig. 1). The primary root of maize seedlings has the ability to continue elongation at very low ψ_w, which helps to maintain adequate plant water status during seedling establishment (Sharp *et al.*, 2004). The physiological mechanisms underlying maintenance of root growth at low ψ_w are important to understand, and have been the subject of intense investigation by Sharp and co-workers (Sharp, 2002; Ober and Sharp, 2007).

Turgor pressure and cell wall extensibility determine cell expansion. Cellular water uptake results from the uptake and synthesis of solutes inside the cell, which decreases the cell 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. At low ψ_w, roots have the ability to continue growth by way of osmotic adjustment (accumulation of solutes in the cells; Sharp and Davies, 1979) and changes in cell wall extension properties (Wu and Cosgrove, 2000). In the maize primary root growing under low soil ψ_w conditions, 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 (Fig. 2). These responses involve complex and differential changes in the cell wall extension properties in the apical and basal region 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 over 40 years ago (Wright and Hiron, 1969). ABA synthesis in plants is linked to cellular dehydration (Wright, 1977), and has been shown in root and leaf tissues. Cellular dehydration caused by soil water shortages has been shown to dramatically increase ABA concentrations in both plant tissues, correlating with stomatal closure. The role of ABA in regulating stomatal closure has been studied in detail and is well understood.

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 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 inhibits primary root growth (Sharp *et al.*, 1994). However, a potential issue in interpreting these results is the assumption that both stressed and non-stressed plants behave similarly in response to increased

concentrations of ABA (Takahashi, 1973; Reid, 1990). A more appropriate approach 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 biosynthesis 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 to be taken, plants were exposed to green light, because in maize, 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 root elongation and promotion of shoot growth, indicating that the normal increase in ABA levels is in fact required to maintain primary root growth, but also causes inhibition of shoot growth (Saab *et al.*, 1990; Sharp et al., 1994). 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. 3). Results with the two methods were very similar. 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 the 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 a newly available 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. 3). Accordingly, *vp14* is a useful resource, because inhibition of ABA biosynthesis takes place downstream of the carotenoid pathway, thus not interfering with carotenoid metabolism. Similarly 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, confirming that ABA accumulation is required for root growth maintenance at low ψ_w.

ABA: interactions with ethylene

The studies described above were important to demonstrate that one particular hormone, in this case ABA, can regulate root growth 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 is commonly involved in regulating plant development and responses to environmental stimuli (Gazzarrini and McCourt, 2001).

In particular, in recent years 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 Zeevart, 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[b]). 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*

thaliana 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 and 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 (Spollen *et al.*, 2000; Sharp, 2002) (Fig. 4). ABA-deficiency under water stress was also associated with root tip swelling, which is a typical symptom of excess ethylene production. 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 the inhibition of 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 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.

To confirm this result genetically, similar experiments using AOA to inhibit ethylene synthesis 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 AOA was supplied only as a pretreatment 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 impairments in ABA biosynthesis. For this reason, in the research reported in this thesis, 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.

ABA: interactions with reactive oxygen species

Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), 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 (Miller *et al.*, 2009; Torres *et al.*, 2005). 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).

Recent studies of maize seedlings under water stress have shown that ABA-deficiency under water stress not only leads to an increase in ethylene production, as detailed above, but also causes an increase in cytosolic ROS production in the primary root growth zone (Fig. 5; Cho, 2006). This was demonstrated using the membrane-permeable dye 5-(and-6)-carboxy-2′7′-dichlorodihydrofluoresceindiacetate (carboxy-H_2DCFDA) to stain for intracellular ROS levels in the *vp14* mutant. 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. 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) 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.

Ethylene: interactions with ROS

Both ethylene and ROS can have positive effects on plant growth and development, and there is much evidence to support their interaction (Fig. 6). For example, ethylene and ROS both play a positive role in lateral root base nodulation of the

semiaquatic legume *Sesbania rostrata* (Haeze *et al*., 2003). In addition, both ethylene and ROS regulate the formation of lysigenous aerenchyma in *Arabidopsis thaliana* (Muhlenbock *et al*., 2007). However, under stress conditions, both ethylene and ROS can increase to excessive levels, with negative consequences for plant growth and metabolism. Under osmotic stress, for example, up-regulation of the ethylene biosynthesis enzyme 1-aminocyclopropane 1 carboxcylic acid oxidase (ACC 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).

Use of oxygenated polyethylene glycol solutions to study maize primary root growth at low ψ_w.

Polyethylene glycol (PEG) is a high molecular weight compound that is often used to impose low ψ_w in solution culture. Because PEG cannot penetrate the cells walls or apoplast of plant tissues (Carpita *et al*., 1979), water is withdrawn both from the cell and the cell wall, thereby mimicking the pattern of cellular water loss in dry soil. However, the viscosity of PEG solutions is also known to limit oxygen availability to levels that can inhibit root growth. To overcome this problem, Verslues *et al*. (1998) demonstrated that adequate oxygen partial pressures in the root growth zone could be achieved in PEG solutions having ψ_w as low as -1.6 MPa by aerating with air supplemented with oxygen. Under oxygen-sufficient conditions, root elongation was maintained at steady rates that were in fact substantially less sensitive to low ψ_w than in vermiculite-grown roots, indicating that PEG solutions did not have other toxic effects. In the research reported in this thesis, the oxygenated PEG culture system was utilized to

allow continuous treatments with inhibitors of ethylene synthesis and action in studies of ABA-ethylene-ROS interactions using the *vp14* mutant system.

Objectives

The focus of this project is to further understand the inter-relationships between ABA accumulation, ethylene and ROS in water-stressed maize primary roots. As reviewed above, it is currently understood that ABA deficiency under water stress causes an increase in ethylene production, which is causally linked with root growth inhibition (Spollen *et al.*, 2000; Sharp, 2002). In addition, ABA deficiency also causes a dramatic increase in intracellular ROS in the root growth zone, which precedes a loss of plasma membrane integrity and cell death (Cho, 2006). The overall objective of this thesis was to determine whether the increase in ROS is the cause or result of the increase in ethylene production in the root growth zone of water-stressed *vp14*. The specific objectives were as follows:

- Confirm that the phenotypes of increased ethylene and ROS occur in the *vp14* mutant growing at low ψ_w in the hydroponic culture system.

- Utilize 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*.

Figure 1. In maize seedlings, root growth is less sensitive than shoot growth to plant water deficits (Sharp, 1990).

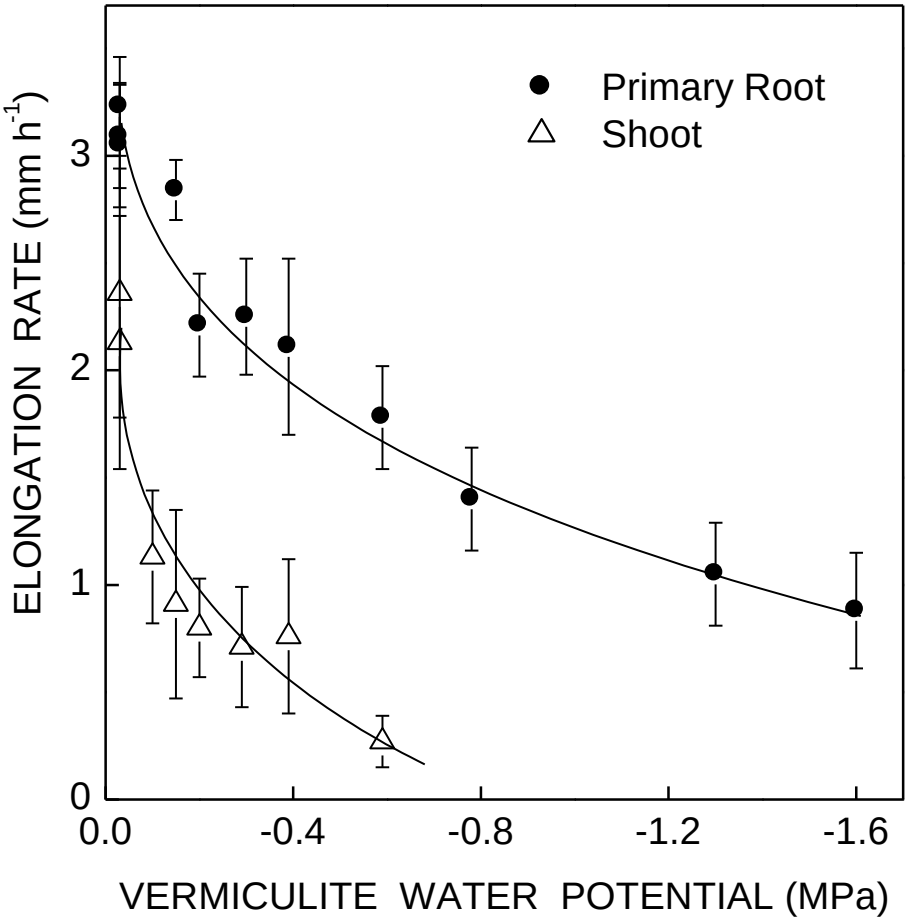


Figure 2. Kinematic analysis illustrating that relative elongation rates are maintained toward the apex of water-stressed roots (modified from Yamaguchi and Sharp, 2010)

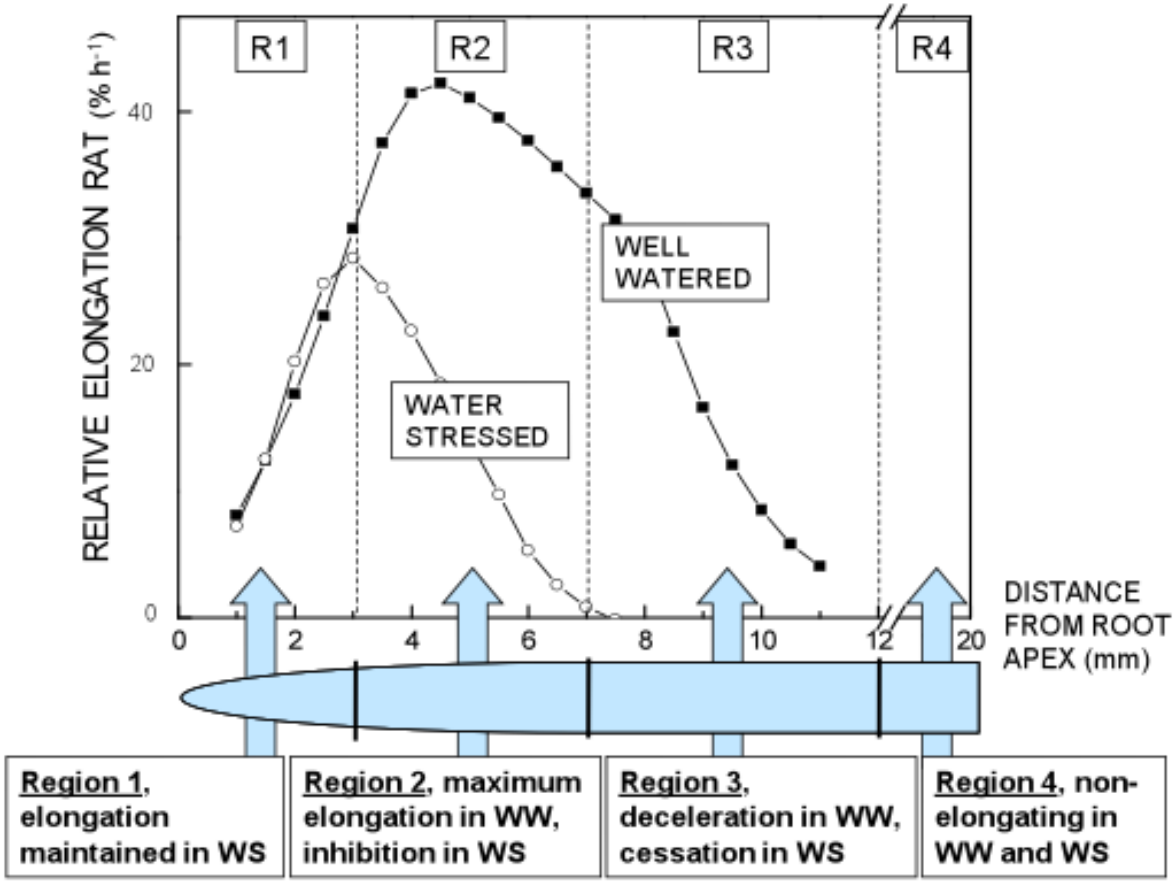


Figure 3. ABA biosynthetic pathway (modified from Taylor *et al.*, 2000). The diagram illustrates where ABA biosynthesis is inhibited in ABA-deficient mutants of maize (*vp5* and *vp14*) and by the chemical inhibitor fluridone (FLU).

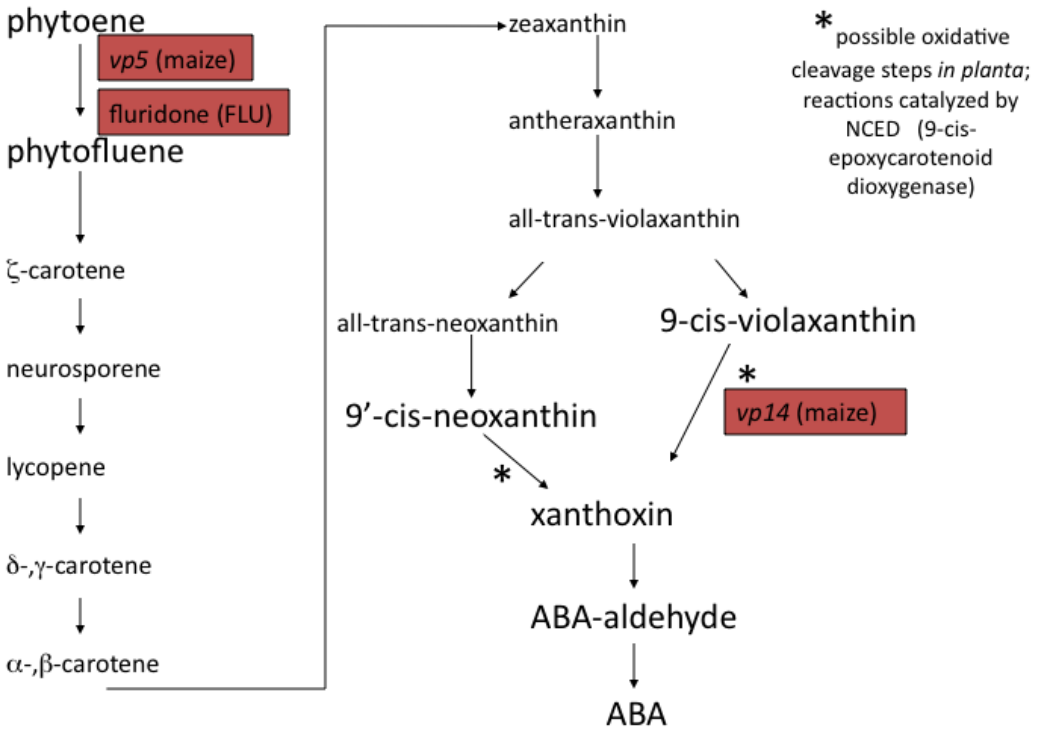


Figure 4. Primary root elongation and seedling ethylene evolution rate as a function of root tip ABA content in *vp5*, *vp14* and fluridone-treated maize seedlings (Sharp 2002)

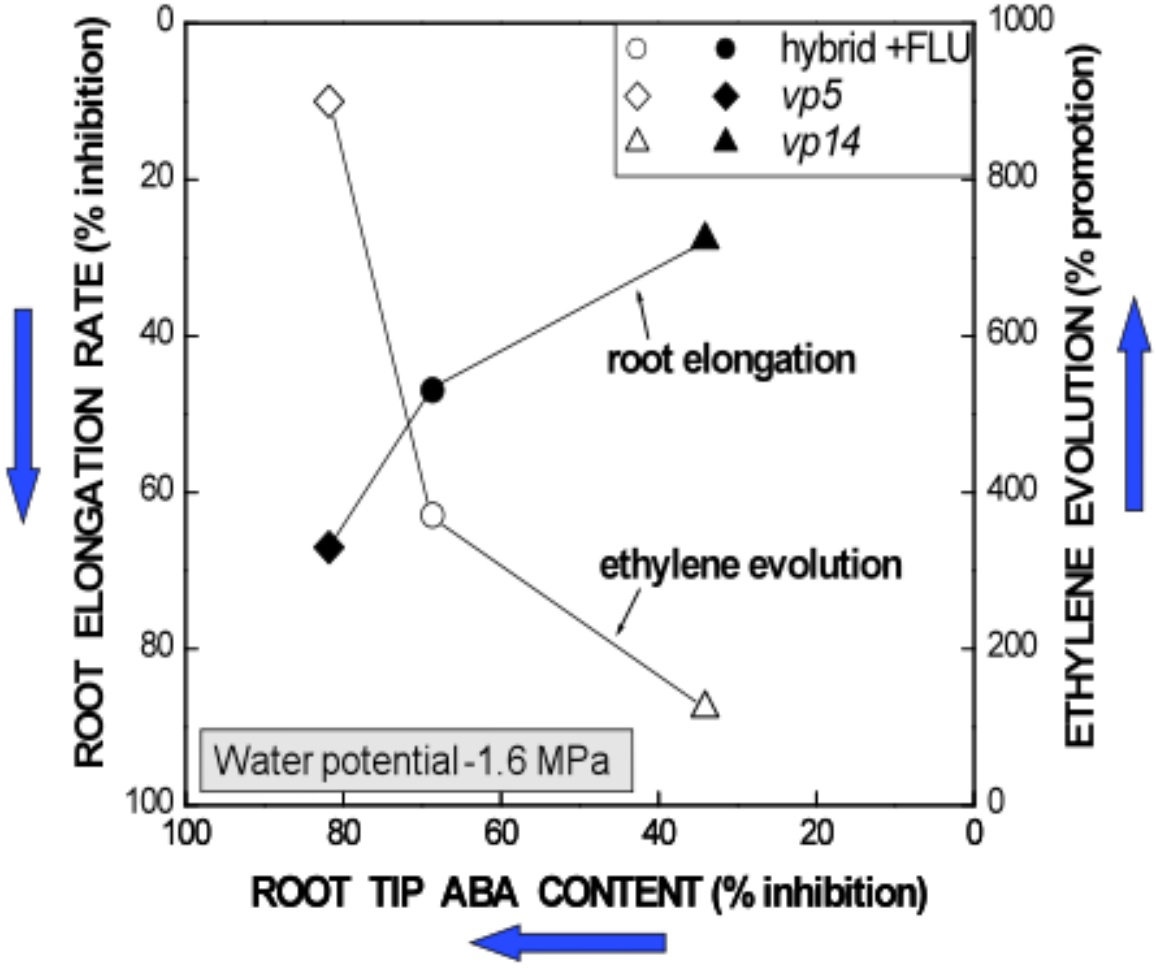
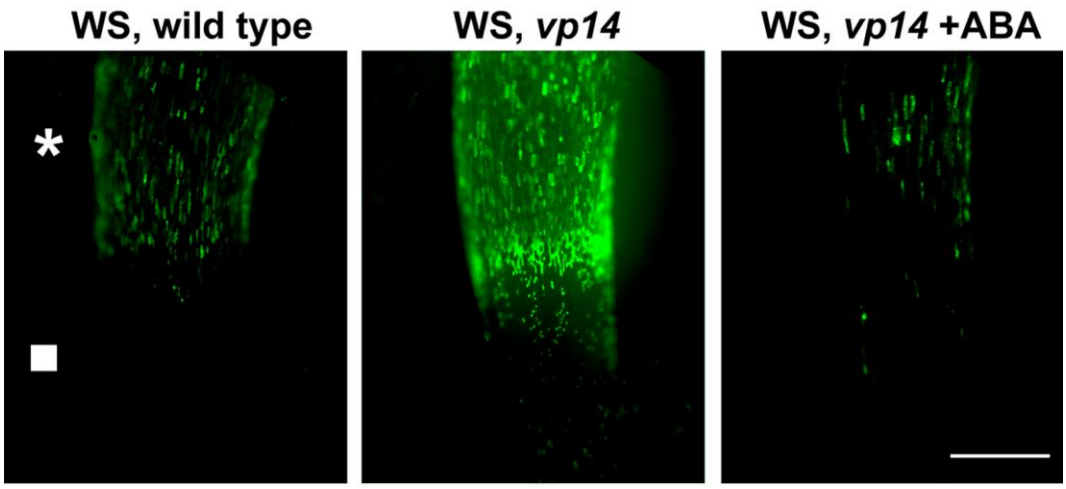
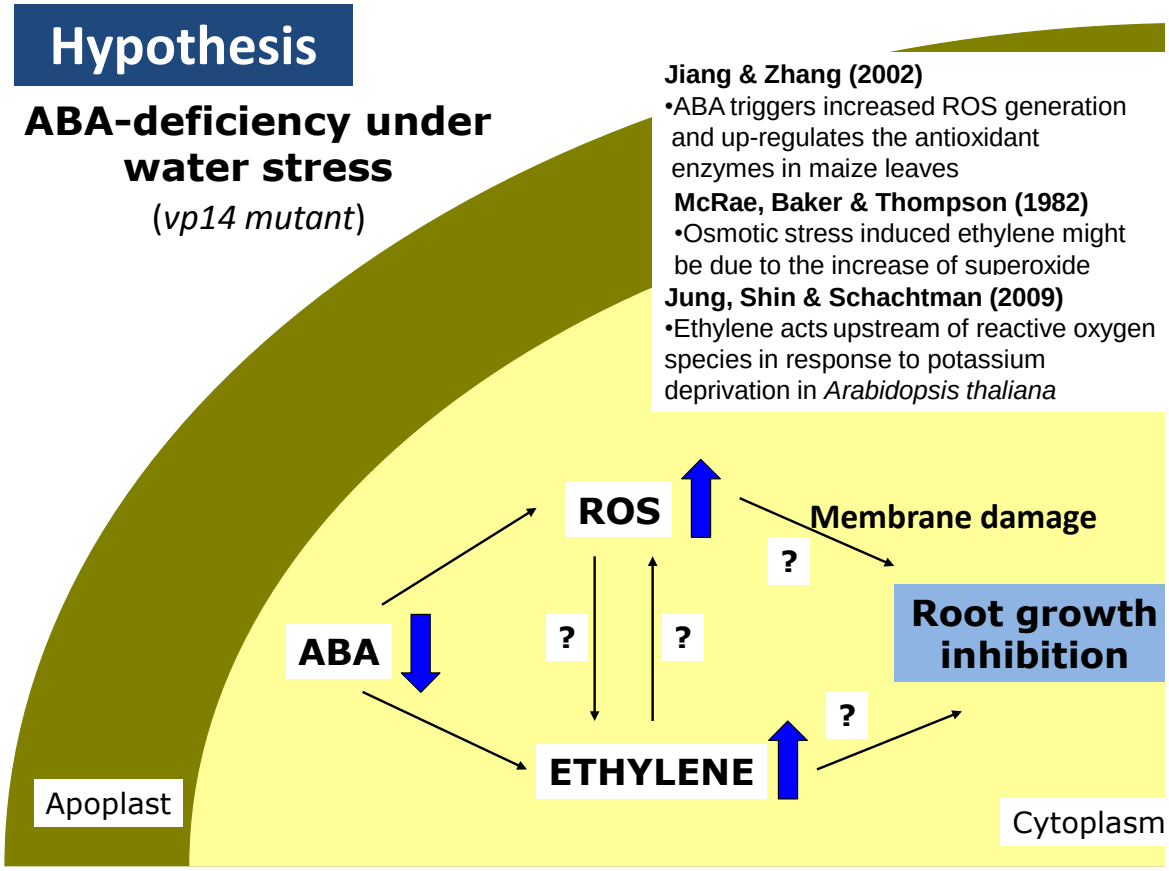


Figure 5. Representative fluorescent stereo-microscope images of the region 0-2 mm from the root apex in water-stressed roots of wild-type and *vp14* mutant seedlings. The dye carboxy-H_2DCFDA was used to image intracellular ROS levels, which were greatly increased in *vp14* and restored to wild-type levels by restoration of ABA levels with exogenous ABA. Root length increases and root tip (apical 10 mm) ABA contents are shown below the images. (From Cho, 2006).



	wild type	*vp14*	*vp14* + ABA
Root Length Increase (mm)	44.0 ± 1.6	31.8 ± 1.6	40.7 ± 0.9
Root Tip ABA Content (ng/g H_2O)	103.2 ± 8.0	68.0 ± 7.9	118.9 ± 9.3

Figure 6. Illustration depicting potential interactions between ABA, ethylene and ROS that take place in the root growth zone of ABA-deficient maize plants under water stress. ABA-deficiency causes an increase in both ethylene evolution and intracellular ROS levels, which either directly or indirectly leads to root growth inhibition.



REFERENCES

Boyer, J.S. (1982). Plant productivity and environment. Science **218**, 443-448

Breusegum, F.V., Bailey-Serres, J., and Mittler, R. (2008). Unraveling the tapestry of networks involving reactive oxygen species in plants. Plant Physiol **147**, 978-984

Burstrom, H. (1953). Physiology of root growth. Plant Physiol **4**, 237-252

Carpita, N., Sabularse, D., Montezinos, D., and Delmer, D.P. (1979). Determination of the pore size of cell walls of living plant cells. Science **205**, 1144–1147

Chaves, M.M., Maroco, J.P., and Pereira, J.S. (2003). Understanding plant response to drought: from genes to the whole plant. Funct Plant Biol **30**, 239–264.

Chernys J.T., and Zeevaart, J.A.D. (2000). Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. Plant Physiol **124**, 343-353

Cho, I. (2006). Function of abscisic acid in maintenance of maize primary root growth under water deficit. (Doctoral Dissertation) Retrieved from ProQuest Dissertations and Theses. (Accession Order No. [3361120])

Desikan, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T., and Neill, S. J. (2004). ABA, hydrogen peroxide and nitric oxide signaling in stomatal guard cells. J. Exp. Bot. **55**, 205-212

Erikson, R.O., and Sax, K.B. (1956). Elemental growth rate of the primary root of *Zea mays*. PAPhS **100**, 487-498

Gazzarrini, S., and McCourt, P. (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr Opin Plant Biol **4**, 387-391

Haeze, W.D., Rycke, R.D., Mathis, R., Goormachtig, S., Pagnotta, S., Verplancke, C., Capoen, W., and Holsters, M. (2003). Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. PNAS **100**, 11789-11794

Henckel, P.A. (1964). Physiology of plants under drought. Ann. Rev. Plant Physiol **15**, 363- 386

Hu, X., Zhang, A., Zhang, J., and Jiang, M. (2006). Abscisic acid is a key inducer of hydrogen peroxide production in leaves of maize plants exposed to water stress. Plant Cell Physiol **47**, 1484-1495

Jiang, M., and Zhang, J. (2002). Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. J. Exp. Bot. **53**, 2401-2410

Jones, H. Sharp, C., and Higgs, K. (1987). Growth and water relations of wilty mutants of tomato (Lycopersicon esculentum Mill.) J. Exp. Bot. **38**, 1848-1856

Jung, J., Shin, R., and Schachtman, D.P. (2009). Ethylene Mediates Response and Tolerance to Potassium Deprivation in Arabidopsis. Plant Cell **21**, 607-621

Kwak, J.M., Mori, I.C., Pei, Z.-M., Leonhardt, N., Torres, M.A.,Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D.G., and Schroeder, J.I. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J. **22**, 2623–2633

Kwak, J.M., Nguyen, V., and Schroeder, J.I. (2006). The role of reactive oxygen species in hormonal responses. Plant Physiol **141**, 323-329

Ke, D.S., Wang, A.G., and Sun, G.C. (2002). The effect of active oxygen on the activity of ACC synthase induced by exogenous IAA. Acta Botanica Sinica **44**, 551-556

Ke, D.S., Wang, A.G., and Sun, G.C. (2003). The role of active oxygen in chilling-induced ethylene production in etiolated mungbean seedlings. Acta Phytophysiol. Sinica **29**, 127-132

Ke, D.S., and Sun, G.C. (2004). The effect of reaction oxygen species on ethylene production induced by osmotic stress in etiolated mungbean seedling. Plant, Growth Regul **44**, 199-206

McRae, D.G., Baker, J.E., and Thompson, J.E. (1982). Evidence for involvement of the superoxide radical in the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by pea microsomal membranes. Plant, Cell Environ **23**, 375-383

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2009). Reactive oxygen species homeostasis and signaling during drought and salinity stresses. Plant, Cell and Environ **10**, 1365-3040

Muhlenbock, P., Plaszczyca, M., Plaszczyca, M., Mellerowicz, E., and Karpinski, S. (2007). Lysigenous arenchyma formation in arabidopsis is controlled by *LESION SIMULATION DISEASE1*. Plant Cell **19**, 3819-3830

Ober, S.E., and Sharp, R.E. (2007). Regulation of root growth responses to water deficit. Advances in molecular breeding towards salinity and drought tolerance, M.A. Jenks *et al*, eds (Netherlands: Springer) pp. 33-53

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. Nature **406**, 731–734.

Quarrie, S.A. (1987). Use of differing genotypes in endogenous abscisic acid levels in studies of physiology and development. In hormone action in plant development – a critical appraisal. Hoad, G.S., Lenton, J.R., Jackson, M.B., and Atkin, R.K. eds (Butterworths, London), pp. 89-105

Reid, J. (1990). Phytohormone mutants in plant research. J Plant Growth Regul. **9**, 97-111

Saab, I.N., Sharp, R.E., Pritchard, J. and Voetberg, G.S. (1990). Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. Plant Physiol **93**, 1329-1336

Sharp, R.E. (1990). Comparative sensitivity of root and shoot growth and physiology to low water potentials. Importance of root to shoot communication in the responses to environmental stress. Davies, W.J. and Jeffcoat, B. eds (Bristol, England), Parchments (Oxford) Ltd, pp. 29-44

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

Sharp, R.E., and Davies, W.J. (1979). Solute regulation and growth by roots and shoots of water-stressed maize plants. Planta **147**, 43-49

Sharp, R.E., Silk, W.K., and Hsaio, T.C. (1988). Growth of the maize primary root at low water potentials. I. Spatial distribution of expansive growth. Plant Physiol **87**, 50-57

Sharp, R.E., Wu, Y., Voetberg, G.S., Saab, I.N., and LeNoble M.E. (1994). Confirmation that abscisic acid accumulation is required for maize primary root elongation at low water potentials. J. Exp. Bot. **45**, 1743-1751

Sharp, R.E., Poroyko, V., Hejlek, L.G., Spollen, W.G. Springer, G.K., Bohnert, H.J., and Nguyen, H.T. (2004). Root growth maintenance during water deficits: physiology to functional genomics. J. Exp. Bot. **55**, 2343-2351

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

Takahashi, K. (1973). Interaction between ethylene, abscisic acid ad giberellic acid in elongation of rice mesocotyl. Planta **109**, 363-364

Tal, M., and Nevo, Y. (1973). Abnormal stomatal behavior and root resistance, and hormonal imbalance in three wilty mutants of tomato. Biochem. Genet. **8**, 291-300

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D., and McCarty D.R. (1997). Genetic control of abscisic acid biosynthesis in maize. Proc. Acad. Sci. U.S.A. **94**, 12235-12240

Taylor, I.B., Burbidge, A., and Thompson, A.J. (2000). Control of abscisic acid synthesis. J. Exp. Bot. **51**, 1563–1574.

Torres, M.A., Jones, J.D.G., and Dangl, J.L. (2005). Reactive oxygen species signaling in response to pathogens. Plant Physiol **141**, 373-378

Trewavas, A.J., and Jones, H.G. (1991). An assessment of the role of ABA in plant development. In abscisic acid: Physiology and biochemistry, Davies, W.J. and Jones, H.G. eds (Oxford, UK) pp. 169-188

Verslues, P.E., Ober, E.S., and Sharp, R.E. (1998). Root growth and oxygen relation at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. Plant Physiol **116**, 1403-1412

Westgate, M.E., and Boyer, J.S. (1985). Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. Planta **164**, 540-549

Wilkins, H., Largué-Saavedra, A., and Wain, R.L. (1974). Control of *Zea* root elongation by light and the action of 3,5-diiodo-4-hydroxybenzic acid. Nature **248**, 449-450

Wu, Y., and Cosgrove, D.J. (2000). Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins. J. Exp. Bot. **51**, 1543-1553

Wright, S.T.C., and Hiron, R. W. P. (1969). (+)-Abscisic acid, the growth inhibitor induced in detached wheat leaves by a period of wilting. Nature (Lond.) **224**, 719

Wright, S.T.C. (1977). The relationship between leaf water potential (Ψleaf) and the levels of abscisic acid and ethylene in excised wheat leaves. Planta **134**, 183–189.

Yamaguchi and Sharp (2010). Complexity and coordination of root growth at low water potentials: recent advances from transcriptomic and proteomic analyses. Plant Cell and Environ. **33**, 590-603

Yamashita, T., and Ueno, C. (1992). Embryo und wurzelentwicklung bei coix lacryma jobi L. (Gramineae). Flora **187**, 79–101.

Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W., and Song, C.P. (2001). Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. Plant Physiol. **126**, 1438–1448.

Zhang, M., Yuan, B., and Leng, P. (2009). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. J. Exp. Bot. **60**, 1579-1588

Zhang, Y., Tan, J., Guo, Z., Lu, S., He, S., Shu, W., and Zhou, B. (2009). Increased abscisic acid levels in transgenic tobacco over-expressing 9 *cis*-epoxycarotenoid dioxygenase influence H_2O_2 and NO production and antioxidant defenses. Plant, Cell and Environ **32**, 509-519

CHAPTER 2

USING THE *vp14* MUTANT IN A HYDROPONIC CULTURE SYSTEM TO ASSESS THE ETHYLENE AND ROS PHENOTYPES AT LOW WATER POTENTIALS

INTRODUCTION

As detailed in Chapter 1, previous work established that in the ABA-deficient mutant *vp14* under water stress, there are increases in both ethylene production and intracellular ROS in the primary root growth zone, which are causally associated with cellular damage and root growth inhibition (Cho, 2006). 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. 3). 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).

The primary objective of this thesis was to utilize inhibitors of ethylene synthesis and action to determine whether the increase in ethylene production causes the increase in intracellular ROS in the roots of water-stressed *vp14*. To definitively investigate this question, it would be preferable to fully prevent the increase in ethylene production in water-stressed roots. The studies of Cho (2006) were conducted in a vermiculite system, which involves transplantation of maize seedlings to media of a pre-determined low ψ_w (-1.6 MPa). A drawback of this system for studies with chemical inhibitors is that the compounds can only be effectively applied as a pre-treatment during germination, since

uptake of chemicals from the dry vermiculite is very limited. As detailed in Chapter 1, using ethylene inhibitors with vermiculite-grown *vp14* resulted in only partial recovery of root elongation, probably because the effectiveness of the inhibitors decreased with time after transplanting to the low ψ_w condition.

This chapter presents the results of initial studies in which the suitability of a hydroponic culture system for studies of *vp14* root responses to water stress was examined. Low ψ_w was imposed using oxygenated PEG solutions as described in Verslues *et al.* (1998). The use of this system has the advantage that chemical inhibitors can be supplied continuously to roots growing under low ψ_w conditions. It is demonstrated that *vp14* exhibits the phenotypes of inhibited root elongation, increased ethylene and increased ROS when exposed to low ψ_w using the hydroponic system, as previously observed in the vermiculite system (Verslues *et al.*, 1998).

MATERIALS AND METHODS

vp14 and wild-type plant material

Homozygous *vp14* and near isogenic wild type (NS2774) seed were derived by selfing the original seed from segregating ears that was supplied by Don McCarty, University of Florida, Gainesville. The seed used for the experiments reported in this thesis were produced in 2002 and 2009 from field-grown plants at the University of Missouri. Homozygosity of *vp14* material was confirmed by genotyping (Cho and Oliver, unpublished).

Oxygenated PEG culture system

Seeds were imbibed for 24 h in aerated 1 mM $CaSO_4$ solution and then germinated for 48 h on germination paper (Anchor Paper, Hudson, WI) that was saturated with the same solution (Spollen *et al.*, 2000). Seedlings with primary roots that were 25-35 mm in length were then transplanted into Plexiglas boxes containing a high ψ_w (-0.02 MPa) solution comprising 1 M MES buffer, 10 mM $CaSO_4$ and 6 mL of a maize micronutrient solution containing 2.3 µM H_3BO_3, 0.9 µM $MnSO_4 \cdot H_2O$, 0.6 µM $ZnSO_4 \cdot 7H_2O$, 0.10 µM $NaMoO_4 \cdot 2H_2O$, 0.11 µM $NiCl_2 \cdot 6H_2O$, 0.01 µM $CoCl_2 \cdot 6H_2O$ and 0.15 µM $CuSO_4 \cdot 5H_2O$, which was adjusted to pH 6.0 with NaOH. The boxes (Fig. 1) were 20 cm long, 1.2 cm wide and 18 or 25 cm tall (the latter were utilized for high ψ_w studies, in which the roots grew to greater lengths). The 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. In well-watered control experiments, the solution was also replaced over the same time course but without the addition of PEG.

Each box held a maximum of twenty seedlings, which were arranged on a Plexiglas holder at the top of the box (Fig. 1). Plexiglas covers encased and maintained a high relative humidity around the shoots. The primary roots grew inside plastic drinking straws (6 mm in diameter), which served as root guides throughout the course of the experiment. The entire surface of the straws was perforated with holes (approx. 0.5 mm in diameter) to allow solution exchange. The solution was aerated via a perforated rubber

tube that extended along the bottom of the box, using a mixture of air (Airgas®) and oxygen at a flow rate of 1100 mL min^{-1} to achieve an oxygen partial pressure in the solution of 43 ± 4.3 kPa (measured with an ISO2 oxygen probe, World Precision Instruments, Sarasota, FL), as recommended by Verslues *et al.* (1998). The ψ_w of the PEG solution was determined by isopiestic thermocouple psychrometry (Boyer and Knipling, 1965). Primary root and shoot lengths were recorded at transplanting and at harvest, and the time course of root elongation rate during the experiments was obtained by periodically marking the position of the primary root apices on the face of the boxes (using a green safe-light as described by Saab *et al.*, 1990).

Ethylene evolution

Ethylene evolution rates were measured from the 0-10 mm region of the primary root, as described by Spollen *et al.* (2000). 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 mins, 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 mins. As a precaution, aeration (with air) was used during the staining period so roots would not be subjected to hypoxic conditions. 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 gives off 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.

RESULTS

Growth measurements of *vp14* and wild-type roots in the solution culture system

Previous studies using the oxygenated PEG culture system were performed using the FR27 x FRMo17 hybrid (Verslues *et al*., 1998; Verslues and Sharp, 1999). In those studies, the ψ_w was lowered to -1.6 MPa over the course of 8 h, which led to severe inhibition of primary root elongation rate during the first 10 h, followed by a period of recovery over the following 30 h before steady root elongation was achieved (Fig. 2, Inset D). When this procedure was followed using *vp14* and its corresponding wild type, the wild type exhibited a similar root growth response to the previous studies. However, root elongation in *vp14* did not recover after the initial period of growth inhibition, but instead continued to decline to negligible rates (Table 1). Since *vp14* roots are able to grow in vermiculite at a ψ_w of -1.6 MPa, these findings suggested that the 8 h stress imposition period may have been too rapid. This hypothesis was tested by extending the period of stress imposition to 24 h. This modified protocol prevented the initial period of severe root growth inhibition in the wild type, and resulted in steady rates of root elongation in *vp14* (Table 1).

The modified stress imposition protocol was used to evaluate the response of primary root elongation of seedlings grown from both the 2002 and 2009 seed. Consistent with previous studies of the response of *vp14* to low ψ_w in vermiculite-grown seedlings, root elongation was not inhibited in *vp14* compared to the wild type when grown at high ψ_w, but was considerably more inhibited in *vp14* than in the wild type when subjected to low ψ_w conditions (Tables 2 and 3).

Ethylene evolution rates

Measurements of ethylene evolution from the apical 10 mm of the primary root of *vp14* and wild-type seedlings were made at 24 h after low ψ_w imposition, and showed that *vp14* exhibits greatly increased ethylene production compared to the wild type (Fig. 3), consistent with previous studies of vermiculite-grown seedlings.

Intracellular ROS staining experiments

In well-watered conditions, both wild-type and *vp14* seedlings exhibited low levels of intracellular ROS in the root growth zone at 24 h after transplanting (Fig. 3). At low ψ_w, wild-type roots did not show an increase in the level of ROS, whereas *vp14* roots exhibited a dramatic increase in ROS levels throughout most of the elongation zone. These results confirm that at low ψ_w in solution culture, as in vermiculite-grown seedlings, ABA-deficiency results in increased intracellular ROS in the root growth zone in association with reduced root elongation and enhanced ethylene production.

CONCLUSION

The results demonstrate that the oxygenated PEG culture system can be used successfully to study the response of root elongation to low ψ_w in *vp14* mutant seedlings. The results confirm that at low ψ_w in solution culture, as in previous studies of vermiculite-grown seedlings, ABA-deficiency causes inhibition of root elongation in association with enhanced ethylene production and increased intracellular ROS in the root growth zone.

Figure 1. Design of the Plexiglas growth boxes for hydroponic culture (original design by Verslues *et al.*, 1998, with modifications by J. Garnett). 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.

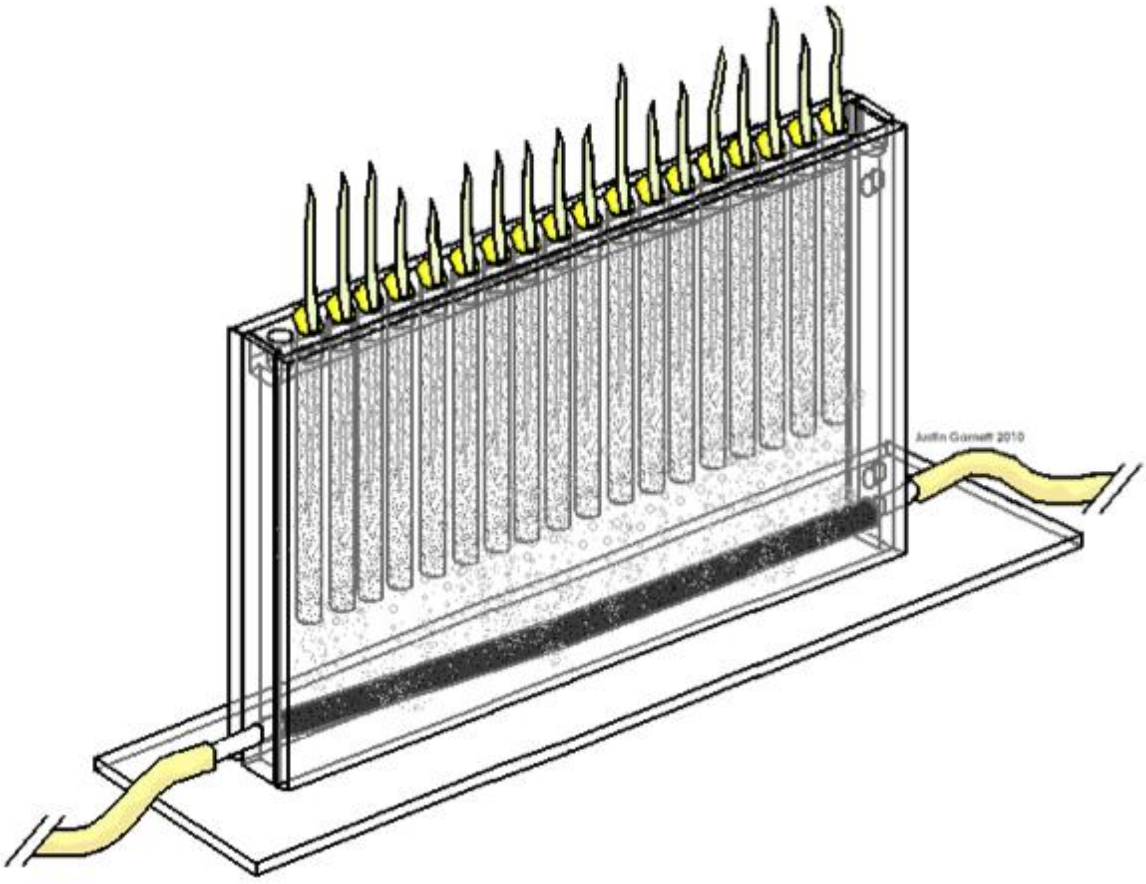


Figure 2. Time courses of primary root elongation rate of FR27 x FRMo17 seedlings in solutions of various ψ_w (imposed by PEG over the course of 8 h) and pO_2. Panel D represents seedlings growing at a ψ_w of -1.6 MPa (Verslues *et al.*, 1998).

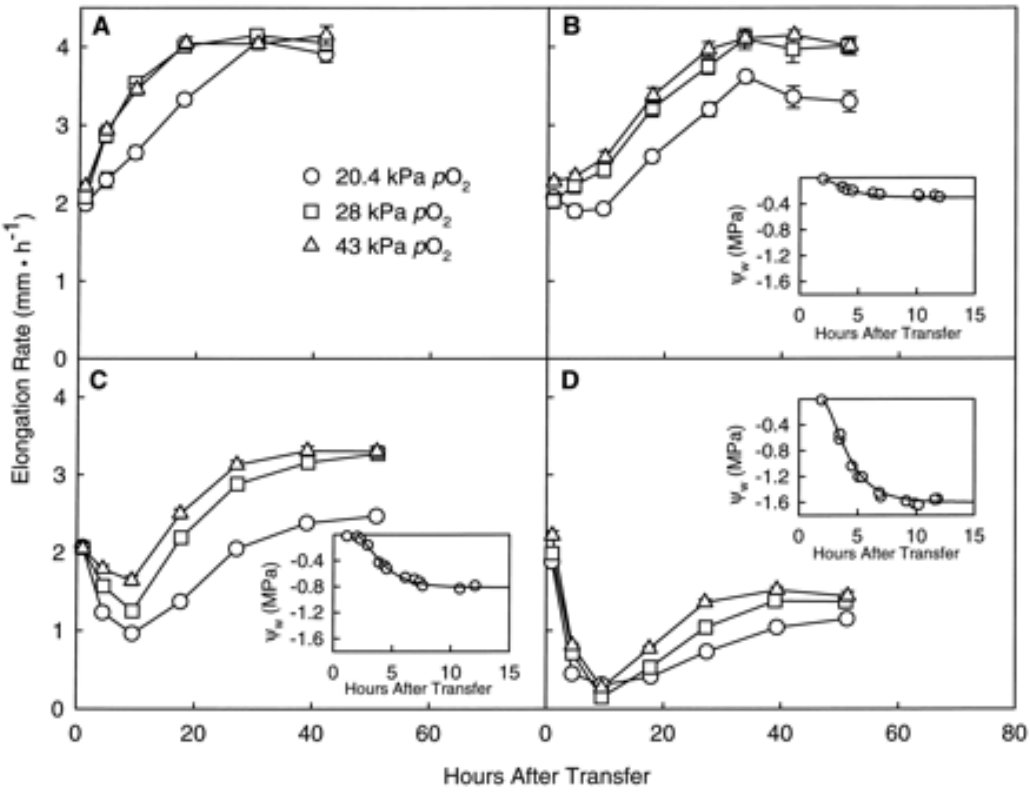


Figure 3. Representative images of intracellular ROS levels and corresponding ethylene evolution rates in *vp14* and wild-type primary root tips under well-watered (WW) and water stressed (WS, ψ_w = -1.6 MPa) conditions in the solution culture system. The images were taken using stereo fluorescent microscopy of the apical 10 mm region after staining with caboxy-H_2DCFDA. Ethylene measurements were made from the apical 10 mm of the primary roots at 24 h after transplanting to solution culture. Results are single measurements.

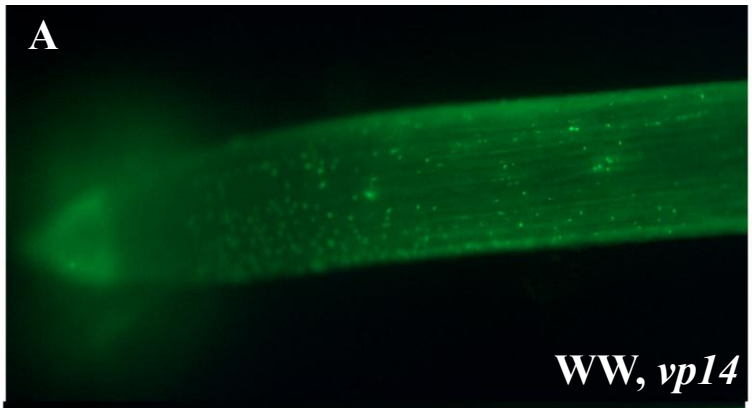
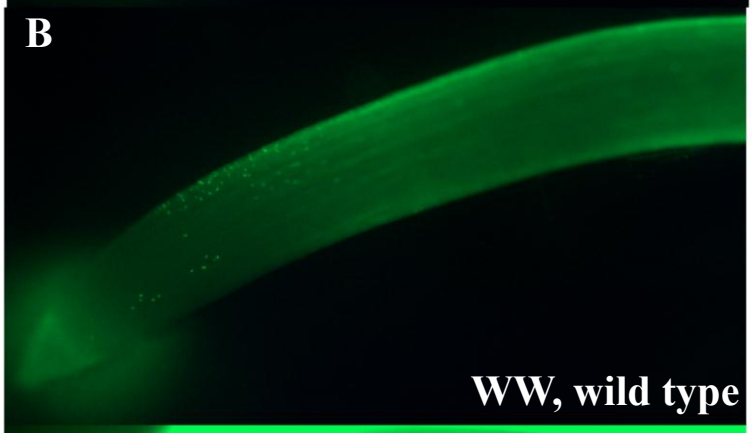
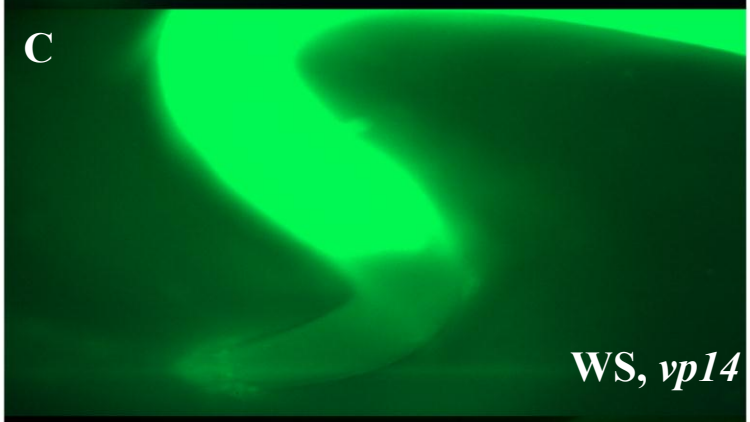
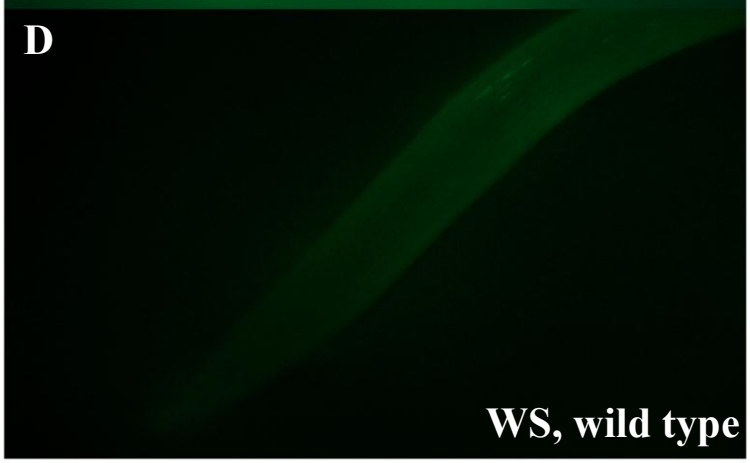
	Ethylene Evolution Rates (pmol/Kg^{-1}/s^{-1})
	19.84
	15.03
	73.22
	19.88

Table 1. Time course comparison of *vp14* primary root elongation rates in PEG solutions following stress imposition over 8 h and 24 h (final ψ_w of -1.6 MPa). Data are means ± SD (n = 20/treatment).

8 h PEG stress imposition

Root elongation rate (mm h^{-1})	Wild type	*vp14*
0-24 h	1.00 ± 0.44	0.25 ± 0.10
24-30.5 h	0.98 ± 0.10	0.19 ± 0.08
30.5-50 h	0.87 ± 0.03	0.03 ± 0.03
50-53.5 h	1.20 ± 0.23	0.00 ± 0.01

24 h PEG stress imposition

Root elongation rate (mm h^{-1})	Wild type	*vp14*
0-24 h	0.98 ± 0.25	0.28 ± 0.14
24-67 h	1.22 ± 0.35	0.32 ± 0.19

Table 2. Average primary root elongation rates of wild-type and *vp14* seedlings grown from 2002 seed during 48 h of growth in high or low ψ_w solutions. Data are means ± SD (n = 20).

Root elongation rate ($mm\ h^{-1}$)	Wild type	*vp14*
High ψ_w (-0.02 MPa)	1.28 ± 0.16	1.56 ± 0.63
Low ψ_w (-1.6 MPa)	0.98 ± 0.19	0.28 ± 0.13

Table 3. Average primary root elongation rates of wild-type and *vp14* seedlings grown from 2009 seed during 48 h of growth in high or low ψ_w solutions. Data are means ± SD (n = 20).

Root elongation rate (mm h^{-1})	Wild type	*vp14*
High ψ_w (-0.02 MPa)	1.48 ± 0.20	1.30 ± 0.32
Low ψ_w (-1.6 MPa)	1.19 ± 0.62	0.89 ± 0.35

REFERENCES

Boyer, J.S., and Knipling, E.B. (1965). Isopiestic technique for measuring leaf water potentials with a thermocouple psychrometer. PNAS **54**, 1044-1051

Cho, I. (2006). Function of abscisic acid in maintenance of maize primary root growth under water deficit. (Doctoral Dissertation) Retrieved from ProQuest Dissertations and Theses. (Accession Order No. [3361120])

De Greef, J.A., De Proft, M., and Veroustraete, F. (1976). Ethylene production and its relation to growth in 8-day old etiolated seedlings. Arch. Int. Physiol. Biochim. **84**, 1062-1063.

Dracup, M., Gibbs, J., and Greenway, H. (1985). Melibiose, a suitable, non-permeating osmoticum for suspension-cultured tobacco cells. J. Exp. Bot. **37**, 1070-1089

Saab, I.N., Sharp, R.E., Pritchard, J., and Voetberg, G.S. (1990). Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. Plant Physiol **93**, 1329-1336

Sharp, R.E., Wu, Y., Voetberg, G.S., Saab, I.N., and LeNoble M.E. (1994). Confirmation that abscisic acid accumulation is required for maize primary root elongation at low water potentials. J. Exp. Bot. **45**, 1743-1751

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

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D., and McCarty D.R. (1997). Genetic control of abscisic acid biosynthesis in maize. PNAS **94**, 12235-12240

Verslues, P.E., Ober, E.S., and Sharp, R.E. (1998). Root growth and oxygen relation at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. Plant Physiol **116**, 1403-141

Verslues, P.E., and Sharp, R.E. (1999). Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. Plant Physiol **119**, 1349–1360.

Chapter 2-Supplement

Hydrogen peroxide accumulation in polyethylene glycol

Measurements of hydrogen peroxide content of polyethylene glycol were made in collaboration with Dr. Mineo Yamaguchi.

INTRODUCTION

As described in Chapter 2, the oxygenated PEG hydroponic culture system was successfully tested for studies of *vp14* and wild-type seedlings. In subsequent experiments, however, both the mutant and the wild type began to exhibit adverse symptoms, including necrotic lesions and severe root growth inhibition. Because the stock of PEG had not changed, and also because tests of the B73xMO17 hybrid did not show adverse symptoms, it was initially thought that the *vp14* and wild-type seed may have degenerated, possibly due to seed-borne disease. However, when *vp14* and wild-type seedlings were grown in vermiculite at both high and low ψ_w, root elongation rates were similar to those previously obtained with this material (Cho, 2006) and lesions were not observed. Accordingly, the possibility of solution toxicity was examined in detail.

Previous studies have shown that under certain storage conditions, including exposure to light and high temperatures, compounds such as polysorbate, polyethylene glycols (PEG) and other polyether detergents can accumulate peroxide (Hamburger *et al.*, 1975; Jaeger *et al.*, 1994; Ha *et al.*, 2002; Kumar and Kolania, 2006). The stock of PEG 8000 that was used for successful testing of the hydroponic system had been stored for several years in the dark at room temperature, and sub-samples were taken immediately before use in experiments. However, the subsequent experiments that caused adverse symptoms used a sub-sample that had been stored in a clear plastic bag in the laboratory for a period of 6 months. Therefore, the possibility that this sub-sample had accumulated toxic levels of peroxide was tested. As a further comparison, the peroxide content of an additional sub-sample from the same stock that had been stored in an opaque white plastic storage bottle in the laboratory for approximately two years was tested. The

results were compared to a new stock of PEG 8000. The original and new stocks of PEG were supplied by Sigma.

MATERIALS AND METHODS

Peroxide assay

The peroxide content of PEG was measured using the ferric iron indicator xylenol orange (FOX) assay (Jiang *et al.*, 1990; Kumar and Kalonia, 2006). The reagent was comprised of the following components: 250 µM ammonium ferrous sulphate, 100 µM xylenol orange and 100 mM sorbitol in 25 mM H_2SO_4. The assay consisted of adding 50 µl of 10% PEG solution to 950 µL FOX reagent. After 30 min incubation at 25°C, solution absorbance was measured at 560 nm based on the study of Jaeger *et al.* (1994) and at 530 nm based on studies of polysorbate 80 by Ha *et al.* (2002) using a spectrophotometer (Spectronic ®, Genesys 5) Absorbance values were then converted into a µM equivalent of H_2O_2 per 375 g of PEG; a representative standard curve using H_2O_2 standards is illustrated in Figure 1. The conversion was based on 375 g of PEG, because 375 g of PEG is necessary to make one liter of -1.6 MPa PEG solution for use in the hydroponic culture system.

RESULTS AND DISCUSSION

The results showed that both the 6-month-old and 2-year-old samples contained large amounts of peroxide; results were similar using absorbances of 530 or 560 nm (Figures 2 and 3). In contrast, the newly-purchased PEG did not contain measurable peroxide levels. Interestingly, the peroxide levels were higher in the samples that had

been stored for only 6 months in the laboratory but in a clear plastic bag than in the samples that had been stored for two years in the laboratory but in an opaque white plastic container. This result suggests that exposure to light was a major factor in the accumulation of peroxide in the stored PEG.

Wild-type and *vp14* seedlings were tested in the hydroponic culture system with the newly-purchased PEG, and toxicity symptoms were not observed. Accordingly, the experiments reported in Chapter 3 were conducted with the new stock of PEG, which was stored in the dark until immediately before PEG solutions were made.

CONCLUSION

These tests indicated that the development of toxicity symptoms in the *vp14* and wild-type seedlings was due to peroxide accumulation resulting from PEG storage under conditions of light exposure in the laboratory. Interestingly, the B73xMO17 hybrid was not adversely affected by the same PEG solutions, indicating some degree of genotypic diversity in root sensitivity to peroxide levels.

Figure 1. Relationship between absorbance at 530 nm and concentration of H_2O_2 standards using the FOX colorimetric assay.

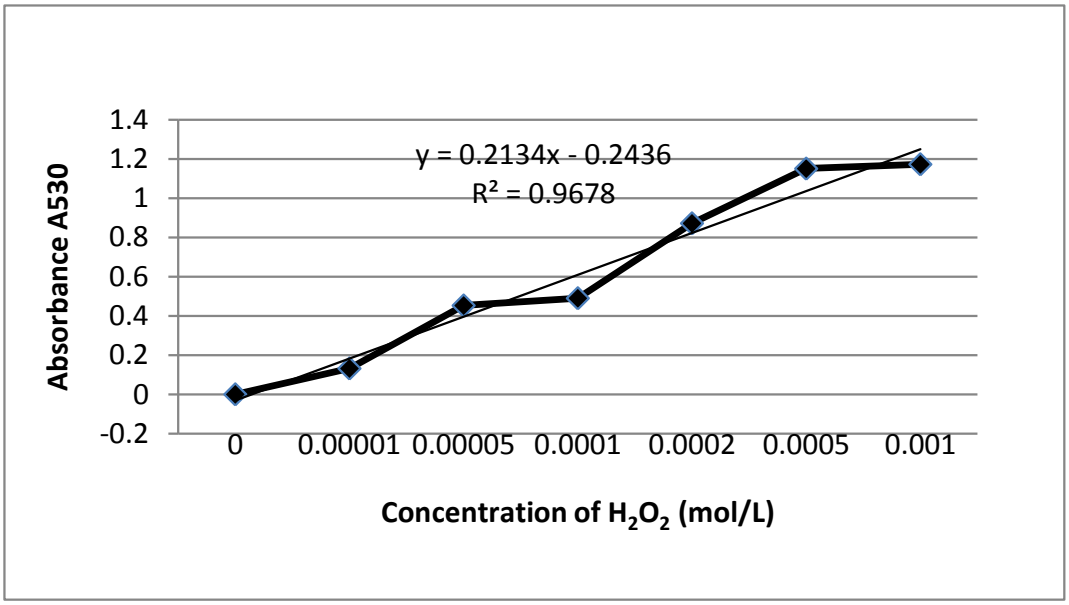
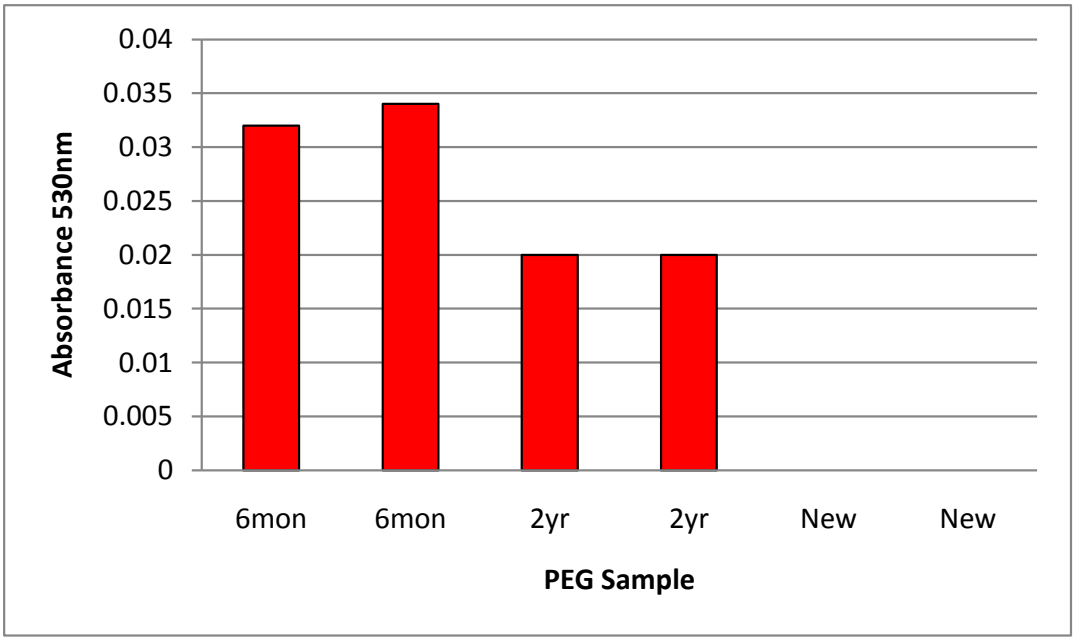


Figure 2. Absorbances at 530 nm and 560 nm of different PEG 8000 solutions using the FOX colorimetric assay. Three samples were tested: 6 mon, PEG that had been stored in a clear plastic bag in the laboratory for about 6 months; 2 yr, PEG that had been stored in an opaque white plastic storage bottle in the laboratory for approximately two years; New, newly-purchased PEG.



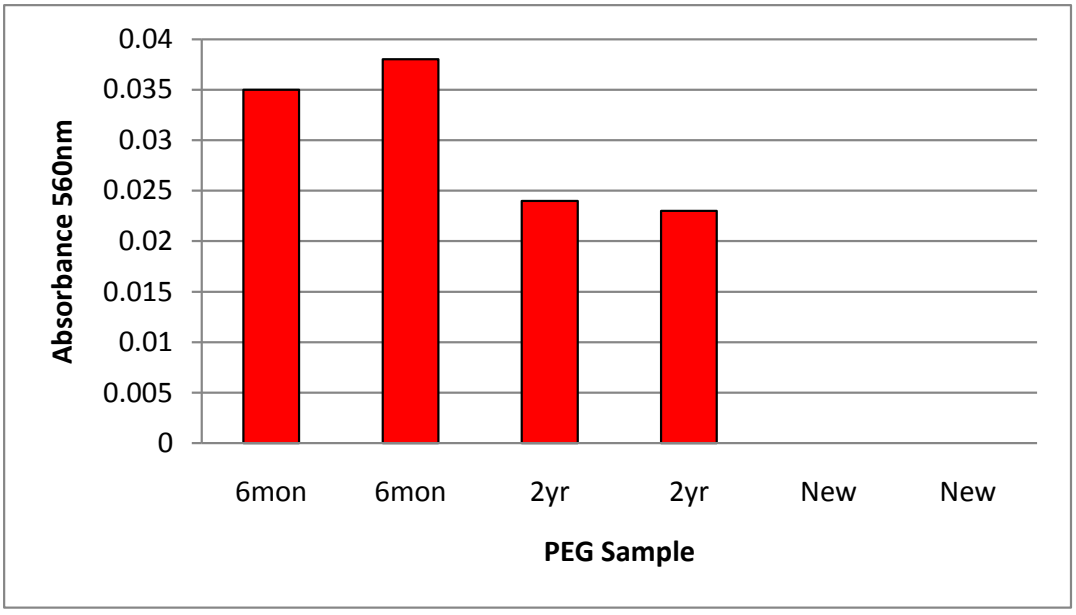
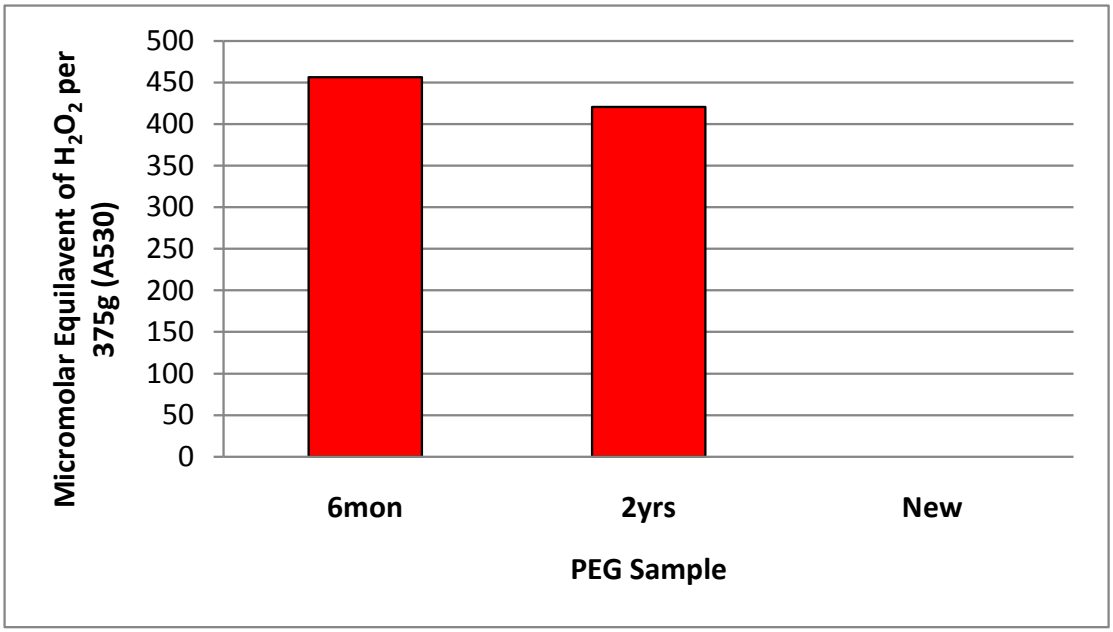
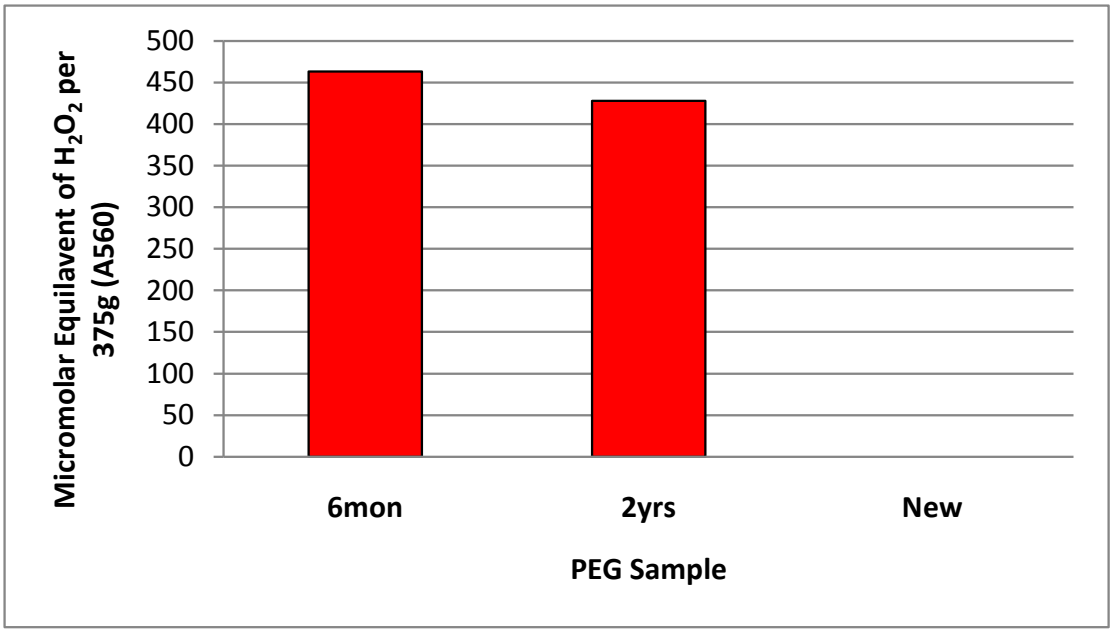


Figure 3. Peroxide contents of different PEG 8000 samples. Three samples were tested: 6 mon, PEG that had been stored in a clear plastic bag in the laboratory for about 6 months; 2 yr, PEG that had been stored in an opaque white plastic storage bottle in the laboratory for approximately two years; new, newly-purchased PEG. PEG contents were calculated from absorbances at 530 nm and 560 nm in the FOX assay.





REFERENCES

Cho, I. (2006). Function of abscisic acid in maintenance of maize primary root growth under water deficit. (Doctoral Dissertation) Retrieved from ProQuest Dissertations and Theses. (Accession Order No. [3361120])

Ha, H., Wang, W., and Wang, J. (2002). Peroxide formation in polysorbate 80 and protein stability. Jour Pharmaceutical Sci **91**, 2252-2264

Hamburger, R., Azaz, E., and Donbrow, M. (1975). Autoxidation of polyoxyethylenic nonionic surfactants and of polyethylene glycols. Pharm Acta Helvetiae **50**, 10-17

Jaeger, J., Sorenson, K., and Wolff, S.P. (1994). Peroxide accumulation in detergents. J. Biochem Biophys Methods **29**, 77-81

Jiang, Z.Y., Woollard, A.C.S., and Woolf, S.P. (1990). Hydrogen peroxide production during experimental protein glycation. FEBS Lett **268**, 69-71

Kumar, V., and Kalonia, D.S. (2006). Removal of peroxides in polyethylene glycols by vacuum drying: Implication in the stability of biotech and pharmaceutical formulations. PharmSciTech **7**, 1-7

CHAPTER 3

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

INTRODUCTION

As detailed in Chapter 1, ABA-deficient roots at low ψ_w exhibit both increased ethylene evolution rates and increased levels of intracellular ROS, and these effects are associated with cellular damage and inhibition of root elongation. Studies in other systems have shown that stress-induced ethylene can trigger increases in ROS (Ke and Sun, 2004), and conversely, that increased ROS levels can cause enhanced ethylene synthesis (Wantanabe and Sakai, 1998). Accordingly, it is likely that the increases in ethylene and ROS in ABA-deficient roots under water stress are causally linked. To further understand the mode of action of ABA in root growth maintenance at low ψ_w, it is crucial to determine whether the increase in ethylene production causes, or is caused by, the increase in ROS levels. In the experiments reported in this chapter, this question was assessed by examining whether inhibition of ethylene synthesis or action prevents the increase in ROS levels in roots of ABA-deficient seedlings under low ψ_w conditions. ABA deficiency was studied using the *vp14* mutant, and low ψ_w was imposed using oxygenated PEG in a hydroponic culture system to allow continuous application of chemical inhibitors of ethylene synthesis or action.

MATERIALS AND METHODS

Plant material, growth conditions, and root elongation measurements

All experiments were conducted with homozygous *vp14* and wild-type seeds that were produced in the field at the University of Missouri in 2009. Seedlings were subjected to low ψ_w (-1.6 MPa) conditions using the oxygenated PEG hydroponic culture system developed by Verslues *et al.* (1998), with modifications as described in Chapter 2.

Average primary root elongation rate during 24 h after the imposition of low ψ_w was determined for all experiments in which ethylene, ROS and ABA were measured. In addition, the time course of root elongation rate during 72 h after low ψ_w imposition was monitored during a series of preliminary inhibitor dose-response experiments.

Inhibitors of ethylene synthesis and action

The hydroponic culture system was utilized to allow the continuous application of aminooxyacetic acid (AOA) to inhibit ethylene synthesis, and of $AgNO_3$ to inhibit ethylene action. AOA was chosen because, among three inhibitors tested by Spollen *et al.* (2000), it was the most effective in restoring elongation of ABA-deficient roots at low ψ_w in vermiculite-grown seedlings. AOA is a non-specific chemical inhibitor, which is required for activity of 1-aminocyclopropane-1-carboxycylic acid (ACC) synthase (Amagasa *et al.*, 1992). Experiments were also conducted using $AgNO_3$, which inhibits ethylene action at the receptor level (Atta-Aly *et al.*, 1987). 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 and intracellular ROS measurements

Measurements of ethylene evolution and intracellular ROS were made in the same experiments in order to obtain the best possible association between the two responses. At 24 h after imposition of the low ψ_w treatment, 15 out of the 20 seedlings per box were harvested for measurements of primary root tip (apical 10 mm) ethylene evolution (three samples of five roots each), and the remaining five roots were used for imaging of ROS

levels in the root tip region. Details of ethylene and ROS measurements were as described in Chapter 2.

ABA assay

In each of two separate experiment, 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, the root segments were immediately frozen in liquid nitrogen and stored at -80°C. 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 has a working range of 125-2000 pg ABA per tube and vials were counted in a Beckman LS 6000IC scintillation counter.

RESULTS AND DISCUSSION

Experiments using AOA to inhibit ethylene synthesis

Determining the appropriate AOA concentration.

A series of preliminary experiments were conducted to determine an appropriate concentration of AOA 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 AOA-

treatment (Spollen *et al.*, 2000), recovery of root elongation in *vp14* was used as the primary indicator of AOA effectiveness in these preliminary experiments. The objective was to identify the lowest AOA 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 AOA and, therefore, was also presumably attributable to excess ethylene. This phenotype provided an additional indicator of the appropriate AOA concentration to be used.

A range of AOA concentrations from 5-915 μM was studied. At 5 μM AOA, root elongation was partially restored in *vp14*, but the root-curling phenotype was not fully prevented. An AOA concentration of 10 μM restored root elongation in *vp14* to a rate that was not significantly different from that in AOA-treated or untreated wild-type seedlings (Table 1), and also fully prevented the "root-curling" phenotype (see Fig. 3). Higher AOA concentrations resulted in less root growth recovery, or caused root growth inhibition.

Accordingly, an AOA concentration of 10 μM was chosen to evaluate the effects of AOA 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), AOA was only supplied as a pre-treatment during germination. Not unexpectedly, the AOA concentration used in that study [732 μM] proved to be much too high for the long-term treatments used in the present experiments.)

Ethylene evolution.

Figure 1 shows that the root tip ethylene evolution rate of *vp14* was approximately four times 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 AOA completely prevented the increase in ethylene evolution in *vp14*, while having no effect on the wild type.

ABA content.

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 AOA treatment were not attributable to an unexpected recovery of ABA levels. If ABA levels were restored in AOA-treated *vp14*, this would have confounded interpretation of the effect of ethylene suppression. The root tip ABA contents of AOA-treated and untreated wild-type and *vp14* seedlings are shown in Table 2. These results confirm that ABA levels were significantly reduced in *vp14* compared to the wild type, and were unaffected by treatment with AOA in both cases.

Intracellular ROS levels.

Figure 2 shows that the increase in root tip ROS levels in water-stressed *vp14* was completely prevented by treatment with 10 μM AOA. The AOA treatment had no effect on the basal levels of ROS in the wild-type roots. For each treatment, all five roots from each of two replicate experiments are presented, confirming the reproducibility of the observations.

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.

Experiments using $AgNO_3$ to inhibit ethylene action

Determining the appropriate $AgNO_3$ concentration.

Although AOA is an effective inhibitor of ethylene synthesis, its action is not specific to ethylene synthesis. Therefore, to strengthen the conclusions from the AOA experiments, similar experiments were attempted using $AgNO_3$ to inhibit ethylene action. Drew *et al.* (1981) successfully used $AgNO_3$, at a concentration of 0.6 μM, to restore the elongation of ethylene-treated primary roots of maize seedlings grown at high ψ_w in a solution culture system. Based on this information, a 0.6 μM concentration of $AgNO_3$ was tested for its ability to restore primary root elongation of water-stressed *vp14*. However, although the treatment did not significantly inhibit the elongation of wild-type roots, primary root elongation in *vp14* was not restored (Table 3). Lower and higher concentrations of $AgNO_3$ were also tested. Lower concentrations were also ineffective in restoring primary root elongation, while higher concentrations caused further growth inhibition, presumably due to toxicity. Beyer (1979) determined that long-term exposure to silver compounds can become toxic in plants, and this could be one reason why growth could not be restored in *vp14* was generally unaffected using our system.

Accordingly, a concentration of 0.6 μM $AgNO_3$ was used for studying the effect of $AgNO_3$ treatment on ethylene, ROS and ABA levels in *vp14* roots under water stress conditions, although the results must be interpreted with caution. The inability to restore

primary root elongation by this treatment suggests that there may have been non-specific and possibly toxic effects.

Ethylene evolution.

When using an inhibitor of ethylene action, ethylene measurements typically are not taken, because the effective action of the inhibitor is downstream of ethylene synthesis. However, Drew *et al.* (1981) showed that 0.6 μM $AgNO_3$ actually caused a substantial increase in ethylene evolution from maize primary roots. Therefore, ethylene evolution rates were examined as an additional method of assessing the effectiveness of the $AgNO_3$ treatment (Fig. 3). Surprisingly, in contrast to the findings of the Drew *et al.* (1981) study, the $AgNO_3$ treatment caused ethylene evolution to decrease in both the wild-type and *vp14* mutant roots. The explanation for the different findings in the two studies is not clear.

ABA content.

Samples were also collected for ABA measurements in the $AgNO_3$ experiments. However, most of the samples proved to be beyond the lower end of the usable range of the standard curve in the assay, and thus did not provide reliable measurements.

Intracellular ROS levels.

Figure 4 shows that the increase in root tip ROS levels in water-stressed *vp14* was completely prevented by treatment with 0.6 μM $AgNO_3$. The $AgNO_3$ treatment had no effect on the basal levels of ROS in the wild-type roots. These results strengthen the conclusion that the effect of ABA deficiency on ethylene production in water-stressed roots is upstream of, and results in, the increase in ROS.

CONCLUSION

The hydroponic culture system has proven to be a useful tool in performing ethylene inhibitor studies. These results demonstrate 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. The studies using $AgNO_3$ to inhibit ethylene action are consistent with and strengthen this conclusion, although the failure of this treatment to restore root elongation in *vp14* seedlings is of potential concern in interpreting the effects of this treatment. Additional studies to investigate the ethylene, ROS and ABA interactions utilizing alternate inhibitors of ethylene synthesis or action are in progress.

Taken together, these findings indicate that the interaction of ABA and ethylene in water-stressed roots is upstream of the production of ROS (Fig. 5). Further analysis with this system will lead to a greater understanding of the signal transduction pathway and primary mechanisms involved in the regulation of root growth by ABA under water deficit conditions. Taken together, these resources will assist us achieving a long-term goal of determining the primary role of ABA in root growth maintenance under water deficit.

Figure 1. Ethylene evolution rates from the apical 10 mm of the primary root of AOA-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 ± SD (n = 3). Different letters indicate significant differences between treatments ($p < 0.05$). The experiment was repeated with similar results.

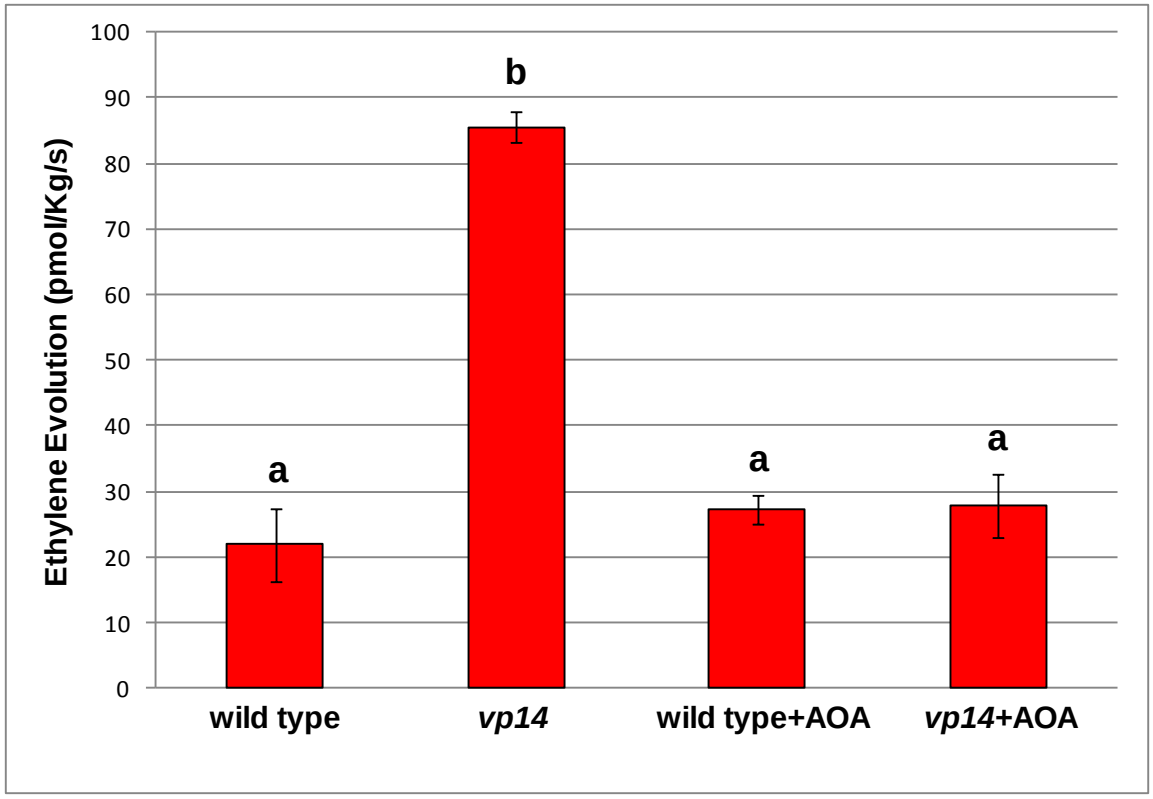
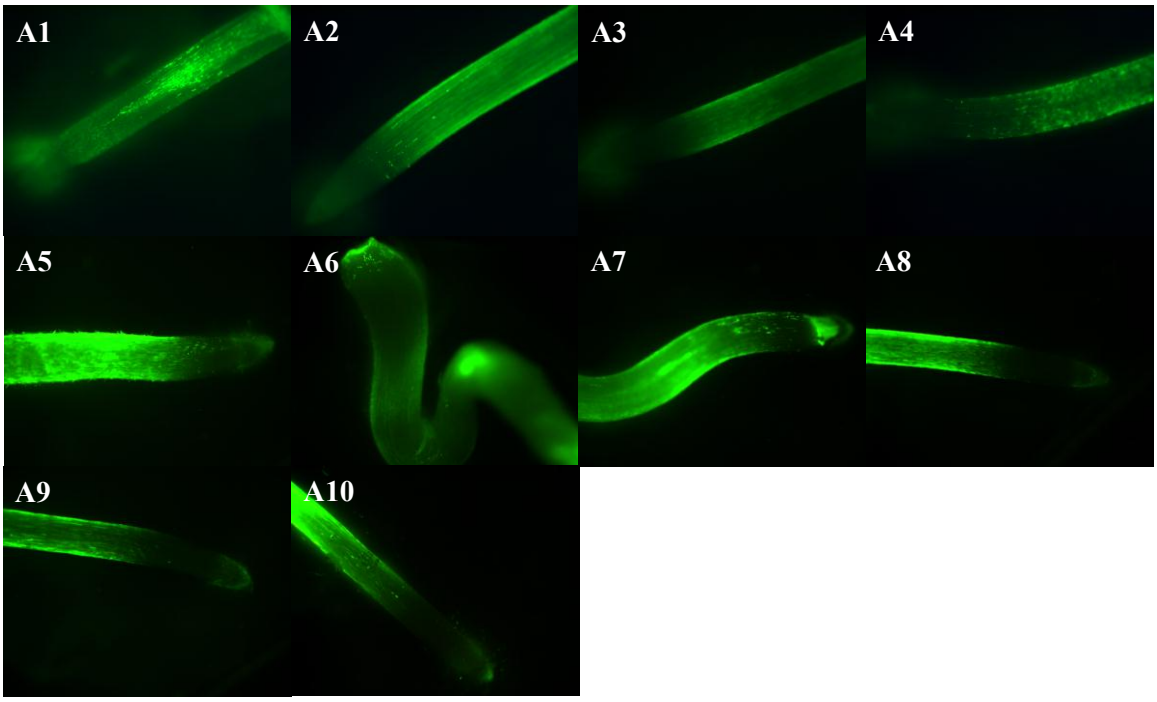
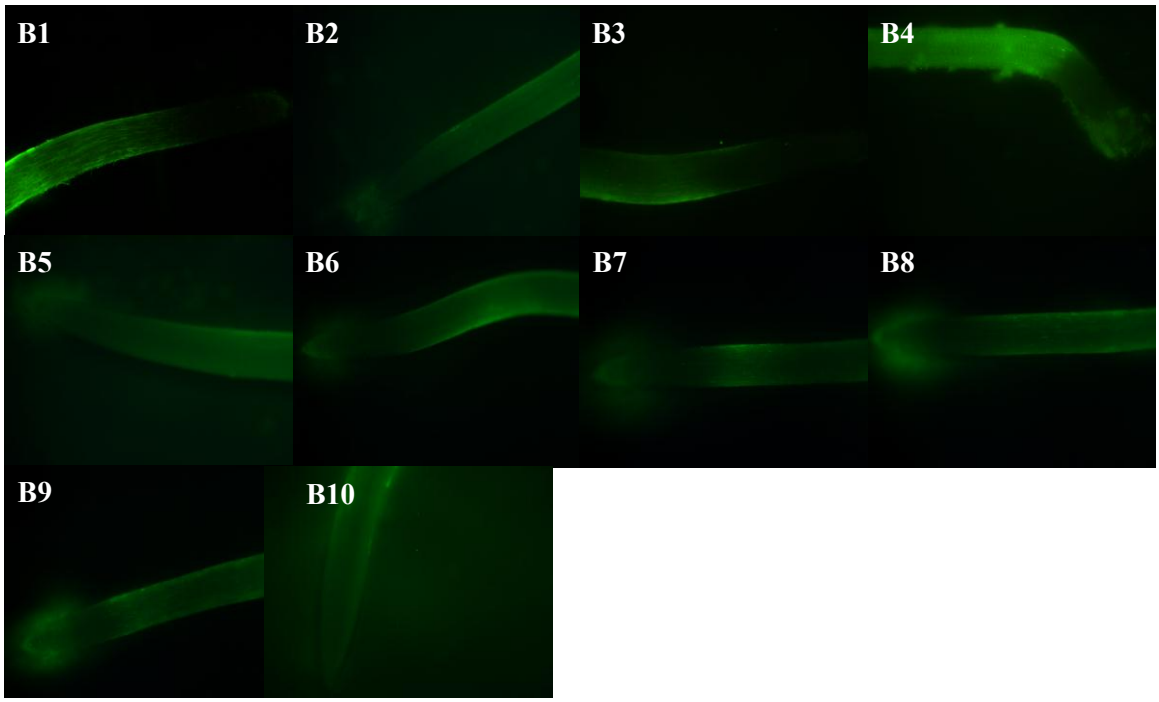


Figure 2. Fluorescent microscopy images of intracellular ROS levels in AOA-treated and untreated *vp14* and wild-type primary root tips. The measurements were made 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. For each treatment, all five roots from each of two replicate experiments are presented. A1-A10 *vp14*; B1-B10 wild type; C1-C10, *vp14* + AOA; D1-D10, wild type + AOA.

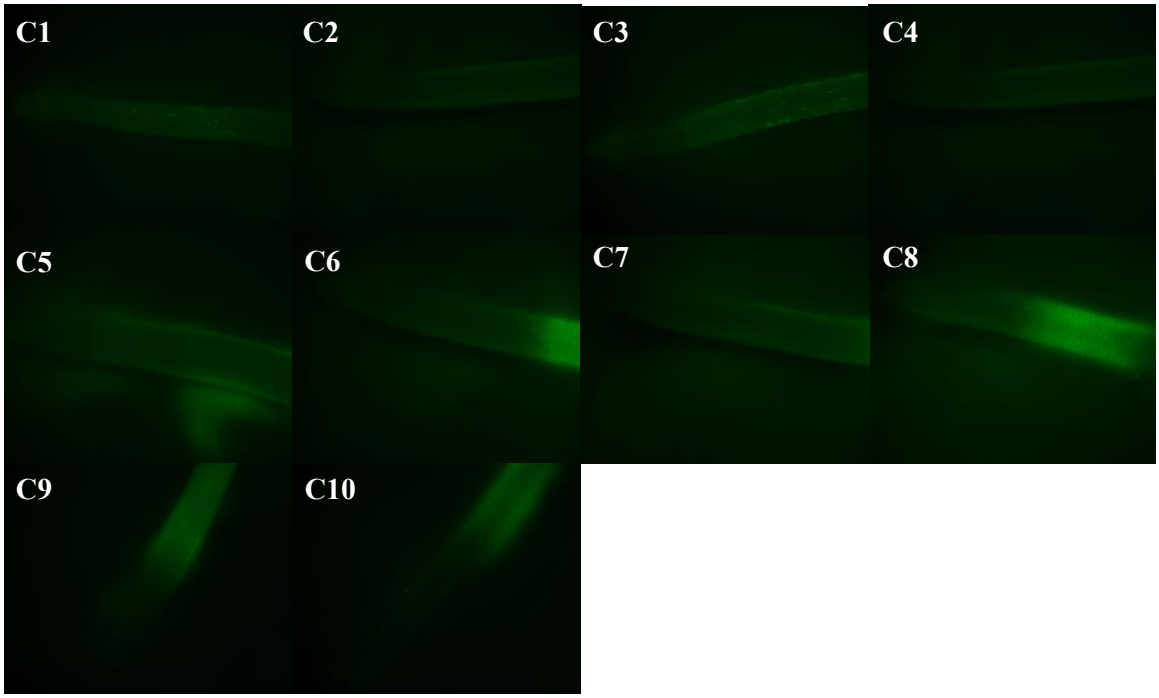
vp14



wild type



vp14 + AOA



wild type + AOA

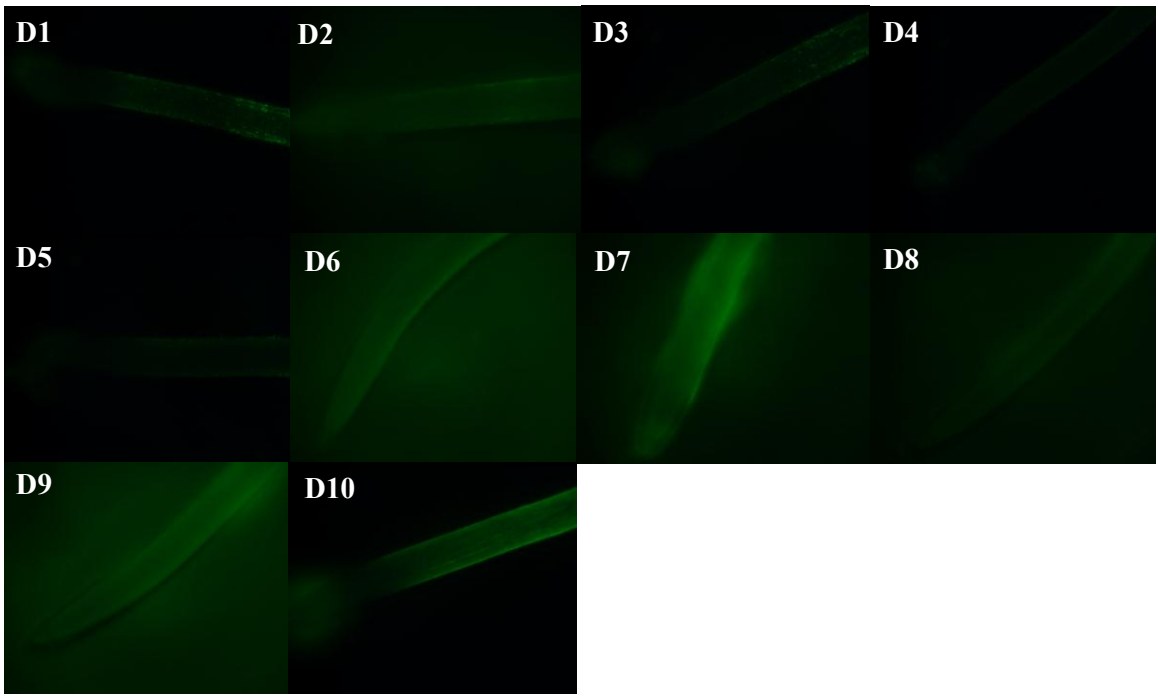


Figure 3. Ethylene evolution rates from the apical 10 mm of the primary root of $AgNO_3$-treated and untreated wild-type and *vp14* seedlings. Samples were taken 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. Data are means ± SD (n = 3). Different letters indicate significant differences between treatments ($p < 0.05$). The experiment was repeated with similar results.

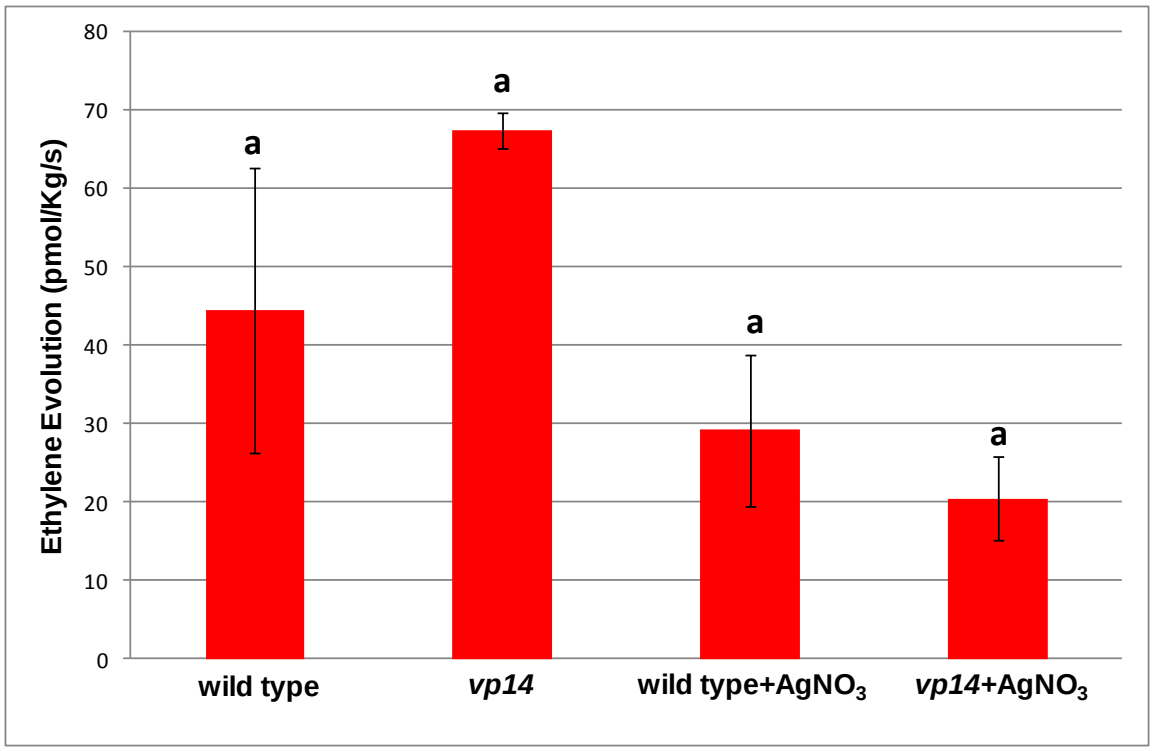
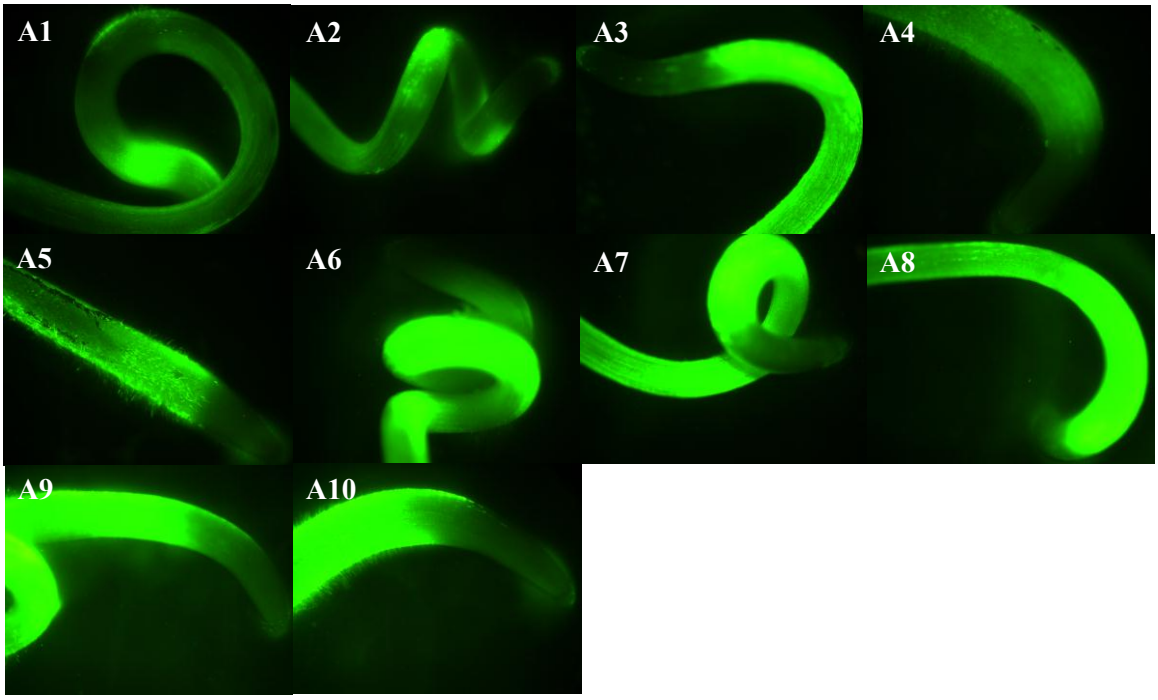
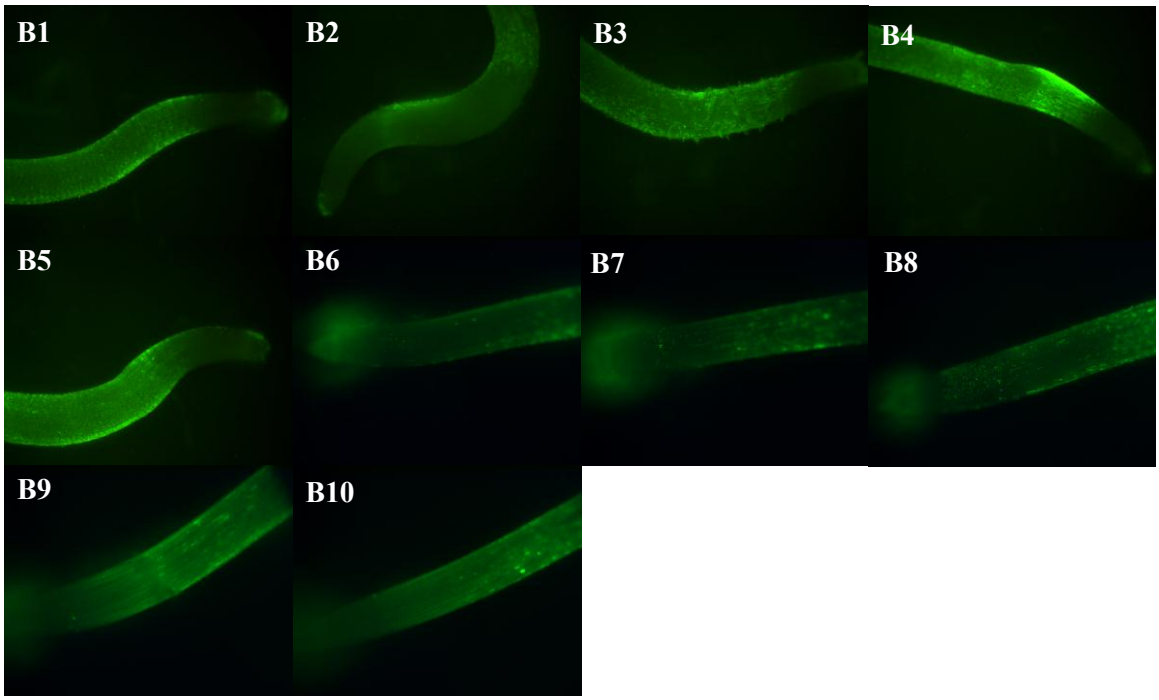


Figure 4. Fluorescent microscopy images of intracellular ROS levels in $AgNO_3$-treated and untreated *vp14* and wild-type primary root tips. The measurements were made 24 h after imposition of low ψ_w, at which time the solution ψ_w in the growth box had decreased to -1.6 MPa. All five roots from each of two replicate experiments are presented for each treatment, with the exception of the wild type + $AgNO_3$ treatment, for which only two roots were measured in the second experiment. A1-A10, *vp14*; B1-B10, wild type; C1-C10, *vp14* + $AgNO_3$; D1-D7, wild type + $AgNO_3$.

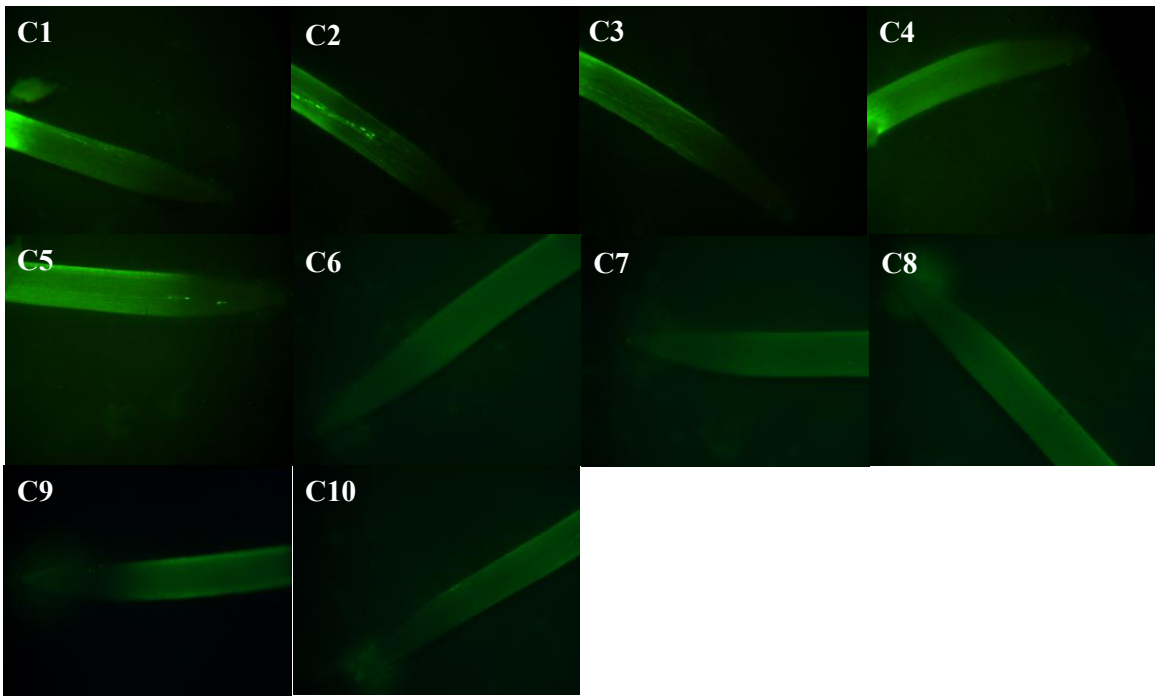
vp14



wild type



vp14 + $AgNO_3$



wild type + $AgNO_3$

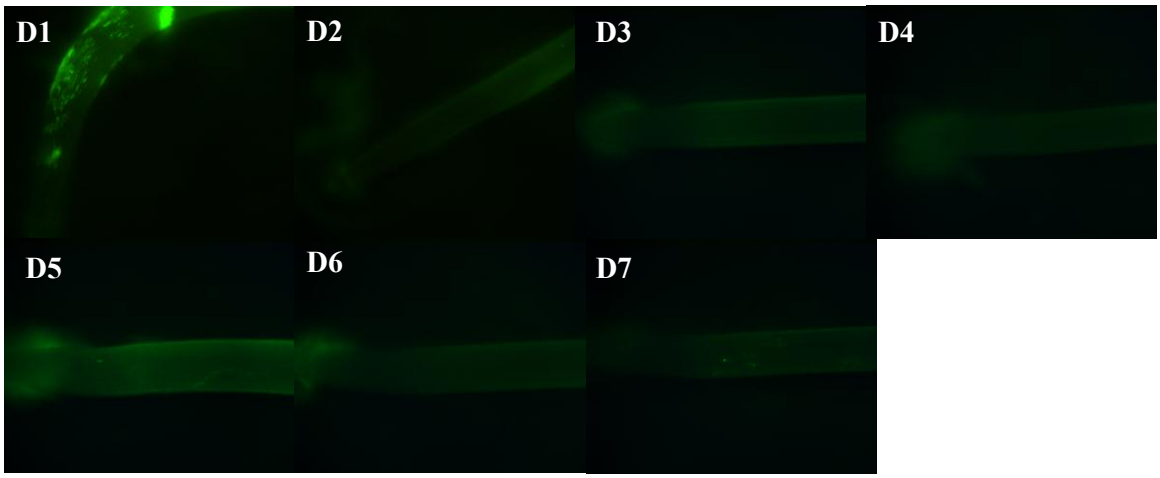


Figure 5. Illustration depicting interactions between ABA, ethylene and ROS that take place in the root growth zone of ABA-deficient maize plants under water stress.

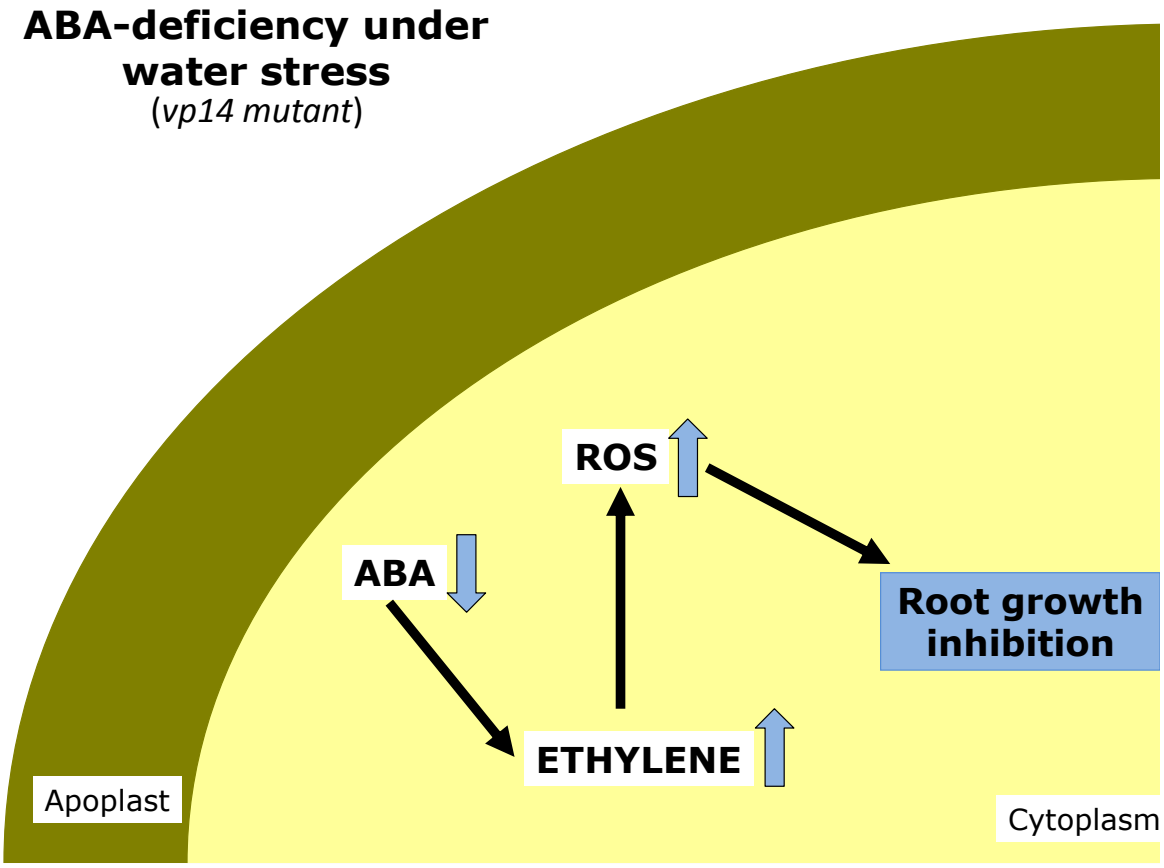


Table 1. Average primary root elongation rate of AOA-treated and untreated wild-type and *vp14* seedlings during 24 h after imposition of low ψ_w, during which time the solution ψ_w in the growth box decreased to -1.6 MPa. Data are means ± SD (n = 80, combined from four experiments. Different letters indicate significant differences between treatments ($p < 0.05$).

	wild type	wild type + AOA	*vp14*	*vp14* + AOA
Root elongation rate (mm h^{-1})	$1.29 \pm 0.29_a$	$0.96 \pm 0.20_a$	$0.58 \pm 0.04_b$	$0.81 \pm 0.23_a$

Table 2. Primary root tip (apical 10 mm) ABA content of AOA-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 = 6-7, combined from two experiments). Different letters indicate significant differences between treatments ($p < 0.05$).

	Root tip ABA content (ng g^{-1} dry weight)
wild type	$309.2 \pm 23.5_a$
vp14	$189.6 \pm 11.7_b$
wild type + AOA	$300.2 \pm 35.4_a$
vp14 + AOA	$149.9 \pm 25.9_b$

Table 3. Average primary root elongation rate of $AgNO_3$-treated and untreated wild-type and *vp14* seedlings during 24 h after imposition of low ψ_w, during which time the solution ψ_w in the growth box decreased to -1.6 MPa. Data are means ± SD (n = 60, combined from three experiments. Different letters indicate significant differences between treatments ($p < 0.05$).

	wild type	wild type + $AgNO_3$	*vp14*	*vp14*+ $AgNO_3$
Root elongation rate (mm h^{-1})	$1.01 \pm 0.13_a$	$0.85 \pm 0.19_a$	$0.55 \pm 0.15_b$	$0.55 \pm 0.25_b$

REFERENCES

Amagasa, T., Ogawa, M., and Sugai, S. (1992). Effects of aminooxyacetic acid and its derivatives on flowering in *Pharbitis nil*. Plant Cell Physiol **33**, 1025-1029

Atta-Aly, M.A., Saltveit Jr, M.E., and Hobson, G.E. (1987). Effect of silver ion on ethylene biosynthesis by tomato fruit tissue. Plant Physiol **83**, 44-48

Beyer, E.M. (1979). A potent inhibitor of ethylene action in plants. Plant Physiol **58**, 268-271

Drew, M.C., Jackson, M.B., Giffard, S.C., and Campbell, R. (1981). Inhibition by silver ions of gas space (aerenchyma) formation in adventitious roots of *Zea* mays L. subjected to exogenous ethylene onto oxygen deficiency. Planta **153**, 217-224

Ke, D.S., and Sun, G.C. (2004). The effect of reaction oxygen species on ethylene production induced by osmotic stress in etiolated mungbean seedling. Plant, Growth Regul **44**, 199-206

Quarrie, S.A. (1987), Use of differing genotypes in endogenous abscisic acid levels in studies of physiology and development. In hormone action in plant development – a critical appraisal. Hoad, G.S., Lenton, J.R., Jackson, M.B. and Atkin, R.K., eds (Butterworths, London) pp. 89-105

Saab, I.N., Sharp, R.E., Pritchard, J., and Voetberg, G.S. (1990). Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. Plant Physiol **93**, 1329-1336

Sharp, R.E., Wu, Y., Voetberg, G.S., Saab, I.N., and LeNoble M.E. (1994). Confirmation that abscisic acid accumulation is required for maize primary root elongation at low water potentials. J. Exp. Bot. **45**, 1743-1751

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

Verslues, P.E., Ober, E.S., and Sharp, R.E. (1998). Root growth and oxygen relation at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. Plant Physiol **116**, 1403-1412

Watanabe, T., and Sakai, S. (1998). Effects of active oxygen species and methyl jasmonate on expression of the gene for a wound-inducible 1-aminocyclopropane-1-carboxylate synthase in winter squash (*Cucurbita maxima*). Planta **206**, 570-576

APPENDIX I

Movement of the *vp14* mutant allele into alternate genetic backgrounds of *Zea mays*

These studies are in collaboration with Dr. Sherry Flint-Garcia and Susan-Melia Hancock.

The *vp14* mutant seed used for the studies reported in this thesis is in a W22 genetic background (Tan *et al.*, 1997). The mutant line was developed by Dr. Don McCarty at the University of Florida, Gainesville. Observations made during seed production of plants grown under Missouri and Puerto Rico field conditions indicate that both the wild-type and mutant plants were often stunted and produced little seed yield at harvest. Because of these phenomena, and because of future plans for studies of *vp14* responses to soil drying under field conditions, it was decided to move the *vp14* mutant allele into an alternate genetic background that is more suitable for plant growth and seed yield under Missouri field conditions, and which exhibits vigorous root growth characteristics under both high and low ψ_w conditions.

As an initial step, seven candidate genotypes representing a range of genetic diversity within maize were suggested by Dr. Flint-Garcia. These genotypes were characterized for primary root growth responses under well-watered (ψ_w of -0.02 MPa) and water-stressed (ψ_w of -1.6 MPa) conditions using the vermiculite-culture system described by Sharp *et al.* (1988) and Spollen *et al.* (2000). The results are shown in Table 1. When the water-stressed growth rates were expressed as a percentage of the well-watered controls, lines KY21, TX303, VA35, OH43 and B97 exhibited the least inhibited responses, with values of 53.4%, 45.7%, 44.4%, 41.3% and 38.7%, respectively. However, KY21 exhibited the slowest elongation rate under well-watered conditions of all the lines tested and, therefore, was not selected for further study. Line CML103 exhibited the slowest elongation rate at low ψ_w (23.9% of well-watered), and was also rejected for further study. Line B73, although exhibiting only a moderate maintenance of root elongation at low ψ_w (28.6% of well-watered), was retained as a

potential candidate because of the advantage that this line has been genetically sequenced (Schnable *et al.*, 2009).

The five selected lines were crossed with *vp14* and will be selfed thereafter. After four to five generations, it must be demonstrated that the *vp14* trait behaves consistently across the various germplasms. This will be accomplished with physiological and molecular testing of the material.

Table 1. Primary root elongation rate of seven genotypes of maize during 72 h after transplanting to high (-0.02 MPa) or low (-1.6 MPa) ψ_w vermiculite. Data are means ± SD (n = 15). Values in parentheses are the water-stressed rates expressed as percentages of the well-watered rates.

	KY21	TX303	VA35	OH43
High ψ_w (-0.02 MPa) Root elongation rate (mm h^{-1})	1.63 ± 0.54	1.99 ± 0.44	2.70 ± 0.49	2.01 ± 0.45
	B97	CML103	B73	
High ψ_w (-0.02 MPa) Root elongation rate (mm h^{-1})	2.35 ± 0.70	2.63 ± 0.37	2.87 ± 0.49	

	KY21	TX303	VA35	OH43
Low ψ_w (-1.6 MPa) Root elongation rate (mm h^{-1})	0.87 ± 0.09 (53.4%)	0.91 ± 0.16 (45.7%)	1.20 ± 0.19 (44.4%)	0.83 ± 0.20 (41.3%)
	B97	CML103	B73	
Low ψ_w (-1.6 MPa) Root elongation rate (mm h^{-1})	0.91 ± 0.09 (38.7%)	0.63 ± 0.45 (23.9%)	0.82 ± 0.20 (28.6%)	

REFERENCES

Schnable *et al*., (2009). The B73 maize genome: complexity, diversity, and dynamics Science **326**, 1112-1115

Sharp, R.E., Silk, W.K., and Hsaio, T.C. (1988). Growth of the maize primary root at low water potentials. I. Spatial distribution of expansive growth. Plant Physiol **87**, 50-57

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

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D., and McCarty, D.R. (1997). Genetic control of abscisic acid biosynthesis in maize. PNAS **94**, 12235-12240