FUNCTION OF ABSCISIC ACID IN MAINTENANCE OF MAIZE PRIMARY ROOT GROWTH UNDER WATER DEFICIT

A Dissertation
presented to
the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

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by
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December 2006

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

FUNCTION OF ABSCISIC ACID IN MAINTENANCE OF MAIZE PRIMARY ROOT GROWTH UNDER WATER DEFICIT

presented by In-jeong Cho

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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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Robert Sharp. Without his patience and guidance, I would have been lost easily since I didn't have any background of plants when I started my study. When I decided to study plant science for my Ph.D. in the US, my goals were to learn critical thinking and to see a forest not a tree. Because of him I have achieved part of my goals. His logic and creative thinking always inspires and reminds me why I am here.

I am really grateful for all of my committee members. Thanks to Dr. Dale Blevins who always cheers me up in either good or bad ways. Dr. Walter Gassmann helps me to solve my research problems. Dr. Steve Pallardy taught me how to see a big picture and reinforced me to remember it. Thanks to Dr. Hari Krishnan for his patience and generosity.

All of Dr. Sharp's lab members deserve my appreciation. Dr. Mary LeNoble Bondra taught me numerous protocols and non-experimental subjects, too. I truly appreciate her time and patience. I cannot express enough thanks to Dr. Eleanor Thorne. She showed what is a true friendship. I appreciate Dr. Mayandi Sivaguru not only because of his work but also his friendship and kindness. All the people who have worked in Dr. Blevin's lab also deserve my many thanks.

I cannot tell how much I adore my family. My parents taught me a discipline and as far as I can tell they have loved me unconditionally. My sister

and my brothers have supported me no matter what. Without my family support I would not be here today. I owe them my degree.

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ABSTRACT

Previous studies suggested that ABA accumulation is required to maintain maize primary root elongation under water deficit and that one of the functions of ABA is to restrict ethylene evolution. These studies were conducted by using carotenoid deficient seedlings to induce ABA deficiency. The first objective of this thesis was to confirm the previous findings by using the recently available ABA-deficient mutant *vp14*. Decreased root growth and increased ethylene production were associated with decreased ABA content in *vp14* under water-stressed conditions. The results confirm that endogenous ABA accumulation maintains root growth under water stress by restricting ethylene synthesis and indicate that the Vp14 gene is an important regulator of the ABA synthesis required for root growth maintenance under water deficit. The second objective was to determine whether ABA regulates ethylene synthesis or sensitivity or both in water-stressed roots. ABA-sufficient and ABA-deficient roots growing at low water potentials showed no difference in root growth response to applied ethylene concentrations, demonstrating that ABA restricts ethylene production and not ethylene sensitivity. The third objective was to investigate whether ABA functions to prevent excess levels of intracellular reactive oxygen species (ROS) in roots growing under

water deficits. Roots of the *vp14* mutant were stained for intracellular ROS with carboxy-H_2DCFDA and with propidium iodide to indicate loss of plasma membrane integrity. The results indicate that ABA deficiency caused excess ROS levels and associated cellular damage in the root growth zone under water stress conditions. The results of simultaneous staining for ROS levels and with propidium iodide indicated that the increase in ROS levels preceded and caused the loss of plasma membrane integrity in *vp14* roots under water deficits. A supplemental objective was to develop a method for imaging apoplastic ROS in order to test the hypothesis that enhanced cell wall loosening in the region 1 of water-stressed roots in associated with increased apoplastic ROS production. Increased apoplastic ROS levels occurred specifically in the region of growth maintenance under water stressed conditions. The results provide conclusive evidence that the maintenance of elongation in the maize primary root requires the accumulation of ABA both to restrict ethylene synthesis and to prevent excess levels of intracellular ROS. Further work is needed to determine the mechanism by which ABA regulates ROS balance in water-stressed roots.

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LITERATURE REVIEW

Water deficit causes major reductions of crop yields (Kramer and Boyer, 1995). In the U.S., drought is the leading cause of crop yield loss among various environmental limitations (Boyer, 1982). Unfavorable environments prevent crops from reaching their full genetic potential; it has been estimated that crops perform at only approximately 20% of their genetic ability (Boyer, 1982). Improvements in crop productivity are essential to feed the increasing human population (Rosegrant and Cline, 2003). To achieve this goal, it is particularly important to gain an understanding of the mechanisms of plant adaptation to water deficits.

Drought adaptation is a very complex trait. Despite much investigation for several decades, the mechanisms of adaptation are not fully understood. New genomics technologies including microarrays can provide information on global gene expression responses, but proper interpretation of these massive data sets requires greater knowledge of the fundamental physiological and biochemical processes involved in adaptation (Miflin, 2000). Indeed, the importance of physiological research to improve the use of advanced genetic techniques was emphasized by Boyer (1982).

Root growth and water deficit

Water uptake into plant cells is essential for plant growth, which is defined as an irreversible increase in cell or tissue volume (Taiz and Zeiger, 2002). Accordingly, plant growth is generally inhibited when water supplies are limited. However, root growth is often less inhibited than shoot growth at low water potentials (Ψ_w), and it has been shown that root elongation can continue at Ψ_w

substantially lower than those which cause complete inhibition of shoot elongation (Westgate and Boyer, 1985; Sharp *et al*., 1988; Spollen *et al*., 1993). The lesser sensitivity to low Ψ_w in roots is considered to be an important adaptation to dry conditions, which facilitates the maintenance of plant water uptake from a drying soil profile (Sharp and Davies, 1989; Spollen *et al*., 1993).

Root growth consists of the production of new cells in the apical meristem and subsequent cell expansion over a restricted zone close to the root apex. In the maize primary root, for example, cell expansion occurs over a distance of approximately 12 mm from the root apex; the accelerating and decelerating regions are approximately equal in length (Burström, 1953; Erickson and Sax, 1956). Turgor pressure and cell wall extensibility determine the rate of cell expansion (Lockhart, 1965). Turgor pressure, which is generated by osmotic water uptake into the cell, creates the driving force to expand the cell walls. At low Ψ_w, turgor pressure can be maintained by osmotic adjustment, i.e., the accumulation of osmotically active solutes in the cells (Sharp and Davies, 1979; Michelena and Boyer, 1982; Verslues and Sharp, 1999). The rate of wall yielding at a given turgor pressure is determined by the cell wall extensibility, which can be metabolically regulated (Wu and Cosgrove, 2000). Previous studies in water-stressed maize roots have shown that both osmotic adjustment and increased cell wall extensibility contribute to root growth maintenance at low Ψ (Sharp *et al*., 1990; Spollen and Sharp, 1991).

ABA and root growth

The hormone abscisic acid (ABA) has been traditionally considered to be a cause of growth inhibition in water-stressed plants. This view developed because ABA accumulates to high concentrations in both roots and shoots under dry conditions, often correlating with growth inhibition (Trewavas and Jones, 1991). In addition, exogenous ABA application to well-watered plants generally causes growth inhibition of both roots and shoots, mimicking the inhibitory effects of water deficits on plant growth (Hartung and Davis, 1991; Trewavas and Jones, 1991). However, interpretation of such results is complicated because exogenously applied ABA may not function in the same way as endogenous ABA, and because the action of the hormone may differ in non-stressed and stressed plants. For example, endogenous hormone balances are known to be altered by environmental stresses (Wright, 1980). Moreover, the ABA concentrations in water-stressed plants are much higher than those in well-watered plants. Therefore, the same concentrations of ABA as in stressed plants may not be physiologically relevant or may even be toxic to plants grown in well-watered conditions (Takahashi, 1973; Reid, 1990).

Accordingly, the preferable approach to study roles of ABA in plant responses to water stress is the manipulation of endogenous ABA levels under water-deficit conditions, via the use of chemical inhibitors of ABA synthesis or by using ABA deficient mutants or transgenics. A complication in the use of these approaches is that ABA-deficient plants have altered water status because of improper stomatal regulation, a major function of ABA. As a result, ABA-deficient mutants typically exhibit a wilty phenotype even under well-watered conditions,

as a result of high stomatal conductance (Tal and Nevo, 1973; Jones *et al*., 1987; Quarrie, 1987). Because the wiltiness itself can result in growth inhibition, it is critical to maintain the water status of ABA deficient compared to *wt* plants in order to accurately interpret effects of ABA deficiency on plant growth.

In studies of the role of ABA in root growth of water-stressed maize seedlings, Sharp and co-workers have developed and used a special growth system designed to prevent differences in water status between ABA-deficient and control plants. To minimize water loss by transpiration, seedlings were grown under near-saturated humidity and darkness (Sharp *et al.*, 1988). Using this growth system, evidence was provided that, in contrast to the prevailing view in the literature, accumulated ABA maintains maize primary root elongation at low Ψ_w (Saab *et al*., 1990; Sharp *et al*., 1994). In these studies, root growth was compared between control seedlings with normal accumulation of endogenous ABA and seedlings which were ABA-deficient at low Ψ_w. ABA deficiency was accomplished by treating seedlings with the chemical inhibitor fluridone (FLU), and by using the viviparous mutant *vp5*. Fluridone blocks the conversion from phytoene to phytofluene in the carotenoid biosynthesis pathway, and thereby decreases ABA biosynthesis (Fig. 2). The same step is impaired in the *vp5* mutant (Fig. 2). The results showed that low endogenous ABA levels in the primary root tip of water-stressed seedlings caused by either fluridone treatment or in the *vp5* mutant were associated with severe inhibition of root growth (Saab *et al*., 1990). Restoration of root tip ABA contents by applying exogenous ABA to the ABA-deficient roots caused complete recovery of root growth (Saab *et al*.,

1990; Sharp *et al*., 1994). The results strongly suggested that ABA accumulation is necessary in order to maintain root growth at low Ψ_w, although further work is needed to confirm that the inhibitory effects of fluridone and the *vp5* mutant on root growth were not due to the impairment of carotenoid metabolism rather than (or in addition to) the deficiency of ABA itself.

In 1997, the *vp14* mutant of maize was characterized at the biochemical and molecular levels (Schwartz *et al*., 1997; Tan *et al*., 1997). *vp14* is impaired in one of the 9-*cis*-epoxydioxygenase (NCED) genes, which code for enzymes catalyzing the cleavage step from 9-*cis*-epoxycarotenoid to xanthoxin (Fig. 2). This step is the first committed step in ABA biosynthesis and only two steps away from the synthesis of ABA. Moreover, the synthesis of xanthoxin is regarded as an important regulatory step in the accumulation of ABA under water stress. For example, NCED genes are up-regulated in water-stressed *vp14* leaves (Tan *et al*., 1997), bean roots and leaves (Qin and Zeevaart, 1999), tomato roots and leaves (Thompson *et al*., 2000), avocado leaves (Chernys and Zeevaart, 2000) and *Arabidopsis* leaves (Iuchi *et al*., 2001). Accordingly, the *vp14* mutant provides the opportunity to manipulate ABA levels in water-stressed roots without potentially confounding effects on carotenoid metabolism. Therefore, my first objective was to evaluate the use of *vp14* in order to confirm the role of accumulated ABA in maize primary root growth maintenance at low Ψ_w. In *Arabidopsis*, five *AtNCEDs* (2, 3, 5, 6, and 9) have been identified and their expressions have been shown to be regulated developmentally and environmentally (Tan *et al*., 2003). Therefore, I also examined the distribution of

Vp14 expression in different parts of maize seedlings growing at high and low Ψ_w.

ABA and ethylene interactions

The importance of hormonal interactions in control of physiological processes has long been recognized (Drury, 1969; Dodd, 2003). Hormone balances are most likely regulated by mutual effects on biosynthesis and/or signal transduction.

Exogenous ABA application decreases ethylene evolution in different plants (Gertman and Fuchs, 1972; Wright, 1980; Yoshii and Imaseki, 1982; Tan and Thimann, 1989), and ABA-deficient mutants often exhibit adventitious rooting and leaf epinasty, which are indicators of excess ethylene evolution. Recent studies of well-watered tomato and *Arabidopsis* confirmed that ethylene evolution rates are increased in ABA-deficient mutants independently of the effects of ABA-deficiency on plant water status (Sharp *et al*., 2000; LeNoble *et al*., 2004). Moreover, it was shown that the increase in ethylene production is a cause of the stunted growth phenotype of the mutants under well-watered conditions.

In earlier studies it was speculated that accumulated ABA functions to restrict ethylene production under water deficit (Bradford and Hsiao, 1982). This idea remained untested until Spollen *et al*. (2000) provided evidence that ABA deficiency causes excess ethylene production in water-stressed maize seedlings. The fluridone and *vp5* mutant approaches described above were used to induce ABA deficiency. When root ABA content was restored with exogenous ABA,

ethylene evolution decreased to the same level as in control seedlings. Moreover, the increase in ethylene production was shown to be a major cause of the inhibition of root elongation in the ABA-deficient seedlings under water deficits. This was demonstrated by adding aminooxyacetic acid (AOA) and aminoethoxyvinylglycine (AVG) to inhibit ethylene synthesis, and silver thiosulfate (STS) to block ethylene reception. All three treatments restored the root elongation rate of ABA-deficient seedlings under water deficits without altering endogenous ABA content. However, as discussed above for root growth, there is a possibility that the effects of fluridone and the *vp5* mutation on ethylene synthesis may be indirect via the impairment of carotenoid metabolism (Fig. 2). Thus, the *vp14* mutant provides an opportunity to confirm that ABA accumulation directly interacts with ethylene synthesis in water-stressed roots.

Ethylene synthesis is regulated tightly by activities of the enzymes aminocyclopropane-carboxylate (ACC) synthase and ACC oxidase (Vriezen *et al*., 1999) or ACC synthase and ACC oxidase gene expressions (Nakatsuka *et al*., 1998). In addition to the control of hormone levels, however, plants can also regulate hormone sensitivity (Drury, 1969), and it has been shown that alterations of ethylene sensitivity are also involved in responses to environmental stresses. For example, nitrogen deficiency induced increases in ethylene sensitivity in association with aerenchyma formation of maize roots and insect-induced volatile emission of maize seedlings (He *et al*., 1992; Schmelz *et al*., 2003). In *Rumex palustris*, a flooding-tolerant plant, low oxygen concentrations triggered increased ethylene sensitivity to enhance petiole elongation (Voesenek

et al., 1997). The ethylene sensitivity was shifted by increased expression of the putative ethylene receptor gene, RP-ERS1. The possibility that accumulated ABA alters the sensitivity to as well as the synthesis of ethylene in water-stressed plants has not been investigated.

Interaction of ethylene and reactive oxygen species (ROS)

Some evidence suggests that ethylene and ROS may interact to regulate each other's production under various environmental stress conditions. An example is ozone-induced ROS generation, especially of the superoxide radical ($^{\bullet}O_2^-$), in tobacco and *Arabidopsis* (Schraudner *et al*., 1998; Rao and Davis, 1999). Ozone activates ethylene synthesis via expression of the ACC synthase or ACC oxidase genes (Tuomainen *et al*., 1997). In fact, in an earlier study, McRae *et al.* (1982) showed that the superoxide radical could regulate ethylene synthesis. On the other hand, stress-induced ethylene can stimulate ROS induction and eventually lead to cell death. When tomato plants were exposed to ozone, ethylene synthesis preceded hydrogen peroxide (H_2O_2) accumulation which triggers cell death (Moeder *et al*., 2002).

The possible involvement of ROS in regulating ethylene production and thus root growth under water deficit has not been investigated. My hypothesis is that ABA accumulation at low Ψ_w stimulates antioxidant systems to block ROS increase which otherwise would trigger excess ethylene evolution.

ROS production under water deficit

ROS can be increased as a result of a shift in balance between prooxidative and antioxidative reactions in living cells (Bartosz, 1997). Various stress conditions including water deficits create unbalanced steady-state ROS levels in plant cells (Price *et al*., 1989; Pastori and Trippi, 1992; Moran *et al*., 1994; Zhang and Kirkham, 1994; Navari-Izzo *et al*., 1996). For instance, leaves experienced increased ROS levels under water deficits, which was predominantly caused by decreased photosynthesis (Smirnoff, 1993). Increased ROS can cause lipid peroxidation which results in membrane damage, protein modification, and DNA breakage, and eventually leads to cell death (Halliwell and Gutteridge, 1989; Gutteridge and Halliwell, 1990).

The sequence of ROS generation and conversion is illustrated in Fig. 3. To neutralize ROS, enzymatic and non-enzymatic antioxidant systems exist in plant cells as well as any other living organisms. The small antioxidant molecules are ascorbate (vitamin C), α-tocopherol (vitamin E), β-carotene, flavonoids, and glutathione. Unfortunately, there are not many studies about the regulation of these small molecules in response to environmental stresses in plants (Arrigoni and Tullio, 2000). In contrast, certain enzymatic systems have been thoroughly scrutinized under various stress conditions (Asada, 1997).

Photosystem II in chloroplasts, the electron transfer system in mitochondria, xanthine oxidase in peroxisomes, and NADPH oxidase in the plasma membrane are all capable of production of $^{\bullet}O_2^-$, which has a short life time and its negative charge does not permit it to cross membranes (Smirnoff, 1993;

Dat *et al*., 2000). Superoxide dismutase (SOD) and/or spontaneous action convert $^{\bullet}O_2^-$ to H_2O_2, which is relatively stable among ROS and is also membrane permeable. H_2O_2 is a precursor for hydroxyl radical ($^{\bullet}OH$) formation by the Haber-Weiss reaction with transition metals such as iron or copper (Fig. 3). The hydroxyl radical is highly destructive to cells and there is no known enzymatic system to scavenge this molecule (Vranova *et al*., 2002). Therefore, it is critical that H_2O_2 is detoxified before hydroxyl radical formation, which can be performed by catalase (CAT) or ascorbate peroxidase (APX).

Superoxide dismutase (SOD) acts as the first line of defense against generation of detrimental $^{\bullet}OH$, and increased SOD activity by over-expression of SOD genes in transgenic plants can increase protection from oxidative stress (Asada, 1999). The SOD enzyme is categorized into three groups depending on its metal cofactors, Cu/Zn, Fe, and Mn. Even though the metal binding site is highly conserved, each isozyme has a distinctive characteristic for location inside a cell, sensitivity to inhibitors, and regulation under different stress conditions (Scandalios, 1997a). Cu/Zn SODs are located in the cytosol, peroxisome, chloroplast, and possibly cell wall (Alscher *et al*., 2002). Chloroplasts and mitochondria contain Fe-SODs and Mn-SODs, which are driven by their targeting sequences at the N-terminal. The compartmentalization of Mn-SOD in peroxisomes is controversial because none of the discovered Mn-SODs have targeting sequences (Scandalios, 1997a). The diverse SOD isoforms and their distinctive compartmentalization indicate complexity of controlling ROS levels in plants (Baum and Scandalios, 1979, 1982). Differential responses of SOD

isozymes to inhibitors and substrates may explain why transgenic plants over-expressing one of the SOD genes are not always resistant to environmental stresses (Scandalios, 1997a). Additionally, maize *Sod4* and *Sod4A* sequences share 95% homology in the coding region, but only *Sod4* was increased by exogenous ABA treatment, probably due to the presence of an ABA response element (ABRE, CACGTGGT) in the promoter sequence (Cannon and Scandalios, 1989). The ABRE in the *Sod4* promoter suggests that endogenous ABA may regulate SOD transcription under stress conditions.

H_2O_2, the end product of the reaction catalyzed by SOD, can react with available metal ions of cellular proteins and is eventually changed to the harmful hydroxyl radical. Alternatively and favorably, H_2O_2 can be converted to H_2O by catalases (CAT) and/or peroxidases, especially ascorbate peroxidase (APX). Even though CAT and APX compete in the same reaction, their specificity to substrate, compartment, and energy dependency totally differ from each other (Dat *et al*., 2000). This suggests that the two enzymes function in a complementary rather than competitive manner. APX requires reducing power from ascorbate for its action, and binds H_2O_2 with a high affinity. On the other hand, CAT neutralizes H_2O_2 without energy consumption and with a rapid reaction rate, but the affinity for the substrate is low, which allows only bulk H_2O_2 removal (Willekens *et al*., 1997). Both CAT and APX are heme-containing enzymes; thereby, the enzymes can be inactivated by their substrate H_2O_2.

Catalase is a tetramer protein and exists in all plants examined (Scandalios *et al*., 1997). Catalases are classified into three categories (Table 1):

class I isozymes (CAT2 in maize) are expressed mainly in leaves, are regulated by light, and are activated during photorespiration (Willekens *et al*., 1995); class II isozymes (CAT3 in maize) are compartmentalized in vascular tissues; class III isozymes (CAT1 in maize) occur in glyoxysomes and are abundant in seeds and young seedlings. In maize, CAT1 and CAT2 isozymes are localized in the peroxisome and cytosol, and CAT3 occurs in the mitochondria (Scandalios, 1994). In addition to their subcellular compartments, the chemical properties of the different isozymes are diverse. CAT1 shows the slowest turnover, but CAT3 is the most resistant to inhibitors including cyanide (CN). The lowest CN sensitivity of CAT3 as well as its specific localization in mitochondria suggests that this isozyme might function under conditions involving activity of CN-resistant (alternative oxidase pathway) respiration (Elthon and McIntosh, 1987).

Environmental stresses regulate CAT at the transcriptional and post-transcriptional levels, and in addition, CAT expression is under developmental and tissue-specific control (Scandalios *et al*., 1997). Under osmotic stress, *Cat1* transcripts accumulated in developing and germinating maize embryos, and in maize leaves in association with high endogenous ABA content. In contrast, *Cat3* in maize leaves was down-regulated by ABA and osmotic stress (Guan and Scandalios, 1998). Most studies have examined CAT in embryos and leaves, and there is little information on roots. It was discussed that analyzing CAT in roots was difficult due to low quantities (Scandalios *et al*., 1997). For example, CAT1 and CAT3 were detected only in young roots, and the assay could not be

performed in mature roots. However, Scandalios *et al*. (1997) mentioned that CAT1 might be the dominant isozyme in roots.

APXs efficiently remove H_2O_2 coupled with the oxidation of ascorbate, especially in photosynthetic tissues. Recently, it was discovered that APX occurs in mitochondria and peroxisomes in addition to the cytosol (Foyer and Halliwell, 1976; Jiménez *et al*., 1997). APX isozymes are classified according to subcellular location: chloroplast (chlAPX), stroma (sAPX), thylakoid membrane-bound (tAPX), microbody (peroxisomes and glyoxysomes) membrane-bound (mAPX), cytosolic (cAPX) and mitochondrial (mitAPX) (Shigeoka *et al*., 2002). Each of the isoforms has different characteristics for specificity of electron donor, lability without ascorbate, affinities for H_2O_2 and ascorbate, and pH optimum. ChlAPX and mitAPX are highly specific for ascorbate as an electron donor, but cAPX and mAPX can oxidize artificial electron donors like guaiacol. Distinguishing properties of APX from other antioxidant enzymes are its instability without ascorbate, and the fact that the enzyme activity is lost when the ascorbate concentration is less than 20 μM ascorbate (Hossain and Asada, 1984). The half-inactivation times of the different isozymes are less than 30 sec in chlAPX and mitAPX, and about 1 h in cAPX and mAPX. The biological meaning of the inactivation by ascorbate absence is not yet clear, but it might function in APX regulation under stress conditions (Shigeoka *et al*., 2002). Various stresses have been shown to induce changes of APX activities and transcription levels. cAPX transcripts were increased under drought in pea (Mittler and Zilinskas, 1994). It was also shown that post-transcriptional regulation was the major control point

during the recovery period after drought. Another example of transcriptional control of APX is barley HvAPX1, a peroxisomal type APX, whose transcripts were increased in response to heat, salt, and 50 μM exogenous ABA (Shi *et al*., 2001).

High levels of reduced ascorbate should be maintained for full function of APX activity under normal and stress conditions. The reduced ascorbate pool is preserved by the ascorbate-glutathione pathway (Fig. 4), which has been extensively studied in chloroplasts (Creissen *et al*., 1994; Creissen and Mullineaux, 2002). However, the discovery of enzymes and substrates in the pathway in non-photosynthetic tissues suggests its role in other cell compartments as well (Creissen *et al*., 1994). Co-regulation of APX and glutathione reductase (GR) activities and transcripts has been discovered under several stress conditions, including drought-exposed pea and ozone-treated *Arabidopsis* (Mullineaux and Creissen, 1997).

GR can participate in scavenging ROS in addition to its function in the ascorbate-glutathione cycle. In the cycle, GR catalyzes oxidized glutathione (GSSG) to reduced glutathione (GSH) by coupling with NADPH to NADP. Glutathione is a small antioxidant molecule, γ-glutamyl-cycteinyl glycine. The reduced GSH can scavenge ROS either alone or with glutathione-S-transferase. However, the ratio between GSH and GSSG must be maintained for cell protection. GR is an NADPH-dependent oxidoredutase and multiple isoforms in plants have been reported which are located in the chloroplast, mitochondria, and cytosol (Creissen and Mullineaux, 2002). The isozymes come from separate

genes rather than post-transcriptional or post-translational modification of a gene because chloroplast, mitochondria and cytosol GR proteins require specific transit peptides for their localization in pea plants. Nevertheless there can be posttranscriptional and/or post-translational regulation of the genes depending on tissue and cell types and stress conditions (Creissen and Mullineaux, 2002).

ABA and regulation of antioxidant systems

There are several reports indicating that ABA up-regulates antioxidant systems either via modification of enzyme activities and/or expression of the genes encoding antioxidant enzymes (Asada, 1997; Mullineaux and Creissen, 1997; Polle, 1997; Richter and Schweizer, 1997; Scandalios, 1997a, b; Scandalios *et al*., 1997). For example, ABA application to leaves of well-watered maize plants increased SOD and CAT activities and expression of their transcripts (Jiang and Zhang, 2002). In addition, both SOD and CAT have abscisic acid-responsive elements (ABRE) in their promoter sequences, indicating that ABA is likely to act as a regulator of the enzymes to control oxidative damage induced by water stress (Scandalios, 1997b). In contrast, there is no direct evidence of APX and GR regulation by ABA. Possibly, ABA may regulate only SOD and/or CAT and this may trigger regulation of other enzymes. There is evidence that antioxidant systems, particularly SOD, CAT, APX, and GR, can be co-regulated under various stress conditions. Maize leaves from different inbred lines showed an inverse relationship between oxidative damage and antioxidant enzyme activities under drought, herbicide treatments

and high oxygen, all of which lead to oxidative stress (Malan *et al*., 1990; Del Longo *et al*., 1993). Transgenic approaches support the co-regulation theory. For instance, when tobacco plants over-expressed *E. coli* catalase in the chloroplast, the plants showed less degradation of chlorophyll and leaf injury than non-transgenic plants under high light conditions imposed with drought. The transgenic plants over-expressing CAT maintained initial levels of SOD activity which, in contrast, was decreased in control plants (Shikanai *et al*., 1998).

Other evidence of ABA alleviation to cellular oxidative injury is that during germination, barley aleurone layers undergo programmed cell death (PCD) which was shown to be slowed by ABA application (Fath *et al*., 2000). Aleurone cells incubated with ABA sustained the ability to scavenge ROS, and this was achieved by increase of both CAT activity and *Cat2* mRNA as well as by maintenance of SOD and APX activities (Fath *et al*., 2001).

The possibility that ABA accumulation functions to up-regulate antioxidant systems and, thereby, to limit ROS production in roots at low Ψ_w has not been investigated.

Apoplastic ROS production

As mentioned above, previous work by Sharp and co-workers has shown that maintenance of cell expansion in water-stressed maize primary roots involves increased cell wall extensibility (Lockhart, 1965). This idea was originally suggested because under severe water stress conditions (Ψ_w of -1.6 MPa), the local rate of cell elongation was fully maintained in the apical few millimeters of

the growth zone (Sharp *et al*., 1988) despite the fact that turgor levels were less than 50% of well-watered values (Spollen and Sharp, 1991). Cell wall loosening is a complex process involving the breaking of covalent bonds in the polysaccharide wall structure by enzymatic or non-enzymatic activities. Understanding of the metabolic basis of the increase in cell wall loosening in water-stressed roots is limited to two studies by Wu *et al*. (1994; 1996), who showed that activities of both xyloglucan endotransglycosylase (XET) and expansins were increased in the apical region of water-stressed compared to well-watered roots. The possible involvement of modifications in non-enzymatic cell wall loosening has not been evaluated.

Evidence has been published in support of the idea that hydroxyl radicals ($^{\bullet}OH$) can directly cause cell wall loosening by cleavage of the polysaccharide structure (Fry, 1998; Liszkay *et al*., 2004). Accordingly, it can be hypothesized that under water deficit, increased ROS levels in the apoplast might loosen the cell wall structure and thereby contribute to the maintenance of cell elongation rate in the root apical region. This hypothesis is supported by recent results in the Sharp laboratory on changes in cell wall protein composition in water-stressed root tips (unpublished data of J Zhu *et al*., NSF Plant Genome grant). Water stress was shown to induce increases in the abundance of ROS-generating proteins in the apoplast of the root apical region, including oxalate oxidase and peroxidases. Oxalate oxidase can generate hydrogen peroxide which can be converted to hydroxyl radicals by cell wall peroxidases (Wojtaszek, 1997; Passardi *et al*., 2004). Accordingly, the proteomics results predicted that higher

production of ROS, especially H_2O_2, occurs in the root tip at low compared to high Ψ_w. This prediction was supported by biochemical measurements of H_2O_2 (Tarpey and Fridovich, 2001) in apoplastic fluid extracted from the root tips by vacuum infiltration and centrifugation (J Zhu and R Sharp, unpublished). However, because of both the instability of ROS and the potential for alteration in ROS production during the extraction procedure, verification of this key result requires confirmation by an independent method that is free of tissue extraction. Therefore, I conducted experiments to visualize apoplast ROS in the apical region of intact well-watered and water-stressed roots using a novel membrane-impermeable fluorescent indicator of ROS.

ROS levels in the apoplast and cytoplasm should be controlled tightly so as not to damage cellular structure and function. The schematic shown in Fig. 5 illustrates my overall hypothesis that ABA is a key regulator of the antioxidant system in the root growth zone, serving to balance ROS levels in order to maintain root growth at low Ψ_w.

Objectives

The overall goal of my research is to confirm that ABA accumulation is required for root growth maintenance at low Ψ_w, and to gain a greater understanding of the physiological and biochemical mechanisms involved in this response. The specific objectives are to:

- Investigate the suitability of the *vp14* mutant to confirm that ABA accumulation maintains maize primary root growth maintenance at low Ψ_w.

- Confirm that the function of ABA in root growth maintenance low Ψ_w involves restriction of ethylene production.
- Determine whether ABA accumulation in roots at low Ψ_w regulates ethylene sensitivity as well as ethylene synthesis.
- Determine whether ABA accumulation in roots at low Ψ_w prevents excess intracellular ROS levels.
- Confirm that water stress induces an increase in apoplastic ROS levels in the apical region of the root growth zone.

Figure 1. Spatial distribution of relative elongation rate in the growth zone of the maize primary root at high and low Ψ_w (-0.03 and –1.6 MPa, modified from Liang *et al*., 1997).

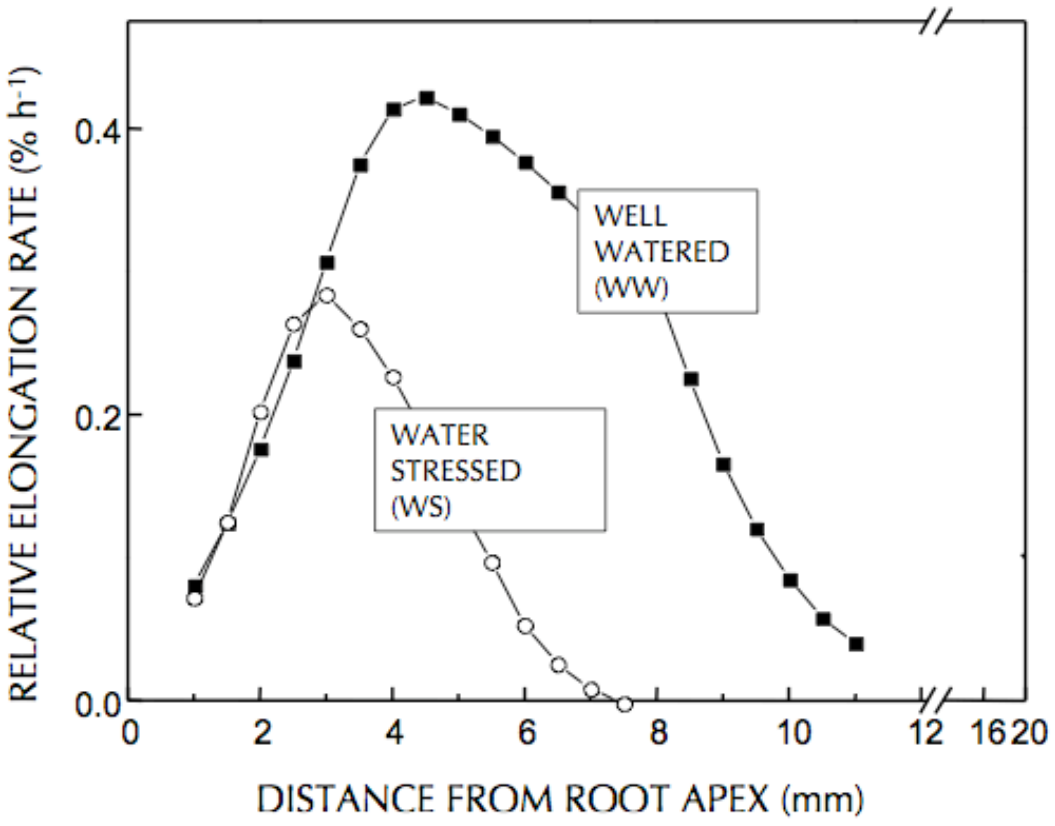

Figure 2. ABA biosynthesis pathway (modified from Taylor *et al*., 2000). FLU, fluridone.

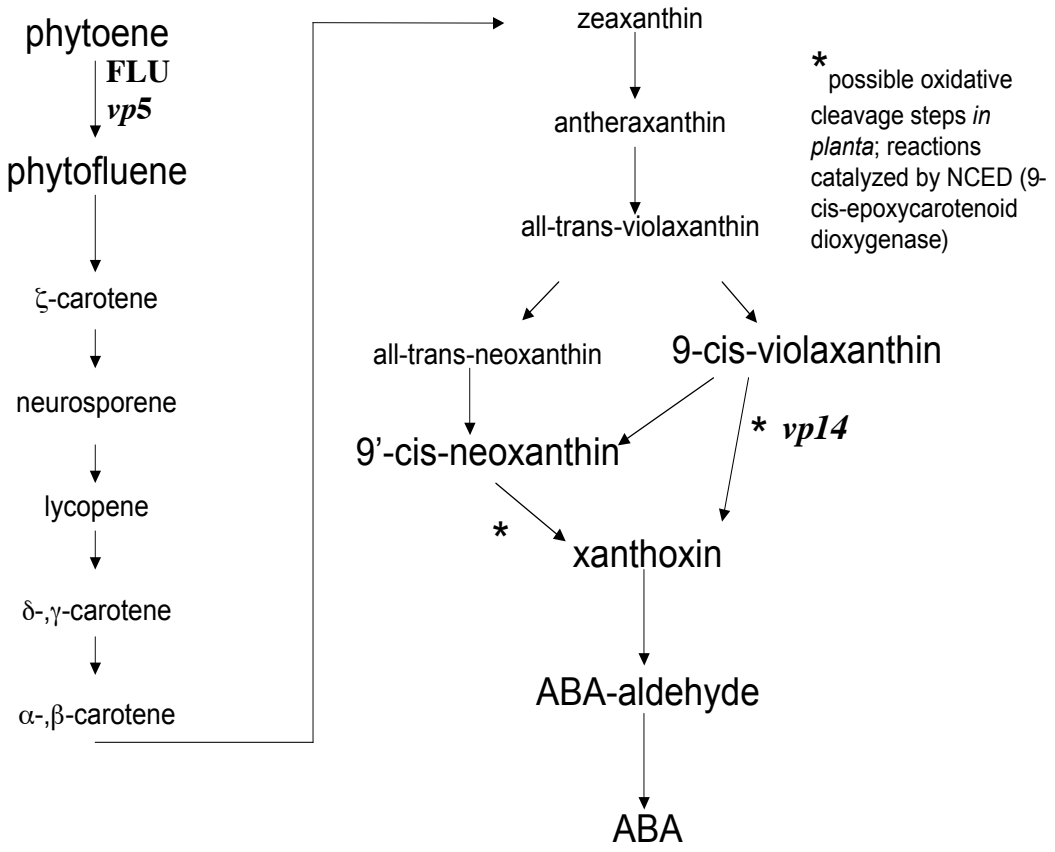

Figure 3. Generation and detoxification of reactive oxygen species (ROS) (modified from Vranova *et al*., 2002).

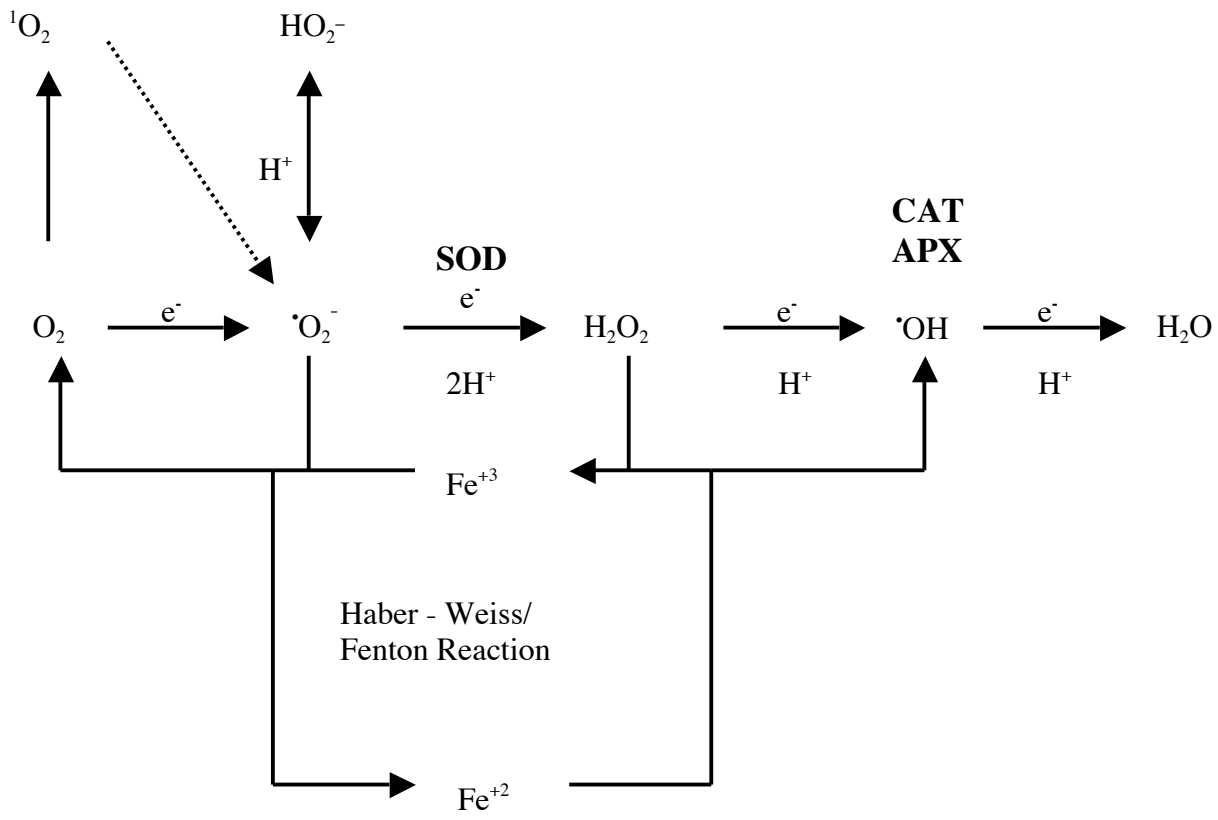

Figure 4. Ascorbate-glutathione cycle (modified from Scandalios *et al*., 1997). Asc. rd, reduced ascorbate; MDA, monodehydroascorbate (oxidized ascorbate); DHA, dehydroascorbate (oxidized ascorbate); GSH, reduced glutathione; GSSG, oxidized glutathione; APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase.

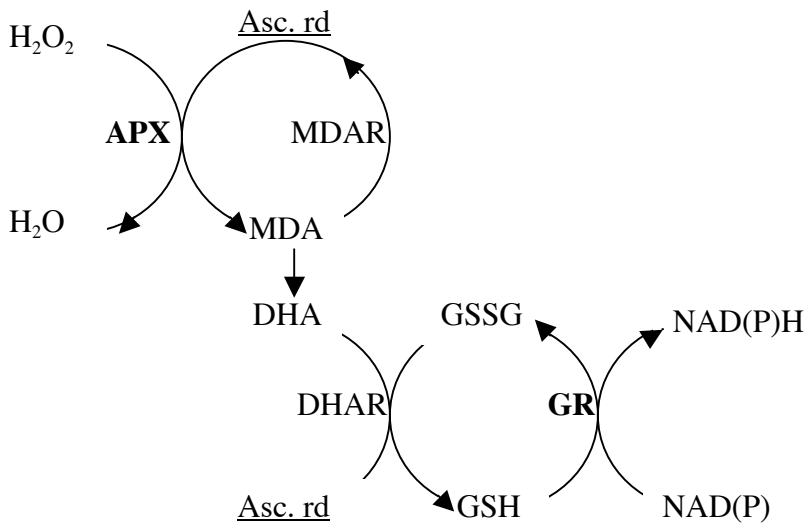

Figure 5. Model depicting function of accumulated ABA in controlling cytosolic and apoplastic ROS levels to maintain root growth under water stress conditions. Red arrows indicate increase.

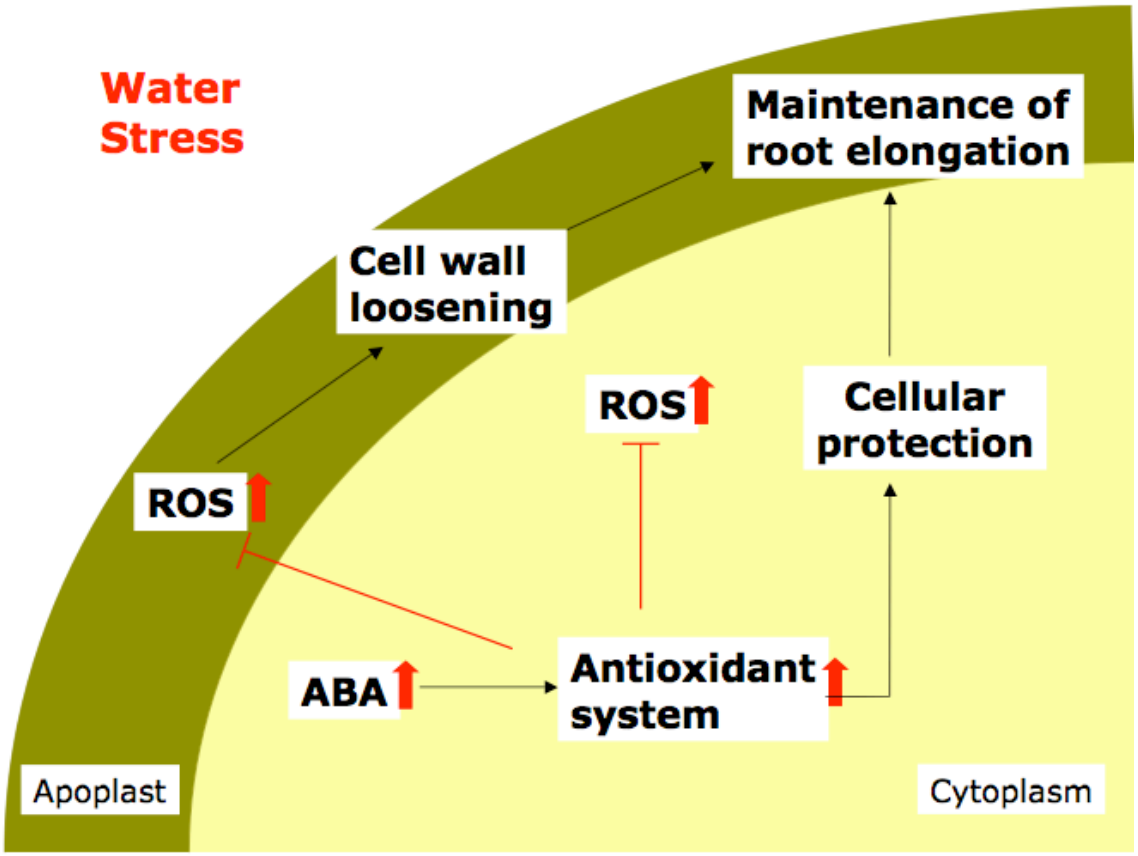

Table 1. Nomenclature of plant catalases (modified from Scandalios *et al*., 1997).

Species	Class I	Class II	Class III
Gossypium hirsutum (cotton)	SU2		SU1
Nicotiana plumbaginifolia (tobacco)	Cat1	Cat2	Cat3
Ricinus communis L. (castor bean)	CAT2	CAT1	
Zea mays (maize)	CAT2	CAT3	CAT1
Arabidopsis thaliana	CAT2	CAT1	CAT3
Lycopersicon esculentum (tomato)		TOMCAT1	
Solanum tuberosum (potato)		Cat2St	

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CHAPTER 2

Use of the *vp14* mutant to confirm that ABA accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production

Real-time RT-PCR was performed by Dr. Bao Cai Tan, a postdoctoral scientist in Dr. Donald McCarty's lab at the University of Florida-Gainesville.

INTRODUCTION

Previous studies by Sharp and co-workers provided evidence that accumulated ABA maintains maize primary root elongation at low Ψ_w (Saab *et al*., 1990; Sharp *et al*., 1994). Decreased endogenous ABA levels in water-stressed roots were achieved by using the chemical inhibitor fluridone and the *vp5* mutant. More recent results using the same approaches indicated that an important role of accumulated ABA in root growth maintenance at low Ψ_w is the prevention of excess ethylene production (Spollen *et al*., 2000). However, because both fluridone and the *vp5* mutant block ABA synthesis indirectly via impairment of carotenoid metabolism, it is important to confirm that the effects of these treatments on root growth and ethylene production are due to ABA deficiency and not to effects on carotenoid precursors (Chapter 1, Fig. 1). In this chapter, the *vp14* mutant (Schwartz *et al*., 1997; Tan *et al*., 1997) was used to confirm the role of ABA in root growth maintenance and restriction of ethylene production under water deficits. In *vp14*, ABA synthesis is impaired in one of the 9-*cis*-epoxydioxygenase (NCED) genes, which code for enzymes catalyzing the cleavage step from 9-*cis*-epoxycarotenoid to xanthoxin. This step is the first committed step in ABA synthesis (Chapter 1, Fig. 2). In *Arabidopsis*, five *AtNCEDs* (2, 3, 5, 6, and 9) have been identified and their expressions are regulated developmentally and environmentally (Tan *et al*., 2003). Therefore, the spatial distribution of *Vp14* expression in different tissues of maize seedlings growing at high and low Ψ_w was also examined.

MATERIALS AND METHODS

vp14 genetic material

Recent discoveries (detailed in Appendix) indicate, unexpectedly, that all of the *vp14* mutant seeds used for this experiment were obtained from segregating ears from heterozygous mother plants. The results indicate that the inheritance of *vp14* appears to follow a maternal pattern with all kernels from a segregating ear on a heterozygous plant displaying the heterozygous genotype (Appendix Table 1). Thus, the use of segregating seeds for physiological studies of the role of ABA in root growth responses to water stress is not compromised. Hereafter, all seed from the segregating ears are referred to as *vp14'*.

Growth experiments

vp14' (vp14-2274) was originally screened in Roberton's Mutator lines via its mild viviparous phenotype and then outcrossed to W22 (Tan *et al*., 1997). Seeds of the near-isogenic wild type (*wt) NS-2274* (nonsegregating) and *vp14'* mutant strains were imbibed for 23 h and germinated for 32 h in vermiculite (Strong Lite, grade 3, Pine Bluff, AR) which was well-moistened with 1 mM $CaSO_4$ (Spollen *et al*., 2000). The seedlings were then transplanted to vermiculite of either high or low water content, corresponding to Ψ_w of approximately –0.02 MPa and –1.6 MPa, as described previously (Sharp *et al*., 1988). The vermiculite was mixed thoroughly with different amounts of 1 mM $CaSO_4$ to obtain the different Ψ_w, which were measured for each experiment by using isopiestic thermocouple psychrometry (Boyer and Knipling, 1965). Transplanted seedlings were grown in Plexiglas boxes or glass beakers at 29 ± 1

ºC and near-saturation humidity in the dark. A green safelight was used for measuring root length and harvesting seedlings (Saab *et al*., 1990). After 48 h, primary root length was measured and the apical 10 mm (encompassing the growth zone; Chapter 1, Fig. 1) was harvested for ABA assay. The root cap was removed and the segments were then immediately frozen in liquid nitrogen. ABA content was measured by radioimmunoassay (Quarrie *et al*., 1988).

For fluridone treatments, 1.5 μM fluridone (Eli Lilly and Company, Indianapolis) was mixed with the vermiculite in which seeds were germinated and into which seedlings were transplanted. Previous work established that 1.5 μM fluridone is the minimal concentration which effectively decreases root tip ABA accumulation under water stress without causing inhibition of root elongation in well-watered roots (Spollen *et al*., 2000). Fluridone solution was prepared as described previously (Ober and Sharp, 1994; Spollen *et al*., 2000). To determine whether exogenous ABA could overcome the inhibition of root growth under water stress in *vp14'*, 0.5 mM (±) ABA (Sigma-Aldrich, St. Louis) was added to the vermiculite into which seedlings were transplanted (Spollen *et al*., 2000).

Ethylene evolution

Ethylene evolution rate of intact seedlings was measured by gas chromatography using a flow-through gas analysis system, as described previously (Spollen *et al*., 2000). After germination, seedlings were transplanted to a Plexiglas chamber filled with vermiculite of high or low Ψ_w, and then the chamber was closed with a gas-tight lid. In one experiment, up to four chambers were prepared. Ethylene-free air was supplied to each chamber at a rate of 49

ml/min. Every 12 h after transplanting, 60 mL of the outlet air from the each chamber was sampled by using a 60 mL gas-tight syringe and injected into the gas chromatograph. The ethylene content of the sample was calculated via a standard series of known amounts of ethylene. The presented unit, ethylene evolution rate/seedling, was calculated as the total ethylene evolution rate of the seedlings in the chamber divided by seedling number (Spollen *et al*., 2000).

***Vp14* expression**

Homozygous *wt* seedlings were transplanted to vermiculite of either high or low Ψ_w. The root tip (0-10 mm), rest of the root, kernel, and shoot were harvested and frozen in liquid N_2 for measurements of *Vp14* expression. There were two well-watered controls in this experiment – a developmental control which was harvested at 22 h after transplanting when the primary roots were of the same length as in the water-stressed roots at 48 h, and a temporal control which was harvested at 48 h after transplanting. Because shoot growth was almost completely inhibited at a Ψ_w of –1.6 MPa (Sharp *et al*., 1988), shoot samples in the water-stressed treatment comprised mainly the tissue which had grown prior to transplanting.

Total RNA was isolated from frozen tissues using the Rneasy Plant Mini Kit (Qiagen, Germany), and the concentration was determined by measuring absorbance at A_{260}. *Vp14* transcript levels were measured by TaqMan™ Real-time quantitative RT-PCR under conditions suggested by the manufacture (Applied Biosystems), using 400 ng total RNA, 800 nM each of *Vp14* forward and reverse primer, and 200 nM of a *Vp14* -specific detection probe. The sequences

of forward and reverse primers as designed by Primer Express™ (Applied Biosystems) were 5'-GCTGGCTTGGCTTGTATACTCTG-T -3' and 5'-CCATCAGTCATATACTGTGAACAAATGT-3', respectively. The TaqMan™ fluorogenic *Vp14* specific probe was 5'-6FAM-CACGCACCGATAGCCACAG GGAA-TAMRA-3'. The conditions for reverse transcription and real-time PCR were 30 min at 48˚C for reverse transcription, 10 min at 95˚C for inactivation of RNA reverse transcriptase and activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions were carried out in the GeneAmp 5700 Sequence Detection (Applied Biosystems, Perkin Elmer). A plasmid containing *Vp14* cDNA was used in a standard curve assay, and all the *Vp14* transcripts were normalized as copy number per ng of total RNA.

Statistical analysis

Analysis of variance was utilized to compare means by using Fisher's LSD test at the P = 0.05 or P = 0.1 level.

RESULTS AND DISCUSSION

Root elongation of *vp14'*

The primary root length increase in *vp14'* and its homozygous *wt* were not significantly different when they were grown at high Ψ_w (-0.02 ± 0.01 MPa). Root length increases over 48 h after transplanting of *wt* and *vp14'* seedlings were 102.6 ± 2.0 mm and 105.0 ± 1.6 mm, respectively (Fig. 2A), corresponding to average elongation rates of 2.14 and 2.19 mm/h. Moreover, ABA content of the

root tip (0-10 mm) was not significantly different between *wt* and *vp14'* seedlings, with mean values of 19.3 and 22.6 ng ABA/g water (Fig. 2B). However, at low Ψ_w (-1.6 ± 0.01 MPa) there were substantial differences between *wt* and *vp14'* in root growth and ABA content (Fig. 2C and D). The root length increase of *wt* seedlings was 44.0 mm at 48 h after transplanting to water stress conditions, in association with a greatly increased ABA content of the root tip (103.2 ng/g water) compared to well-watered seedlings. In contrast, the root length increase of *vp14'* was only 31.8 mm, in association with a root tip ABA content of 68 ng/g water. Thus, root elongation and root tip ABA content were reduced by 28% and 34%, respectively, in *vp14'* compared to *wt* seedlings. These findings are consistent with previous reports using fluridone-treated or *vp5* mutant seedlings (Saab *et al*., 1990; Sharp *et al*., 1994; Spollen *et al*., 2000). Moreover, when 1.5 μM fluridone was applied to *wt* seedlings at a Ψ_w of –1.6 MPa, root elongation and root tip ABA content were inhibited to the same degree as in *vp14'* (Fig 2B and D). These results indicate that the effects of fluridone result from insufficiency of ABA accumulation rather than possible side effects.

In previous studies (Spollen *et al*., 2000), an exogenous concentration of 0.5 mM (±) ABA was optimal for root growth restoration of 1.5 μM FLU-treated seedlings growing at a Ψ_w of -1.6 MPa. This high concentration was necessary to restore the root tip ABA content to *wt* levels because of limited uptake of ABA from the dry vermiculite (Sharp *et al*., 1994). Therefore, to confirm that the root growth inhibition of *vp14'* under water stress was due to ABA deficiency, 0.5 mM

(±) ABA was applied. This treatment resulted in root lengths and root tip ABA contents which were not significantly different to those of *wt* seedlings.

To examine whether further decrease in root tip ABA content caused a more severe phenotype, 1.5 μM fluridone was applied to *vp14'* at low Ψ_w. This treatment induced further inhibition of root elongation in association with further reduction of ABA content compared to untreated *vp14'* (Fig. 2C and D). The elongation rate and root tip ABA content of fluridone-treated *vp14'* were inhibited by 51% and 55%, respectively, compared to *wt* seedlings, and the severity of the phenotype was similar to results obtained with fluridone-treated or *vp5* mutant roots at low Ψ_w in previous studies (Sharp *et al*., 1994). Taken together, the results with *vp14'* confirm previous conclusions that maintenance of root elongation under water stress depends on and is tightly associated with the level of accumulated ABA (Sharp, 2002).

Ethylene evolution of *vp14'*

At 12 h and 24 h after transplanting to a Ψ_w of -1.6 MPa, the ethylene evolution rate of *vp14'* seedlings was approximately twice that of the *wt* (Fig. 3). Consistent with previous studies (Spollen *et al*., 2000), the ethylene evolution rate of both *wt* and *vp14'* seedlings was somewhat lower at 24 h compared to 12 h after transplanting. Ethylene evolution rate returned to the *wt* level when 0.5 mM (±) ABA was added to *vp14'*, in association with the restoration of root tip ABA content at 12 and 24 h (Fig. 2D). The ethylene evolution rate of fluridone-treated *wt* seedlings was also measured, and was not significantly different from

that of *vp14'* at either time (Fig. 3). This finding is consistent with the similar degrees of ABA deficiency in the two treatments (Fig. 2D).

The results with *vp14'* confirm that ABA accumulation under water stress functions to restrict ethylene evolution. The similarity in the responses of fluridone-treated and *vp14'* seedlings indicates that the previously-reported increases in ethylene evolution caused by fluridone or in the *vp5* mutant (Spollen *et al*., 2000) resulted from ABA deficiency rather than side effects caused by the impairment of carotenoid metabolism.

Gene expression of *Vp14*

Tan *et al.* (2003) investigated the expression of five NCED genes in *Arabidopsis*, and found that the genes are regulated differently in various tissues and at different developmental stages. In maize, Southern blot analysis indicates that NCED genes also belong to a small gene family (Tan *et al*., 1997). To determine in which tissues *Vp14* gene transcription is a major regulation point of ABA synthesis under water deficit, real-time RT-PCR was performed to quantify *Vp14* transcripts in various parts of *wt* seedlings at high and low Ψ_w: the root tip (0-10 mm), rest of the root, shoot and kernel. Fig. 4 illustrates that *Vp14* gene expression at low Ψ_w was stimulated in all parts of the seedling, especially in the root tip (encompassing the growth zone) which showed a 10-fold increase of transcript abundance at low compared to high Ψ_w. The results suggest that the increase in expression of *Vp14* in the root tip probably makes a major contribution to the accumulation of ABA in the root growth zone, and thus to the maintenance of root growth at low Ψ_w. The increase in *Vp14* expression at low

Ψ_w is consistent with other studies. For example, NCED gene expression was increased under water stress conditions in *Vp14* maize leaves, *arabidopsis* leaves, and bean leaves and roots (Qin and Zeevaart, 1999; Tan *et al*., 1997; Tan *et al.* 2003). In bean leaves and roots, NCED transcripts directly contributed to the amount of NCED enzymes (Qin and Zeevaart, 1999).

However, the expression of *Vp14* in all parts of the seedling suggests that expression of *Vp14* is not tissue specific under water stress conditions. Furthermore, there was virtually no difference in expression in any tissues between the developmental and temporal well-watered controls, indicating that expression of *Vp14* is not regulated developmentally.

It should be noted that the absolute duplicated number of *Vp14* transcripts in the mature (non-growing) region of the root (>10 mm from the apex) was larger than the number in the apical 10 mm region (Fig. 4). The root tip maintained the ability for cell elongation even at low Ψ_w, which probably reflects the sum result of vigorous metabolism including solute accumulation, gene expression, and enzyme regulation. Therefore, transcripts in the growing zone might be more diverse than transcripts in the mature zone. Since *Vp14* transcripts were represented based on total RNA content, the relative amounts of *Vp14* transcripts per ng total RNA in the root tip were lower than in the mature zone due to possible dilution effects.

Interestingly, NCED genes that respond to water stress share the characteristic of one exon without introns. Since severe stress conditions can alter RNA splicing, which can cause improper protein folding or protein

malfunction, the one exon strategy might be an advantage for adaptation to drought (Bournay *et al*., 1996; Marrs and Walbot, 1997; Tan *et al*., 2003).

CONCLUSION

The results indicate that *vp14'* is a suitable tool to elucidate ABA functions under water deficits not only due to its specific site of blockage in the ABA synthesis pathway (Chapter 1, Fig. 2) but also because, at least at the seedling stage, the roots are ABA deficient under water-stressed but not well-watered conditions (Fig. 2). This permits the study of the role of ABA accumulation under stress without confounding effects of growth impairment under control conditions.

By using *vp14'*, the results of this study establish that accumulated ABA constrains excess ethylene evolution and thereby maintains primary root growth of maize seedlings at low Ψ_w, confirming previous studies which used either fluridone treatment or the *vp5* mutant to induce ABA deficiency (Saab *et al*., 1990; Sharp *et al*., 1994; Sharp *et al*., 1998; Spollen *et al*., 2000). It is possible that accumulated ABA at low Ψ_w can regulate ethylene sensitivity as well as synthesis, and this question is addressed in the following chapter. Further studies about the relationships between ABA and ethylene at the gene and protein levels will be required to gain a comprehensive understanding of this hormone interaction.

Figure 1. Flow-through gas analysis system to measure ethylene evolution rate of intact maize seedlings. The black arrow indicates the sampling point for ethylene produced by seedlings.

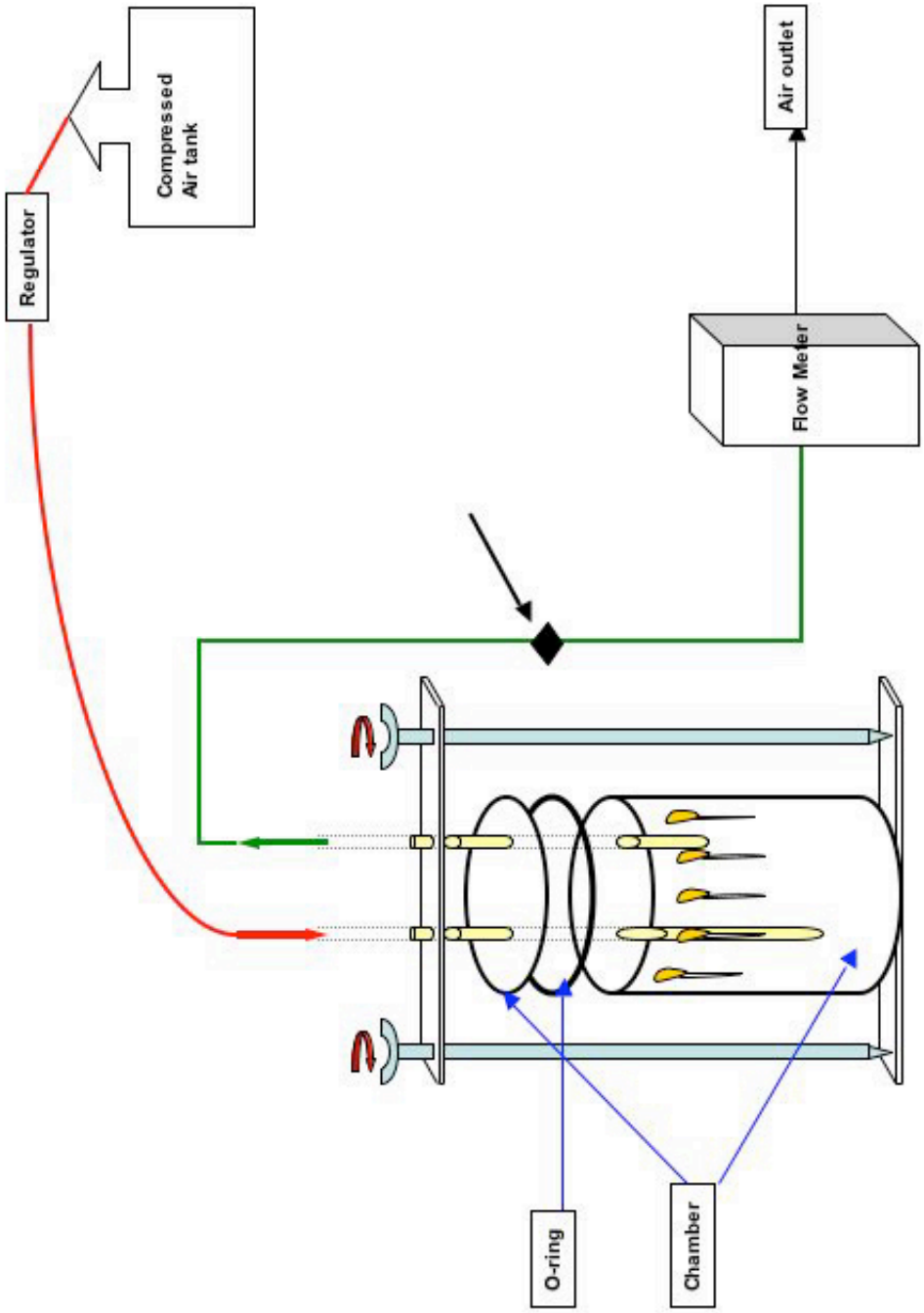

Regulator
Compressed Air tank
Flow Meter
Air outlet
O-ring
Chamber

Figure 2. Primary root length increase (A, B) and root tip ABA content (C, D) of maize seedlings 48 hours after transplanting to vermiculite at high Ψ_w (-0.03 MPa) or Ψ_w (-1.6 MPa). Data are means ± SE (root length, *n* = 25-50; ABA contents, *n* = 5-10). The results for root elongation were similar in several replicate experiments. For ABA contents, results from three experiments were combined. WT, *wt*; +FLU, treated with 1.5 μM fluridone to inhibit ABA synthesis; +ABA, treated with 0.5 mM ABA to restore the root tip ABA content of *vp14'* to *wt* levels. Within each panel, bars with different letters are significantly different at the 0.05 level (panels A-C) or 0.1 level (panel D).

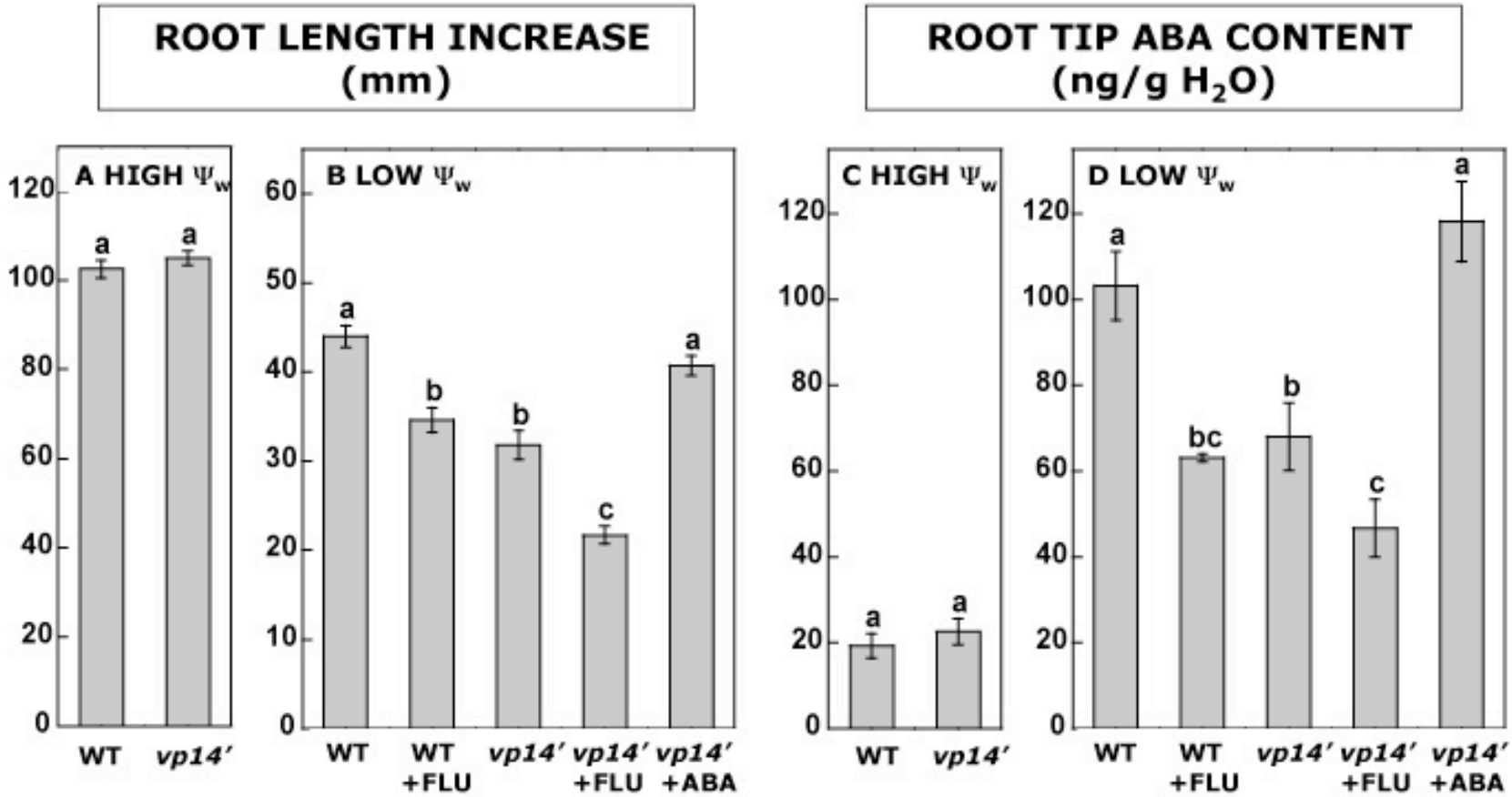

ROOT LENGTH INCREASE (mm)
ROOT TIP ABA CONTENT (ng/g H2O)
A HIGH Ψw
B LOW Ψw
C HIGH Ψw
D LOW Ψw
WT
vp14′
WT +FLU
vp14′ +FLU
vp14′ +ABA
a
b
bc
c

Figure 3. Ethylene evolution rate of intact maize seedlings at 12 h (A) and 24 h (B) after transplanting to vermiculite at a Ψ_w of -1.6 MPa. Data are means ± SE (*n* = 13-20, combined from three experiments). Within each panel, bars with different letters are significantly different at the 0.05 level.

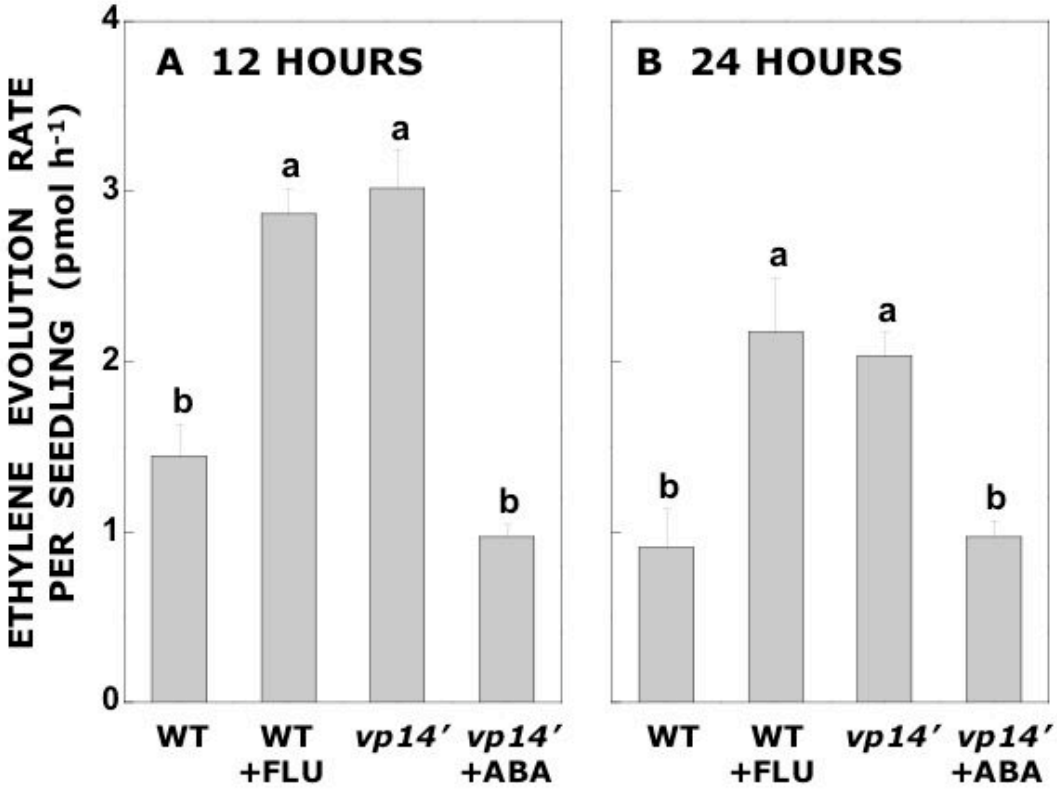
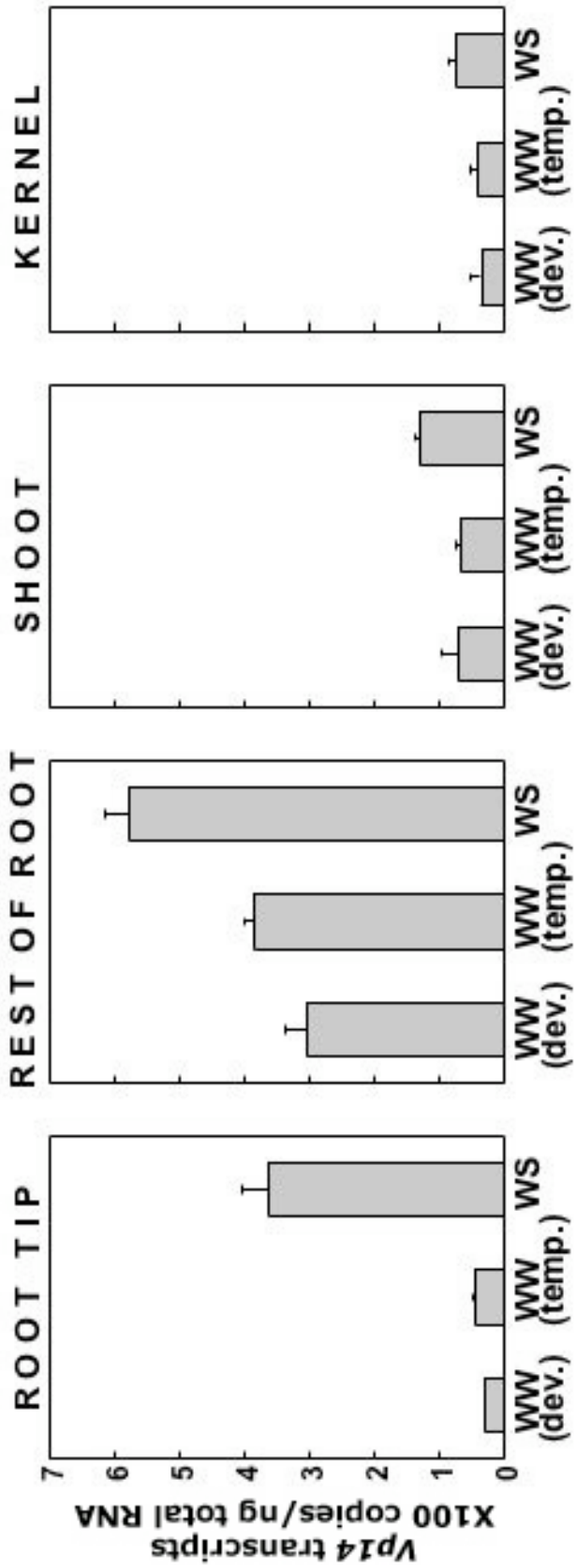


Figure 4. Expression of *Vp14* in *wt* seedlings under well watered (WW, Ψ_w = -0.03 MPa) or water stressed (WS, Ψ_w = -1.6 MPa) conditions. Both developmental (dev.) and temporal (temp.) well-watered controls were sampled. Data are means $\pm$ SE of four replicates for each sample.


Vp14 transcripts
X100 copies/ng total RNA
7
6
5
4
3
2
1
0
ROOT TIP
REST OF ROOT
SHOOT
KERNEL
WW (dev.)
WW (temp.)
WS

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CHAPTER 3

Does ABA accumulation regulate ethylene sensitivity as well as ethylene synthesis in water-stressed roots?

INTRODUCTION

Hormone sensitivity can be modified by several factors including environmental conditions, tissue specificity, developmental stage and the levels of other hormones (Davies, 1995). There are many reports of modifications in ethylene sensitivity in response to environmental conditions. For example, reduced sensitivity to ethylene in tomato fruit inhibits ripening at high temperatures (Yang, 1990), whereas increased sensitivity to ethylene is involved in maize root developmental responses to nitrogen deficiency and in the stimulation of petiole growth of Rumex under flooding (He *et al.*, 1996; Voesenek *et al*., 1997). Ethylene sensitivity can be affected by regulation of either turnover rate or synthesis rate of ethylene receptors. For example, Voesenek *et al*. (1997) showed that expression of a putative ethylene receptor gene was increased in response to flooding.

There is some evidence for interactions between ABA responses and ethylene sensitivity. Ghassemian *et al*. (2000) showed that the *era3* (enhanced response to ABA) mutant of *Arabidopsis* is in fact *ein2* (ethylene insensitive). Their results indicated that ethylene sensitivity could alter responses to ABA during germination and root growth of well-watered seedlings. The results presented in Chapter 2 confirm previous studies that the maintenance of maize primary root growth at low Ψ_w by ABA accumulation involves restriction of ethylene synthesis. However, it is not known whether ABA accumulation also alters the sensitivity of growth to ethylene in water-stressed roots.

MATERIALS AND METHODS

The experimental design involved exposing control and ABA-deficient seedlings growing at low Ψ_w (-1.5 MPa) to a range of applied ethylene concentrations. The study was complicated, however, because of the increase in endogenous ethylene production in the ABA-deficient seedlings. Thus, in order to compare the responses of control and ABA-deficient seedlings to the same total ethylene concentrations, it was necessary to restore the endogenous ethylene production rate of ABA-deficient seedlings to the control level. This was accomplished by applying an ethylene-synthesis inhibitor, aminooxyacetic acid (AOA), as used effectively in previous studies of water-stressed maize seedling roots (Spollen *et al*., 2000). However, root growth of *vp14'* could be only partially restored (approximately 50%) with AOA, presumably due to incomplete prevention of the increase in ethylene evolution, whereas in hybrid seeds (cv FR27 X FRMo17) treated with fluridone to induce ABA deficiency, AOA treatment fully restored the rates of root growth and ethylene production to the levels of untreated seedlings (Fig. 2, Table 2). These findings are consistent with those reported for the *vp5* mutant by Spollen *et al*. (2000), who suggested that the difference in effectiveness of AOA between mutant and fluridone experiments might be explained by the mutations causing a more pervasive inhibition of ABA content, such that AOA does not penetrate to all the ABA-deficient cells. Accordingly, for this study hybrid seeds with and without fluridone treatment to induce ABA deficiency were used instead of *vp14'*.

Growing conditions, fluridone treatment (1.5 μM) and exogenous ABA treatment (0.5 mM) were the same as for the *vp14'* experiments described in Chapter 2. AOA (732 μM) was applied during germination as described previously (Spollen *et al*., 2000). When primary root lengths were 20-30 mm, seedlings were transplanted to the Plexiglas chambers of the flow-through ethylene analysis system described in Chapter 2. The system was modified to supplement the inflowing air stream with a range of applied ethylene concentrations (Abeles et al., 1992). The air stream was passed through a section of Tygon tubing which was sealed inside a Mason jar containing various volumes of pure ethylene (>99.5%, Linweld). Diffusion of the ethylene across the Tygon tubing provided a constant ethylene concentration in the air stream. A range of ethylene concentrations was obtained by varying the length of the Tygon tubing, the Mason jar volume, and the volume of pure ethylene, at a constant airflow rate of 50 ml/h. For low ethylene concentrations (0.018 ppm to 0.64 ppm), the tubing length was 10 cm, the Mason jar volume was 200 mL, and ethylene volumes were 50 to 150 mL. For the highest ethylene concentration of 0.93 ppm, tubing length was 15 cm and the jar and ethylene volumes were 1 L. Applied ethylene concentrations were measured for each experiment by gas chromatography as described in Chapter 2.

After 42 h, primary root length was measured, root tips were sampled for ABA analysis as described in Chapter 2, and the Ψ_w of the vermiculite was measured using isopiestic thermocouple psychrometry (Boyer and Knipling, 1965). To determine whether exogenous ethylene application affected

endogenous ethylene synthesis in the root tips, 20 root tip segments (apical 10 mm) were harvested and sealed inside a 10 mL gas-tight syringe at near saturation humidity (pre-equilibrated with humid air). The syringe was then incubated for 15 min at 29 ± 1 ºC in the dark. The resulting ethylene concentration inside the syringe was measured by gas chromatography and used to calculate the rate of ethylene production by the root segments; procedures were modified from those previously described for leaf ethylene production by Sharp *et al*. (2000). Preliminary experiments showed that the rate of ethylene evolution was steady for about 20 mins after excision and then increased substantially, presumably due to wounding. Therefore, the reported measurements are considered to be good estimates of the rate of evolution by the root tips of intact plants (Jackson and Campbell, 1976).

RESULTS AND DISCUSSION

Ethylene sensitivity of root growth

There were four treatments: 1) control, 2) control plus AOA, 3) treatment with fluridone to induce ABA deficiency and with AOA to restore endogenous ethylene production to the control rate, and 4) treatment with fluridone, AOA and 0.5 mM (±) ABA to restore the root tip ABA content to the control level. Root tip ABA contents are shown in Table 1. Fluridone treatment reduced root tip ABA content by approximately 50% compared to control roots, and the addition of 0.5 mM (±) exogenous ABA restored the ABA content to control levels, as reported

for the *vp14'* experiments described in Chapter 2. AOA treatment did not affect the ABA content of the root tips, as reported previously (Spollen *et al*., 2000),

As shown in Fig. 2, the increase in primary root length of all treatments was the same when no ethylene was applied, indicating a) the effectiveness of AOA in preventing excess ethylene production in fluridone-treated roots (Table 2; Spollen *et al*., 2000), and b) the lack of effect of the same AOA concentration on the basal level of ethylene production in control roots (Table 2). Moreover, root length increases of all treatments were affected similarly by increased ethylene concentrations; in all cases, applied ethylene concentrations greater than 0.1 ppm resulted in progressive inhibition of root elongation. The sensitivity of the root growth response to ethylene was similar to that reported in maize and white mustard roots (Jackson, 1982; Whalen and Feldman, 1988).

It has been reported that ethylene can alter ABA content under various conditions. For example, flooding-induced ethylene restricts ABA content in deep water rice (Hoffmann-Benning and Kende, 1992). If such an effect occurred in the present study this could have complicated comparisons of the response to applied ethylene between treatments. However, root tip ABA content showed no trends with increasing ethylene concentration in any treatment; the results shown in Table 1 are the average ABA contents across applied ethylene concentrations.

Ethylene synthesis can be controlled in either a positive or negative way by feedback regulation in which ethylene either stimulates or inhibits 1-aminocyclopropane-1-carboxylate synthase (ACC synthase, ACS) or 1-

aminocyclopropane-1-carboxylate oxidase (ACC oxidase, ACO) at the transcriptional and/or post-transcriptional levels (Kende, 1993). It has been well studied that exogenous ethylene promotes ethylene production by activating ACS and/or ACO in ripening fruits (Liu *et al*., 1985) or carnation flowers (Wang and Woodson, 1989). Negative feedback regulation was reported in auxin- or stress-induced ethylene increase (Yang and Hoffman, 1984). If such an effect occurred in the present study this could also have complicated comparisons of the response to applied ethylene between treatments. Therefore, the endogenous ethylene production rates of the root tips were measured at the various applied ethylene concentrations. The results showed no trends with increasing ethylene concentration in any treatment; Table 2 shows that average ethylene production rates across applied ethylene concentrations were not significantly different between treatments.

CONCLUSION

The similar response of root elongation to applied ethylene in control and ABA-deficient seedlings indicates that ethylene sensitivity is not regulated by ABA accumulation in maize roots at low Ψ_w. Thus, the role of accumulated ABA in root growth maintenance at low Ψ is largely accounted for by the prevention of excess ethylene synthesis (Spollen *et al*., 2000). Additional studies are required to investigate whether ABA directly or indirectly controls ethylene production.

Figure 1. Flow-through system for continuous supply of various ethylene concentrations to intact maize seedlings. Red and green arrows indicate the sampling points for the measurement of background and post-sample ethylene concentrations of the air stream, respectively.

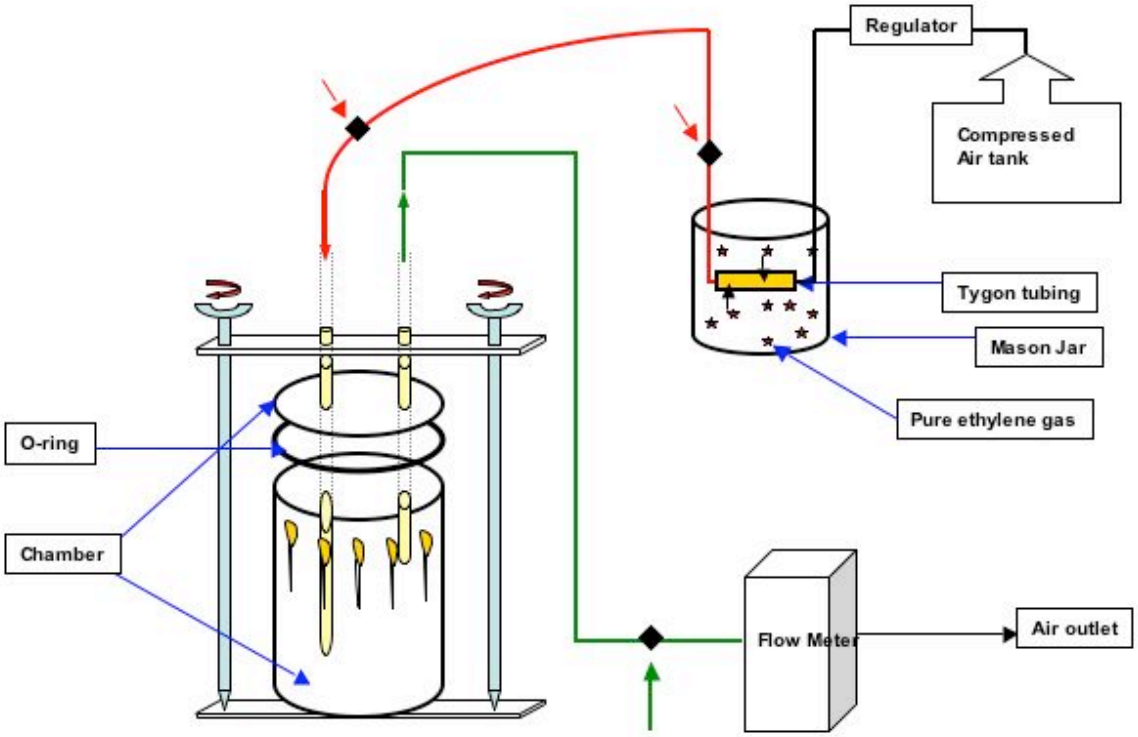

Figure 2. Effect of continuous treatment with various concentrations of exogenous ethylene on root length increase over 42 h after transplanting to a Ψ_w of –1.5 ± 0.15 MPa. Data are means ± SE (n = 20-40). Control, no treatment; +AOA, treated with 732 μM aminooxyacetic acid; +AOA+FLU, treated with AOA and 1.5 μM fluridone (FLU); +AOA+FLU+ABA, treated with AOA, fluridone and 0.5 mM ABA.

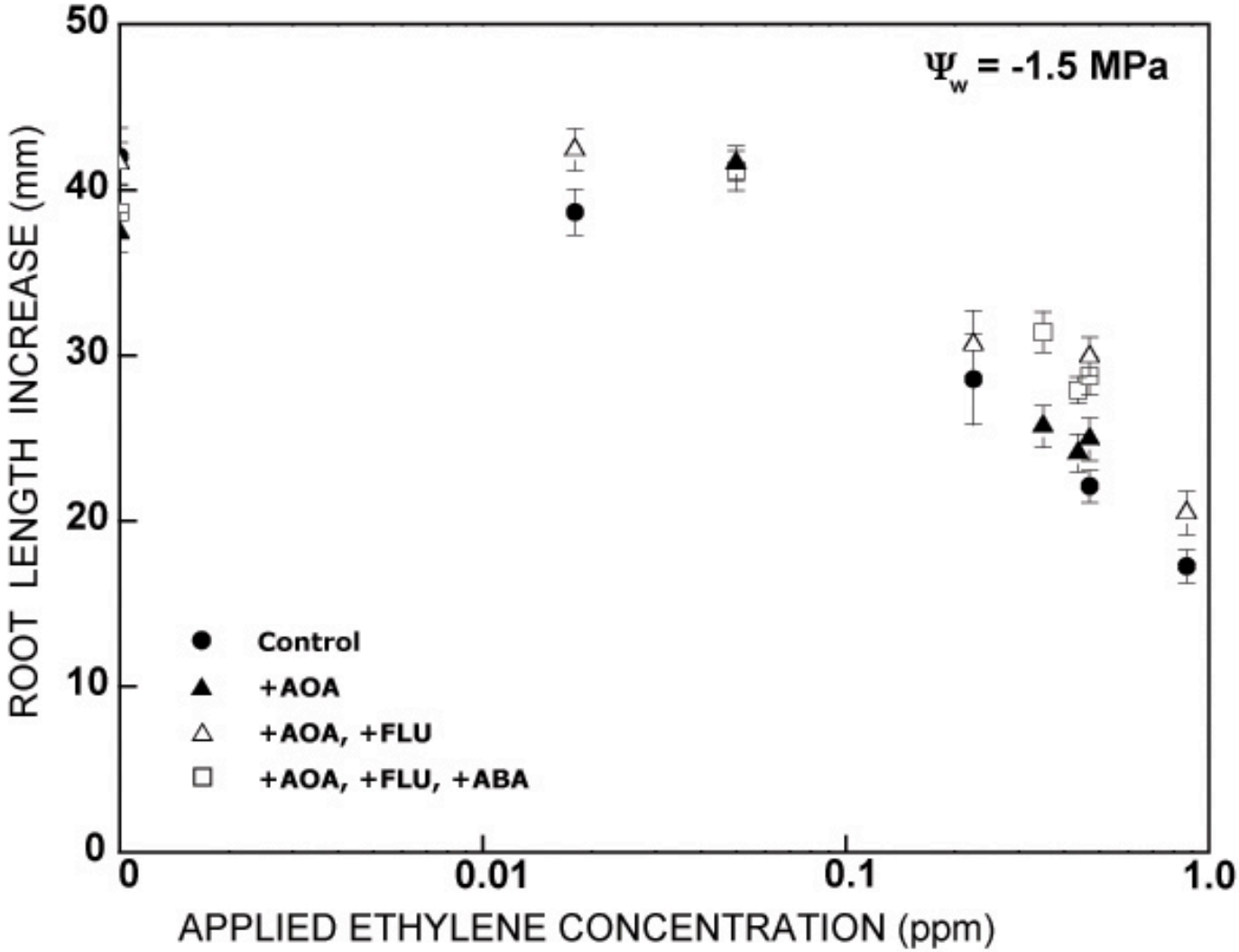
Ψ_w = -1.5 MPa
ROOT LENGTH INCREASE (mm)
50
40
30
20
10
0
0
0.01
0.1
1.0
APPLIED ETHYLENE CONCENTRATION (ppm)
Control
+AOA
+AOA, +FLU
+AOA, +FLU, +ABA

Table 1. Average root tip (0-10 mm minus the root cap) ABA contents across all applied ethylene concentrations. Measurements were made 42 h after transplanting to low Ψ_w (-1.5 ± 0.15 MPa). Data means ± SE of 12 -16 samples from six independent experiments. Values followed by different letters are significantly different at the 0.05 level (Fisher's LSD test).

	Control	+AOA	+AOA+FLU	+AOA+FLU+ABA
ABA content (ng/g H_2O)	71.7 ± 11.27[a]	70.13 ± 6.94[a]	36.39 ± 10.89[b]	70.20 ± 13.28[a]

Table 2. Average endogenous ethylene evolution rates from excised root tips (apical 10 mm) across all applied ethylene concentrations. Measurements were made 42 h after transplanting to a low Ψ_w (-1.5 ± 0.15 MPa). Data are means ± SE of 4-6 measurements of 20 roots from two independent experiments. Values followed by the same letter are not significantly different at the 0.01 level (Fisher's LSD test).

	Control	+AOA	+AOA+FLU	+AOA+FLU+ABA
Ethylene conc. (pmol / gFW h)	4.56 ± 0.74[a]	5.08 ± 0.48[a]	6.05 ± 0.57[a]	5.99 ± 0.55[a]

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CHAPTER 4

ABA deficiency causes excess intracellular levels of reactive oxygen species (ROS) in the growth zone of maize roots under water deficits

Dr. Mayandi Sivaguru (Molecular Cytology Core, MU) contributed substantially to this particular project.

INTRODUCTION

ROS production and detoxification should be tightly controlled for the plant's survival (Mittler, 2002). Various stress conditions result in unbalanced ROS levels in plant cells (Price *et al*., 1989; Pastori and Trippi, 1992; Moran *et al*., 1994; Zhang and Kirkham, 1994; Navari-Izzo *et al*., 1996), which can lead to cellular damage and eventually cell death (Halliwell and Gutteridge, 1989; Gutteridge and Halliwell, 1990). To keep the ROS levels in balance both in normal and stress conditions, cells activate antioxidant systems, particularly enzymatic systems (Chapter 1, Fig. 2). There are several reports indicating that ABA up-regulates antioxidant systems either via modification of enzyme activities and/or expression of the genes encoding the enzymes (Scandalios, 1997). Thereby, ABA can play an important role in maintaining appropriate ROS levels, as shown in barley aleurone layers and in water stressed maize leaves (Fath *et al*., 2001; Jiang and Zhang, 2001). In addition, ABA treatment has been shown to alleviate cellular damage in various tissues and conditions. For example, when somatic embryos of alfalfa (*Medicago sativa* L.) underwent slow drying, membrane integrity and α-helical protein secondary structure were retained by ABA, and hence the embryos showed improved survival rate during germination (Sreedhar *et al*., 2002).

There are few studies about ROS generation and elimination in roots compared to leaves (Mano, 2002), and there are no studies concerning the possible inter-relationship between ROS levels, antioxidant systems, and ABA accumulation in the root growth zone under any stress conditions. This chapter

presents evidence that ABA accumulation is required to maintain plasma membrane integrity by preventing high levels of ROS in the growth zone of the maize primary root under water stress.

MATERIALS AND METHODS

ROS staining experiments

Seedlings of the ABA-deficient mutant *vp14'* and its wild type were grown in either well-watered (Ψ_w = -0.2 MPa) or dry (Ψ_w = -1.6 MPa) vermiculite, as described in Chapter 2. To confirm that effects of *vp14'* were attributable to ABA deficiency, 0.5 mM ABA was applied to restore the root tip ABA content (Chapter 2, Fig. 2).

After 18 h, the seedlings were removed from the vermiculite and the apical region of intact roots was stained for 30 mins with either 15 µM carboxy-H_2DCFDA (DCFDA; 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate) prepared in 1 mM $CaSO_4$, or with 100 µg/mL propidium iodide (PI) in 1 mM $CaSO_4$. Both dyes were obtained from Molecular Probes (Eugene, OR). DCFDA is a fluorescent indicator of intracellular ROS, presumably H_2O_2 (Maxwell *et al*., 1999). The dye readily crosses the plasma membrane because of diester moieties. Once the dye is inside the cell, intracellular esterase cleaves the diester moieties, which results in longer retention times because the cleavage creates a negative charge, and which exposes the oxidation site. Once the dye is oxidized by ROS, it emits a green fluorescence. PI, which emits a red fluorescence, was used to assess loss of plasma membrane integrity. This dye is known to

penetrate across the plasma membrane and label the cell nuclei only when the plasma membrane integrity is compromised (Sauch *et al*., 1991). In some experiments both dyes were applied simultaneously in order to assess co-localization of ROS and membrane integrity.

Immediately after staining, the root tips (apical 10 mm) were harvested and the epidermal cells were imaged using either stereo- or confocal-microscopy. For stereomicroscopy of entire root apices, the segments were observed under a Leica fluorescence stereoscope (SMZ III, Leica, Germany) using either a GFP filter (for DCFDA, excitation 488 nm, emission 515/30 nm) or a Texas red filter set (for PI, excitation 568 nm, emission 600 nm). A confocal laser scanning microscope (IX 70, Olympus, Tokyo, Japan) coupled with Radiance 2000 (Bio-Rad, Hercules, CA) was used to capture high resolution images of interesting regions along the root apex. The confocal images were obtained using either a 20x (UPlanApo, 0.8 NA) or 60x (UPlanApo, 1.2 NA) water immersion objective. Due to the three-dimensional nature of the surface of the root apex and the individual cell files, in most cases confocal optical sections were taken at either 0.7 micron (20x) or 0.3 micron (60x) intervals. A total of 80-120 images were obtained for a given region of interest using two different lasers. DCFDA was excited by the 488 nm line (emission 515/30 nm) and PI by the 568 nm line (emission 600 LP) of the Kr/Ar mixed gas lasers. The laser power was tuned down to lowest levels and the gain was increased, which minimized the photo-toxicity of the laser and hence the live cells were protected during the acquisition period. Raw confocal data were analyzed using Metamorph 3-D analysis

software (version 4.9, Universal Imaging Corporation, NY), including the analysis of total ROS intensity. The Metamorph program allowed the creation of 3-D movies from the individual sections of the 3-D stacks (results are linked to this report).

To assess the sequence of effects of ABA deficiency on ROS levels and plasma membrane integrity, additional experiments were conducted in which roots were stained with both DCFDA and PI at various times after transplanting to low Ψ_w vermiculite.

Iso-osmotic and aeration control experiments

To determine whether the staining patterns of the water-stressed roots were influenced by effects of rehydration during the staining period, experiments were also conducted using staining solutions which were iso-osmotic to the root Ψ_w at the time of removal from the vermiculite (–1.3 MPa). The use of polyethyleneglycol (PEG), melibiose or mannitol to lower the solution Ψ_w was tested. The possibility of effects of oxygen depletion during staining was also tested by comparing staining with or without aeration of the solution.

Wild type root treated with fluridone experiment

Wild type seedlings were germinated in wet vermiculite with 1.5 μM FLU and transplated to a dry vermiculte (-1.6 MPa). After 24 h, the roots were stained with carboxy-H_2DCFDA and PI and then imaged by using a stereo-microscope. Staining procedures and imaging processes were the same as the above.

Peroxidase distribution experiment

Total peroxidase (POX) activity in the root tip was tested using 3,3'-diaminobenzidine (DAB, tetrahydrochloride enhanced liquid substrate system for membrane applications, Sigma, St. Louis) and 3,3',5, 5'-tetramethylbenzidine (TMB, liquid substrate system for membranes, Sigma, St. Louis). DAB and TMB are substrates of POX that can oxidize to a brown insoluble precipitate and a blue soluble end product, respectively, coupled with hydrogen peroxide reduction (Liszkay *et al*., 2004).

Wild type and *vp14'* roots were hand-cut into 1-mm serial sections and rapidly blotted onto nitrocellulose membranes (Schopfer, 1994) . The membranes were stained with either DAB or TMB and then briefly rinsed with distilled water. The dried membranes were photographed under a 50X dissecting microscope.

Assay for ABA content

To measure the spatial distribution of ABA content in the root tip, three sections (0-1, 1-3, 3-6 mm from the tip) were harvested after briefly rinsing with distilled water and blotting with facial tissue to remove adhering mucilage (preliminary experiments showed that mucilage interfered with ABA assays of the apical regions). ABA was measured by radioimmunoassay (Quarrie *et al*., 1988) as described previously.

RESULTS

ROS levels

Stereoscope images illustrating DCFDA staining patterns of the apical 5 mm of wild type (*wt*) and *vp14'* roots 18 h after transplanting to high and low Ψ_w are shown in Fig. 1. In well-watered conditions, both *wt* and *vp14'* roots showed virtually undetectable levels of ROS throughout the apical 5 mm (Fig. 1A and B). At low Ψ_w, in contrast, ROS levels were slightly increased between 1-5 mm from the apex in *wt* roots (Fig. 1C), and were dramatically increased specifically in the 1-3 mm region of *vp14'* roots (Fig. 1D). Interestingly, the 0-1 mm region (encompassing the meristematic zone) showed no increase in ROS levels in either *wt* or *vp14'* roots. ABA measurements showed that the 0-1, 1-3 and 3-6 mm regions were equivalently ABA-deficient in *vp14'* compared to *wt* roots (Table 1), suggesting that ABA accumulation is particularly required to maintain ROS levels in the 1-3 mm region under water stress conditions. Application of exogenous ABA confirmed that the increase in ROS in the 1-3 mm region of *vp14'* roots under water stress was due to ABA deficiency. Fig. 2 C1 shows that restoration of the root tip ABA content (see Chapter 2, Fig. 2) recovered ROS to the *wt* level.

Higher magnification stereoscope images (Fig. 2 A2-C2 and A3-C3) and confocal images (Fig. 2 A4-C4) revealed that cellular ROS levels were not uniformly increased in either *wt* or *vp14'* roots under water stress. Instead, individual cells showed very different levels of ROS. The confocal images allowed quantification of ROS intensity (Table 2). In the region 1-2 mm from the

apex of roots at low Ψ_w, the ROS intensity of *vp14'* increased to 188% of the *wt* level, and *vp14'* roots treated with ABA showed a 65% lower intensity than untreated *vp14'*. The intensity data revealed that both the ROS level in individual cells as well as the number of cells with high ROS were increased in *vp14'* compared to *wt* roots.

Membrane integrity

It is well known in plants as well as in animals that oxidative stress due to high levels of ROS can cause cellular damage including lipid peroxidation (Halliwell and Gutteridge, 1989). Thus, the high ROS levels that occurred in *vp14'* roots under water stress could have caused cellular damage and, thereby, have been causally related to growth inhibition. In support of this possibility, it is important to note that the inhibition of cell elongation in ABA-deficient (fluridone-treated) roots at low Ψ_w occurs predominantly in the same region of the growth zone, 1-3 mm from the apex, as the increase in ROS levels that occurred in water-stressed *vp14'* (Saab *et al*., 1992; Sharp *et al*., 1994; Ober and Sharp, 2003). However, it is also possible that the increase in ROS was involved in cellular signaling in response to ABA deficiency. For example, H_2O_2 has been shown to be involved in ABA signaling in stomatal guard cells (Neill *et al*., 2002; Laloi *et al*., 2004). Therefore, PI staining was used to assess whether the increased ROS levels in *vp14'* roots at low Ψ_w were associated with cellular damage. At high Ψ_w, both *wt* and *vp14'* roots exhibited uniform red staining of the cell wall, and showed no indication of loss of membrane integrity (Fig. 3A and B). At low Ψ_w, *wt* roots showed a limited amount of nuclear PI staining, indicating

loss of membrane integrity in a few cells (Fig. 3C). In *vp14'*, in contrast, evidence of loss of membrane integrity in a much larger number of cells was observed starting at 1 mm from the apex (Fig. 3D), spatially correlating with the onset of increased ROS levels. Restoration of the ABA content completely prevented this effect (Fig. 3E).

Co-localization and sequence of increase in ROS and loss of membrane integrity

Combined staining with DCFDA and PI was used to further assess the correlation between increased ROS levels and membrane damage in water-stressed *vp14'* roots. Confocal imaging in the region 1-2 mm from the apex revealed that cells with high ROS levels also displayed PI-stained nuclei (Fig. 4). Next, to determine whether the increase in ROS preceded the loss of plasma membrane integrity, time course experiments were conducted in which roots were stained and imaged at 1, 3 and 6 h after transplanting to low Ψ_w. From the results, the projected sequence of events is illustrated by confocal images of individual cells shown in Fig. 5. In the first stage (Fig. 5,I), ROS levels are not increased and PI staining is restricted to the cell wall, indicating that the plasma membrane is fully functional. In the next stage, cellular ROS levels are increased but there is no PI penetration into the cell, indicating that impairment of plasma membrane integrity has not yet occurred (Fig. 5,II). Once ROS reached a threshold level and/or duration, however, impairment of membrane integrity occurs as evident from nuclear staining (Fig. 5,III). Subsequently, ROS levels begin to decrease and the cell contents show signs of disruption (Fig. 5,IV).

Finally, ROS staining disappears leaving just the PI-stained nucleus of, possibly, dead cells (Fig. 5,V). Because PI staining of the nucleus is irreversible, the existence of cells in phase II, which exhibited ROS staining but no PI staining of the nucleus, provides compelling evidence that the increase in ROS precedes and probably causes the loss of plasma membrane integrity in *vp14'* roots under water stress. Accordingly, the results indicate that ABA accumulation is essential to maintain ROS at non-damaging levels in the growth zone of water-stressed roots.

Iso-osmotic and aeration controls

When water-stressed roots were stained in solutions which were iso-osmotic to the root Ψ_w (–1.3 MPa), the results were similar to those obtained with staining solutions of high Ψ_w. Figure 6 illustrates confocal images from the 1-3 mm region of the roots after staining with both DCFDA and PI in 1 mM $CaSO_4$ and with the addition of either melibiose or mannitol to lower the solution Ψ_w. ROS levels were consistently greater in *vp14'* compared to *wt* roots. However, the results also showed evidence that the number of cells in *wt* roots which exhibited high ROS levels and PI penetration were increased in the presence of either osmoticum, suggesting that these treatments caused some side effects on cellular metabolism and membrane integrity. This effect was more pronounced with the use of mannitol. (PEG was also tested but interfered with the staining success, possibly because of high solution viscosity.) Therefore, staining solutions without the addition of osmotica were used routinely. Aeration of the staining solution during the 30 min staining period had no effect on the DCFDA or

PI staining patterns in either *wt* or *vp14'* roots (Fig. 7) and, therefore, was not used routinely.

Osmotic swelling observations

The use of high Ψ_w staining solutions proved to be of unexpected benefit. The confocal images collected during the time course experiments with water-stressed *vp14'* roots revealed that some of the epidermal cells in phase II, i.e. cells which had high intracellular ROS levels but did not yet show evidence of loss of plasma membrane integrity, exhibited pronounced osmotic-induced swelling and cell deformation (Fig. 8). 3-D imaging revealed that the swelling projected from the center of the cells outward from the surface of the root (Fig. 9, movie). These observations suggest that there might be excessive weakening of the cell wall in these specific cells prior to the impairment of membrane integrity, leading to cell deformation in response to turgor increase after immersion of the root tip in the high Ψ_w staining solution.

ROS staining of *wt* + FLU roots

Since *vp14'* seeds were not genetically homogenous, ROS staining of FLU-treated *wt* roots was important. The roots of *wt* + FLU showed increased ROS and bleached membranes, which supports that ABA deficiency causes the symptoms (Fig. 10).

POX activities of *wt* and *vp14'* at high and low Ψ_w

Because all ROS dyes requires POX activities, POX activities of *wt* and *vp14'* root segments were observed. DAB staining illustrated that total POX

activities were similar in all sections in both *wt* and *vp14'* roots under water-stressed and well-watered conditions (Fig. 11).

DISCUSSION

It has been reported that under water deficits, plants often show increased ROS levels especially in leaves, which is presumably associated with reduction of photosynthesis (Smirnoff, 1993). Roots have not been studied as extensively as leaves. In addition to the potentially damaging effects of excess ROS levels, the functioning of ROS as a signaling molecule is becoming increasingly recognized, for example in plant pathogen relationships (Mittler, 2002), stomatal regulation (Kwak *et al.*, 2003) and root hair growth (Foreman *et al.*, 2003). In this study, *wt* roots showed slightly increased levels of ROS in the elongation zone at low Ψ_w, and our results cannot distinguish whether this effect was due to excess ROS production and/or decreased scavenging capacity, or was due to potential ROS signaling responses. It is noteworthy that cell elongation is normally fully maintained in the first 3 mm of water-stressed roots (Chapter 1 Fig. 1). Concurrently with growth maintenance, it can be expected that there is a large amount of energy consumption associated with mechanisms of adaptation (Greenway, 1970; Collier and Cummins, 1992; Verslues *et al.*, 1998). Consequently, the large energy requirement might result in increased ROS production in the elongation zone at low Ψ_w due to electron transport over-reduction in mitochondria (Moller, 2001).

In *vp14'*, in contrast, it is clear that the much higher ROS levels observed in the 1-3 mm region were associated with cellular damage as indicated by loss of both plasma membrane and cell wall integrity. Thus, the results indicate that ABA accumulation is essential to maintain appropriate regulation of ROS levels and, therefore, cell elongation, in the growth zone of water-stressed maize primary roots. There are several studies showing that ABA can up-regulate antioxidant systems either via enzyme activities or expression of genes encoding the enzymes (Scandalios, 1997). Additionally, ROS can be scavenged by non-enzymatic antioxidants (Smirnoff and Cumbes, 1989; Hare and Cress, 1997) such as proline, which is accumulated to high levels in the root growth zone under water deficit in association with ABA accumulation (Ober and Sharp, 1994). Further studies are required to determine the function of ABA accumulation in regulating ROS levels in the root growth zone under water stress conditions.

Carboxy–H_2DCFDA has frequently been used as an indicator of intracellular ROS levels (Maxwell *et al*., 1999). However, several studies have questioned the reliability of the dye as an independent ROS indicator and its specificity for substrates (Hempel *et al*., 1999). Those studies suggest that all ROS indicators including carboxy-H_2DCFDA require peroxidase (POX) activity as well as ROS. Therefore, it was important to confirm that the higher staining with carboxy-H_2DCFDA observed in the 1-3 mm region of water-stressed *vp14'* compared to wild-type roots (Fig. 1) was not because of differential POX activities by using DAB staining (Fig. 11). Accordingly, the uniform POX activities

of *wt* and *vp14'* indicate that the higher ROS staining in the 1-3 mm region of water-stressed *vp14'* was indeed due to increased ROS levels.

ROS and ethylene production are commonly related under stress conditions. Previous studies (Spollen *et al*., 2000) and results reported in Chapter 2 showed that ethylene evolution is restricted by accumulated ABA to maintain growth in the maize primary root at low water potential. The detailed mechanisms of how ABA regulates ethylene synthesis in this system have not been studied. According to McRae *et al*. (1982) the superoxide radical could regulate ethylene synthesis. This suggests that the increased ROS levels in *vp14'* may regulate ethylene synthesis, and thereby indirectly inhibit root growth. In addition, the increased ROS may result in growth inhibition via direct cellular damage. Rapidly increased hydrogen peroxide levels led to less water transport in cucumber roots exposed to low temperature (Lee and Lee, 2000), and it was assumed that the hydrogen peroxide caused protein conformational changes of H^+-ATPase and aquaporins required for water transport and located in the plasma membrane. However, ethylene can also induce ROS generation. For example, ethylene synthesis was shown to occur prior to hydrogen peroxide accumulation in tomato exposed to ozone, and thus to trigger cell death (Moeder *et al.*, 2002). It is difficult to interpret the sequence of ROS and ethylene generation in maize primary roots at low water Ψ_w without further studies of the relationships between ROS and ethylene in this system.

Recent studies indicate that controlled ROS levels are necessary for cell elongation not only through cellular signaling roles but also via direct action in

cell wall loosening. Evidence has been published for the involvement of ROS in cell elongation growth in maize coleoptiles (Schopfer, 2001; Schopfer *et al.*, 2002), maize leaves (Rodriguez *et al.*, 2002), and maize roots (Liszkay *et al.*, 2004). It is suggested that plasma membrane NAD(P)H oxidase generates superoxide radical ($^{\bullet}O_2^-$) that is converted to hydroxyl radical ($^{\bullet}OH$) by cell wall peroxidase coupling with hydrogen peroxide (H_2O_2). The $^{\bullet}OH$ is then responsible for breaking cell wall polysaccharides without enzymatic reaction (Fry, 1998). Moreover, ROS is required for regulation of Ca^{2+} acquisition that is essential for the elongation of roots and root hairs in *Arabidopsis* (Foreman *et al.*, 2003). All these studies showed that a certain amount of controlled ROS in the cell wall is necessary for the elongation of leaves or roots. However, the ROS levels involved in this function must be controlled tightly by regulating either generation or degradation to avoid impairment of cell wall structure. The osmotic swelling results we obtained in water-stressed *vp14'* suggest the possibility that ABA deficiency causes excess apoplastic as well as cytosolic ROS levels. This hypothesis cannot be tested with the standard DCFDA dye used in these studies because it indicates specifically intracellular ROS levels, but could be examined using the novel apoplast-specific ROS indicator described in Chapter 5.

Figure 1. Representative stereo-microscope images of ROS levels in the apical 5 mm of wild-type and *vp14'* roots under well-watered (WW, Ψ_w = -0.3 MPa) and water-stressed (WS, Ψ_w = -1.6 MPa) conditions. ROS levels were visualized by the fluorescent dye carboxy-H_2DCFDA. Results are representative of roots imaged in 10 independent experiments.

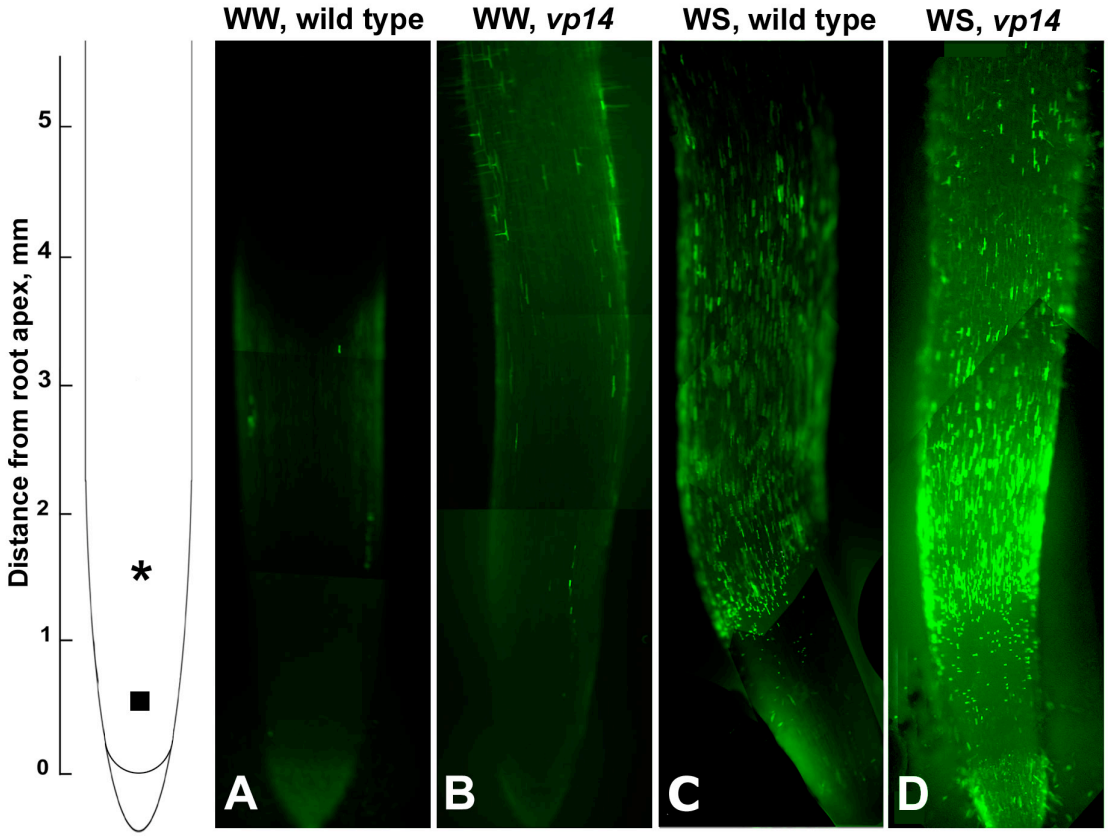


Table 1. ABA contents in apical segments of *wt* and *vp14'* roots at low Ψ_w (1.6 MPa). The root cap was removed from the 0-1 mm segment. Data are means ± SE of 3-6 replicate samples from six independent experiments. Each measurement contained 161-185 *wt* and 163-188 *vp14'* root segments. Analysis of variance was performed using Fisher's LSD test at the P = 0.05 level.

	ABA content (ng / g dry weight)		
	0-1 mm	1-3 mm	3-6 mm
wild type	424 ± 44[b]	577 ± 11[a]	461 ± 47[b]
vp14'	312 ± 33[c]	329 ± 38[c]	323 ± 57[c]

Figure 2. Images of ROS levels in wild-type, *vp14'*, and *vp14'*+ABA root tips under water-stressed conditions (WS, Ψ_w, -1.6 MPa) taken by stereo microscopy of the 0-2 mm region (A1 to C1, n = 10), or by laser scanning confocal microscopy in the 1-2 mm region (A2 to C2, n = 3-5; A4 to C4, n = 1) and the 0-1 mm region (A3 to C3). Restoring the ABA content of *vp14'* roots prevented the high levels of ROS (C1 to C4). High magnification images (A4 to C4) show ROS production within individual cells. Bars in C1, 500 μm; in C3, 100 μm; and in C4, 10 μm.

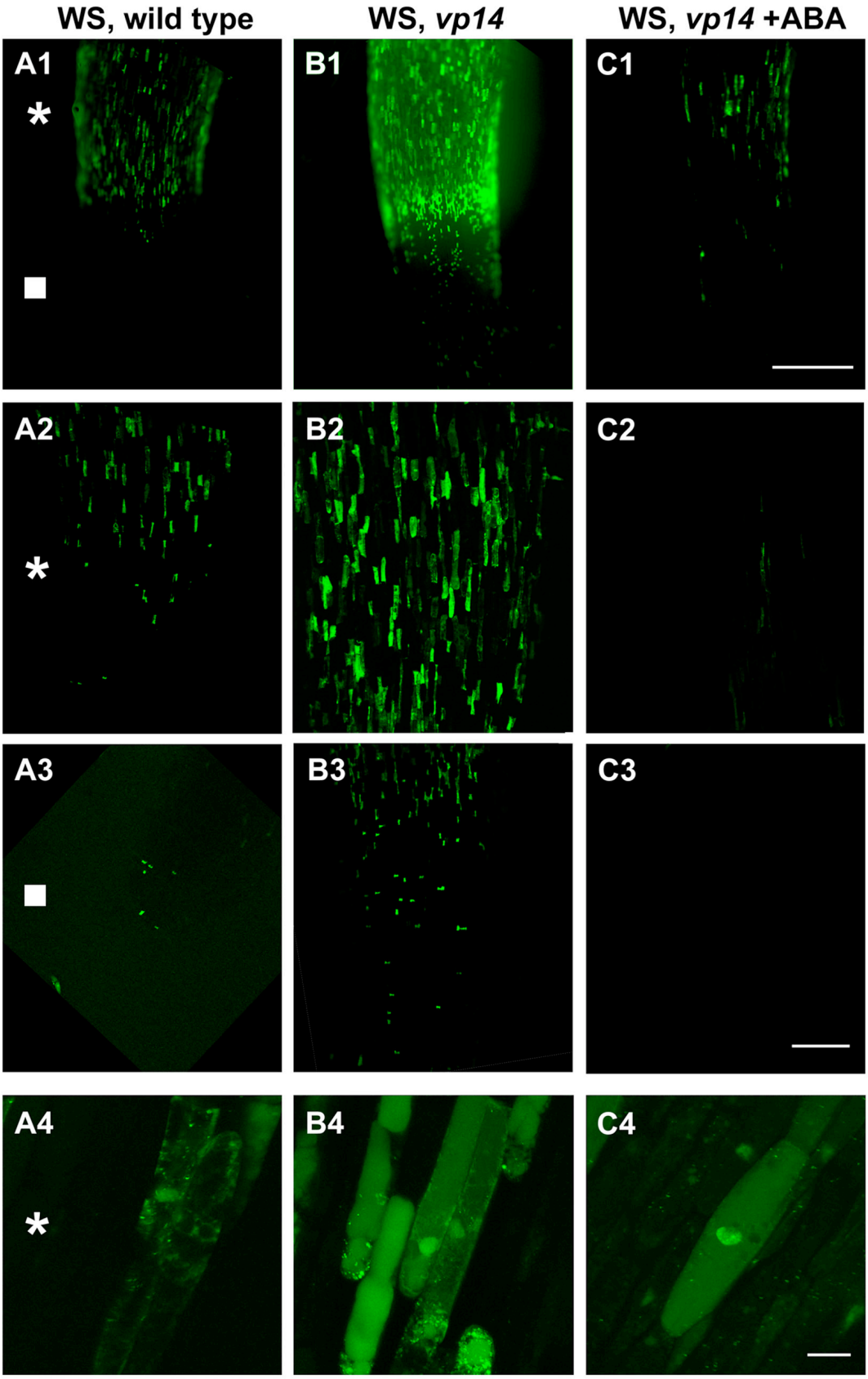

WS, wild type
WS, vp14
WS, vp14 +ABA
A1
B1
C1
A2
B2
C2
A3
B3
C3
A4
B4
C4

Table 2. Relative intensity of DCFDA staining in the 1-2 mm region of *wt, vp14'*, and *vp14'*+ABA root tips at low Ψ_w (-1.6 MPa). Two separate confocal images from two roots were quantified by using Metamorph program. The unit is arbitrary (± SE).

	wt	*vp14'*	*vp14'* + ABA
ROS intensity	58.8 ± 33.8	110.4 ± 52.4	77.1 ± 24.6

Figure 3. Stereo-microscope images of propidium iodide (PI) staining patterns of the apical 3 mm of roots under well-watered (WW, Ψ_w = -0.03 MPa) and water-stressed conditions (WS, Ψ_w = -1.6 MPa). Loss of plasma membrane integrity of *vp14'* at low Ψ_w, indicated by nuclear staining, started at 1 mm from the apex (D), which was alleviated when the ABA content of the root tip was restored (E). Due to intense PI staining, the short exposure time was necessary for water-stressed *vp14'* root (360 ms vs. 720 ms). Bar in E is 500 μm.

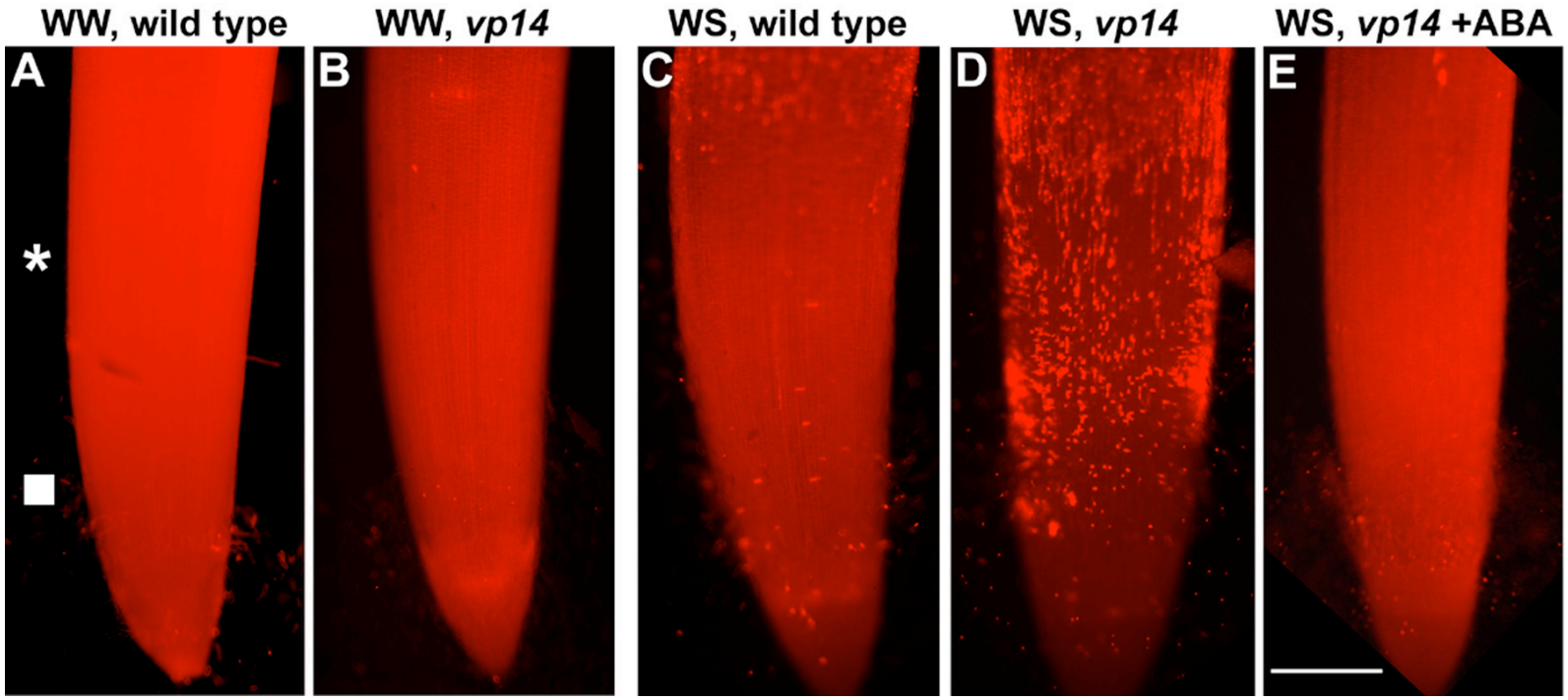

WW, wild type
WW, vp14
WS, wild type
WS, vp14
WS, vp14 +ABA
A
B
C
D
E
*

Figure 4. Confocal images of the 1-2 mm region of the root tip in *vp14'* under water-stressed conditions (WS, Ψ_w = -1.6 ± 0.2 MPa). Simultaneous staining for ROS (DCFDA, A) and plasma membrane integrity (PI, B) shows close association between increased ROS levels and loss of plasma membrane integrity in individual cells. C is a merged image of DCFDA and PI images. Images are representative of $\leq$4 separate roots. Bar in C, 10 µm.

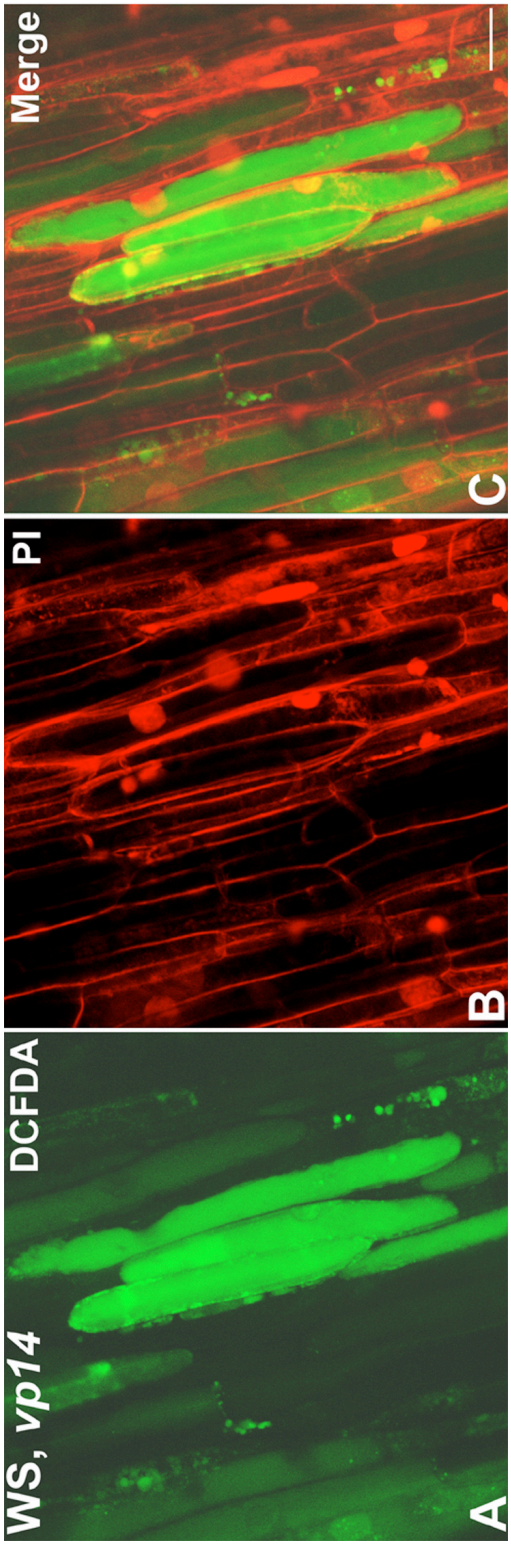

WS, vp14
DCFDA
A
PI
B
Merge
C

Figure 5. Confocal images showing the projected sequence of ROS increase and loss of plasma membrane integrity in cells in the root tip of *vp14'* under water-stressed conditions (WS, Ψ_w = -1.6 MPa) (see text for further details). Images were collected from roots grown for 1, 3, and 6 h after transplanting to low Ψ_w. Images are representative of six separate roots. Scale bar represents 10 µm.

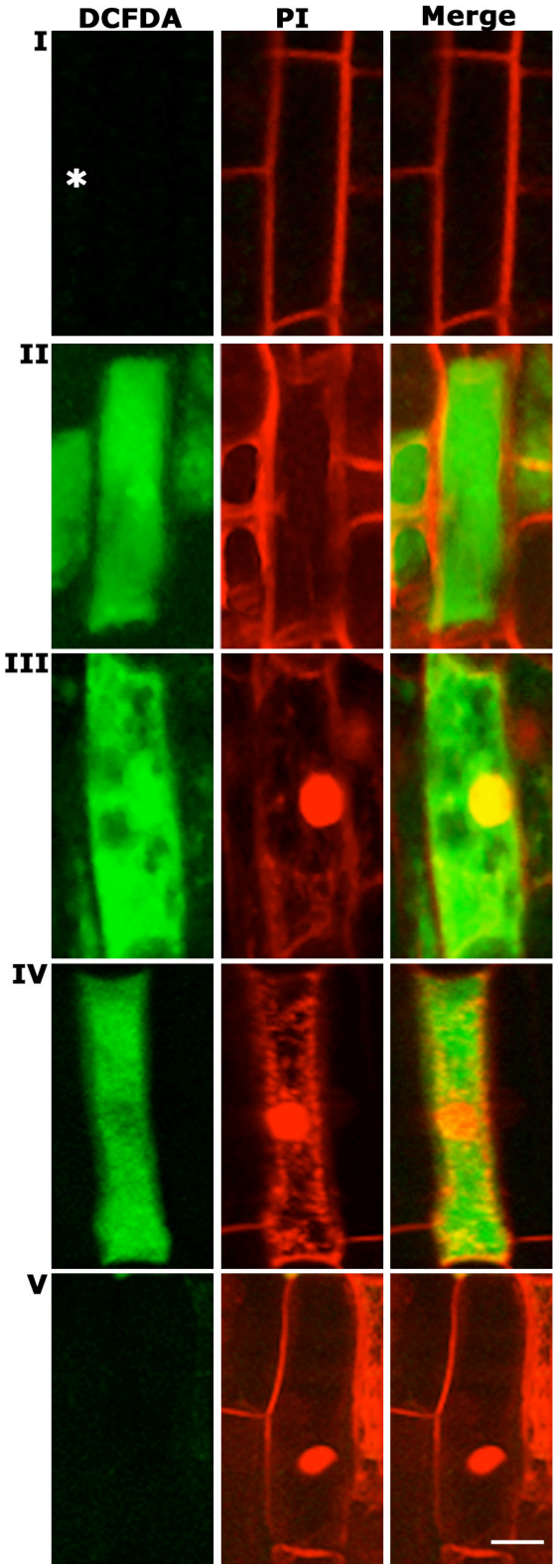

DCFDA
PI
Merge
I
II
III
IV
V

Fig. 6. Confocal images from the 1-2 mm region of *wt* and *vp14'* root tips after 18 h transplanted to low water potential (-1.3 MPa). The roots were staining with a mixture of dyes (DCFDA and PI) and various iso-osmotic solutions achieved by using melibiose and mannitol.

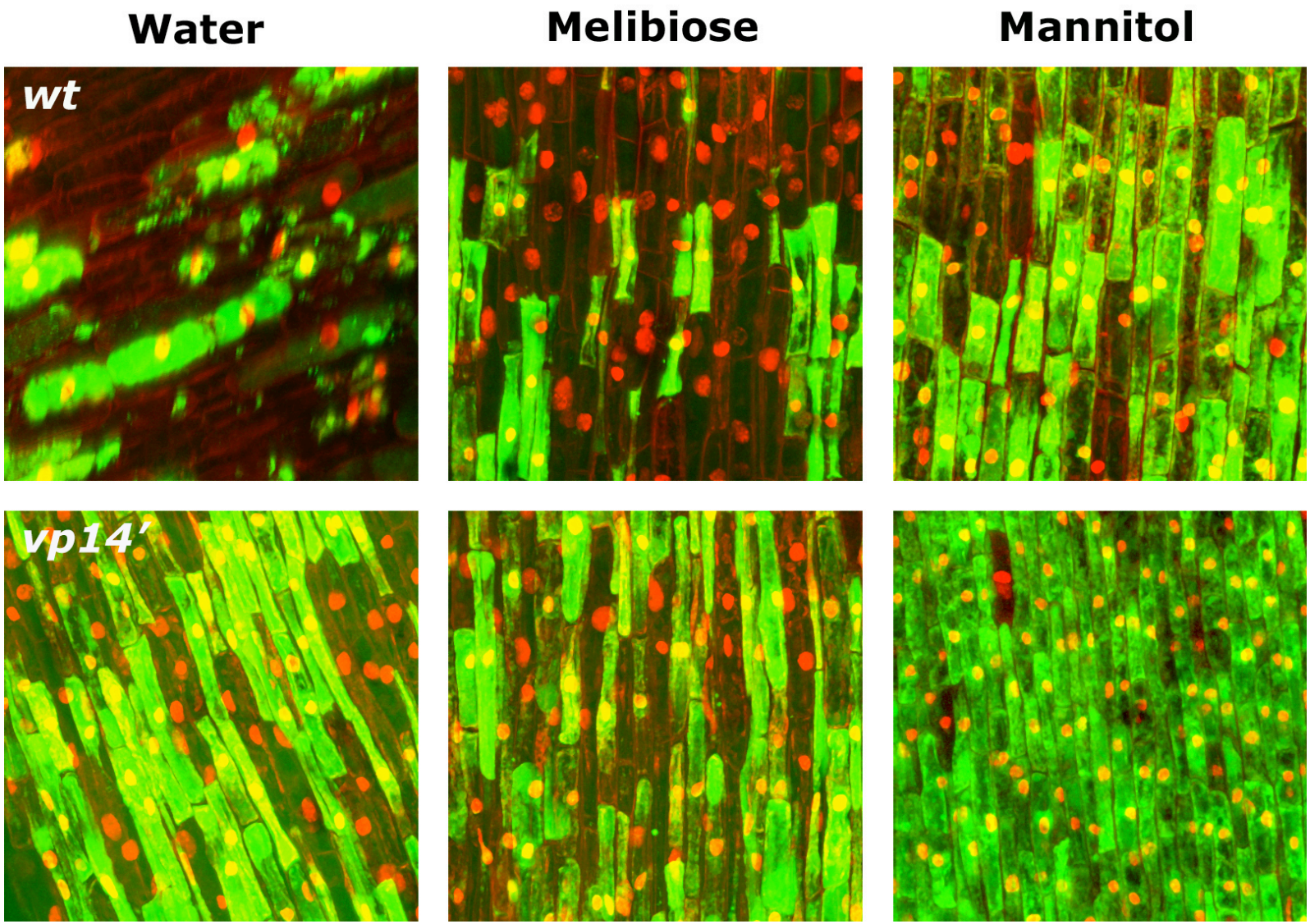

Water
Melibiose
Mannitol
wt
vp14'

Figure 7. Stereo-scope images of *wt* and *vp14* roots 18 h after transplanting to dry vermiculite (Ψ_w = -1.6 MPa). The roots were stained with carboxy-H_2DCFDA and PI and during the staining air was supplied constantly to the staining solution.

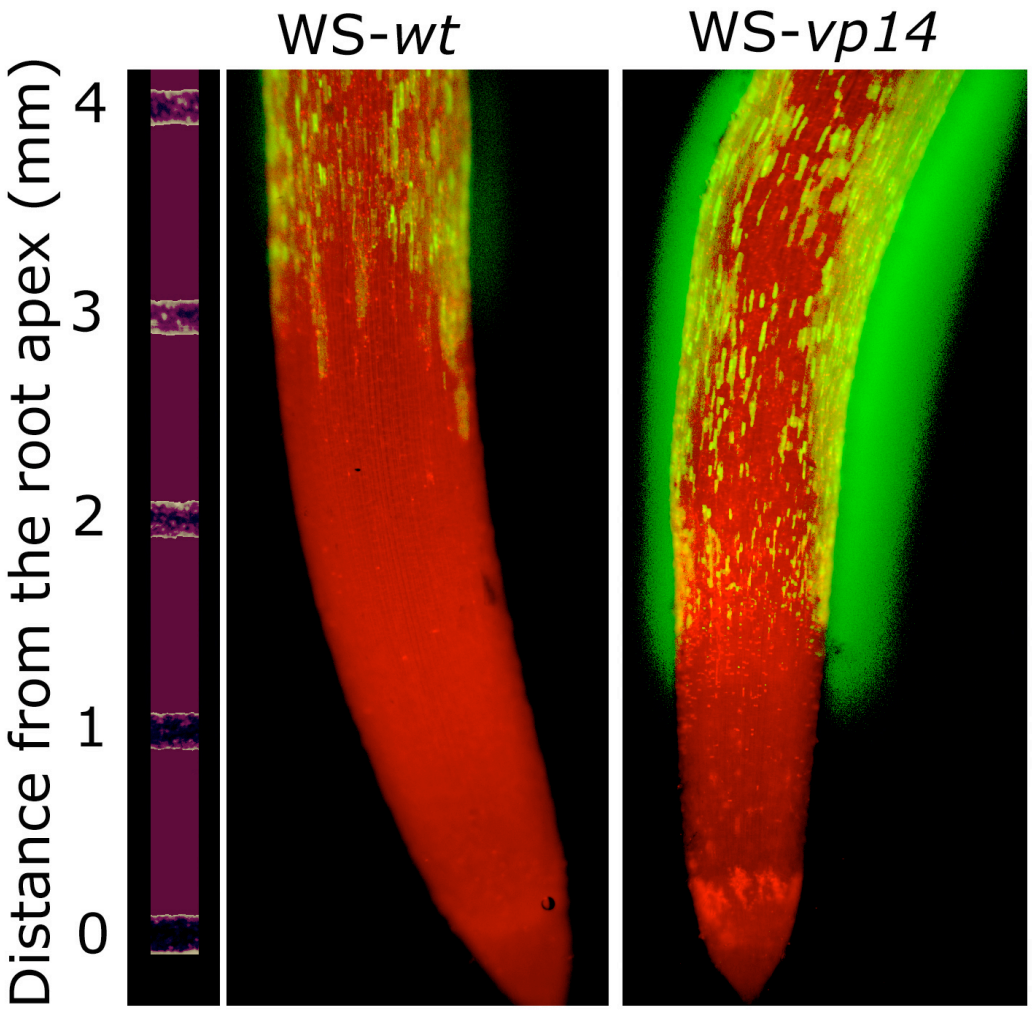


Fig. 8. Three-dimensional reconstructed confocal images of *vp14'* root tip (1-3mm) 6 h after transplanting to water deficit (-1.6 MPa). Using high Ψ_w staining solution of carboxy-H_2DCFDA and PI showed epidermal cell swelling. White arrows indicate the swollen cells.

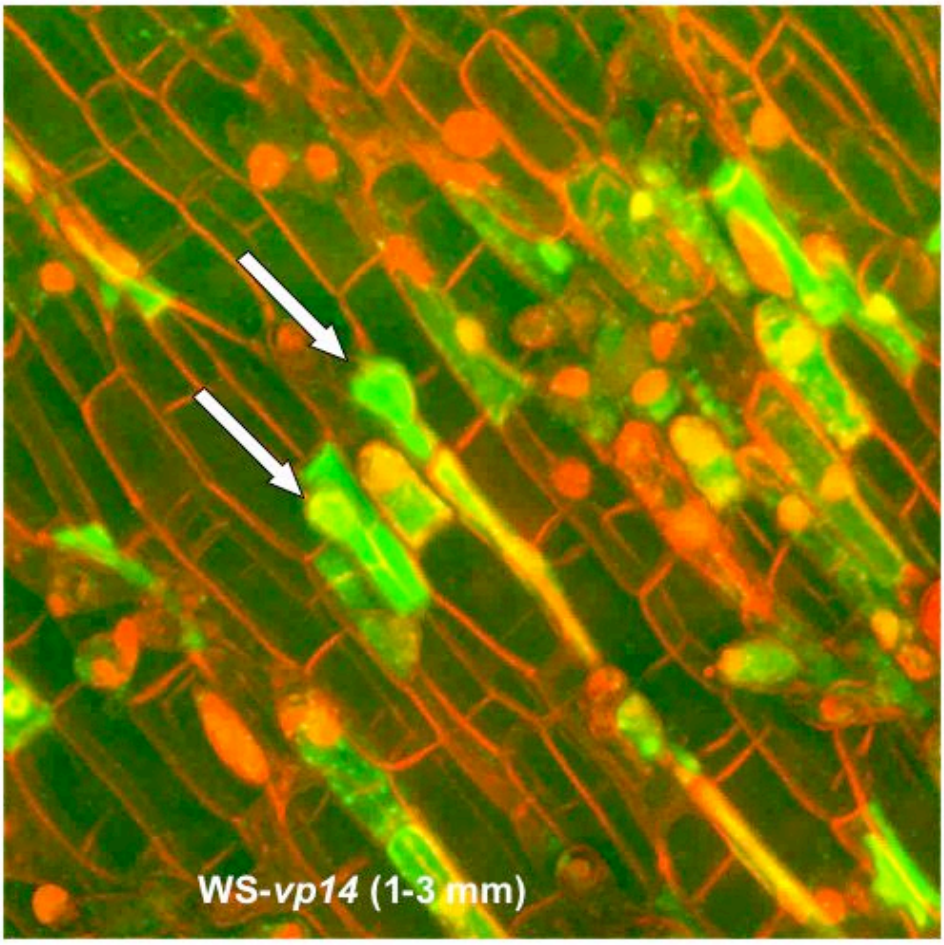


Fig. 9. Three-dimensional reconstructed movie of the confocal images of water-stressed *vp14'* roots shown in Fig. 8.

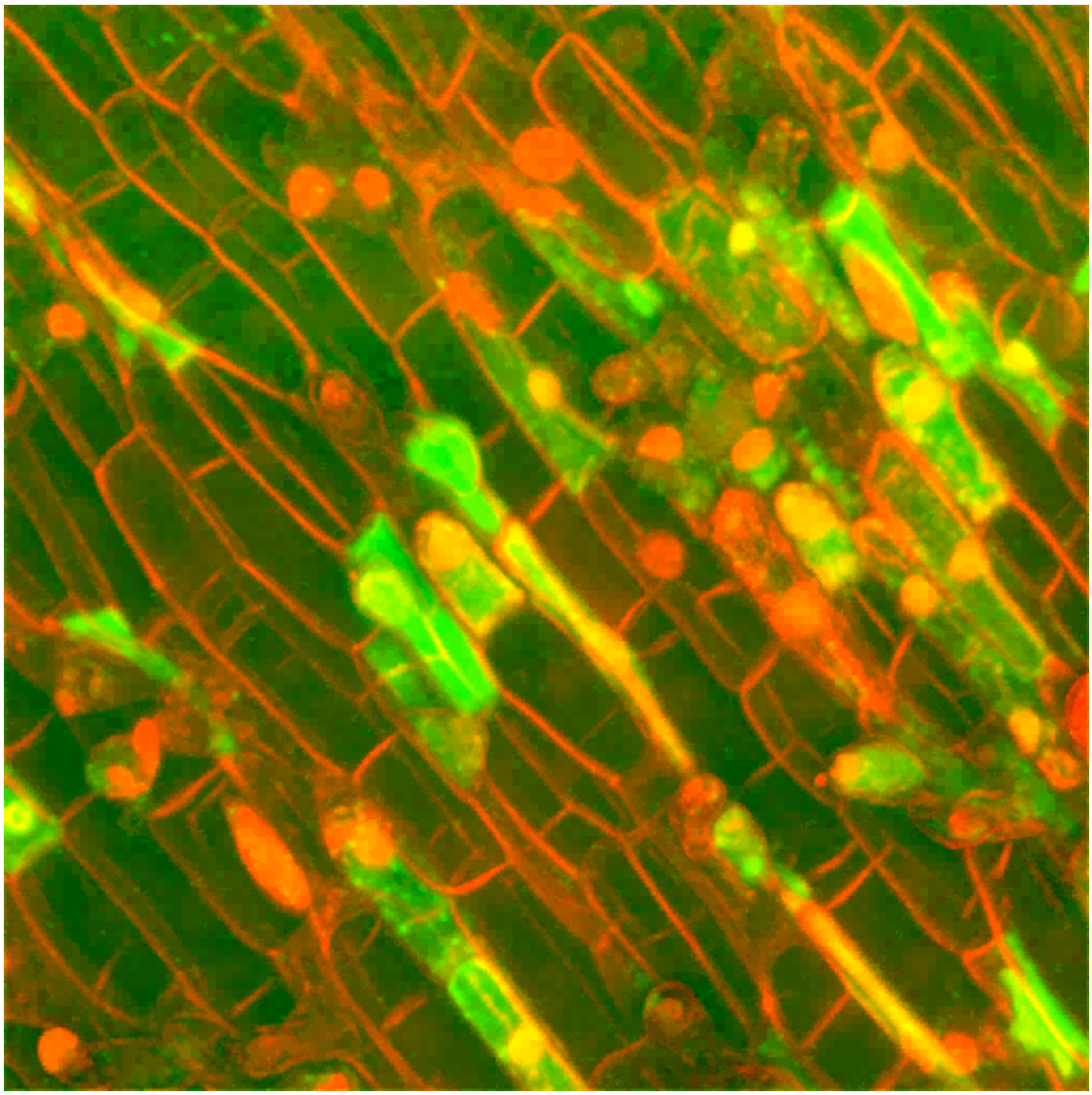


Figure 10. Stereoscope images of FLU-treated wild type roots (0-6 mm from the tip) stained with carboxy-H_2DCFDA and PI 24 h after transplanting to water stress conditions (Ψ_w = –1.6 MPa).

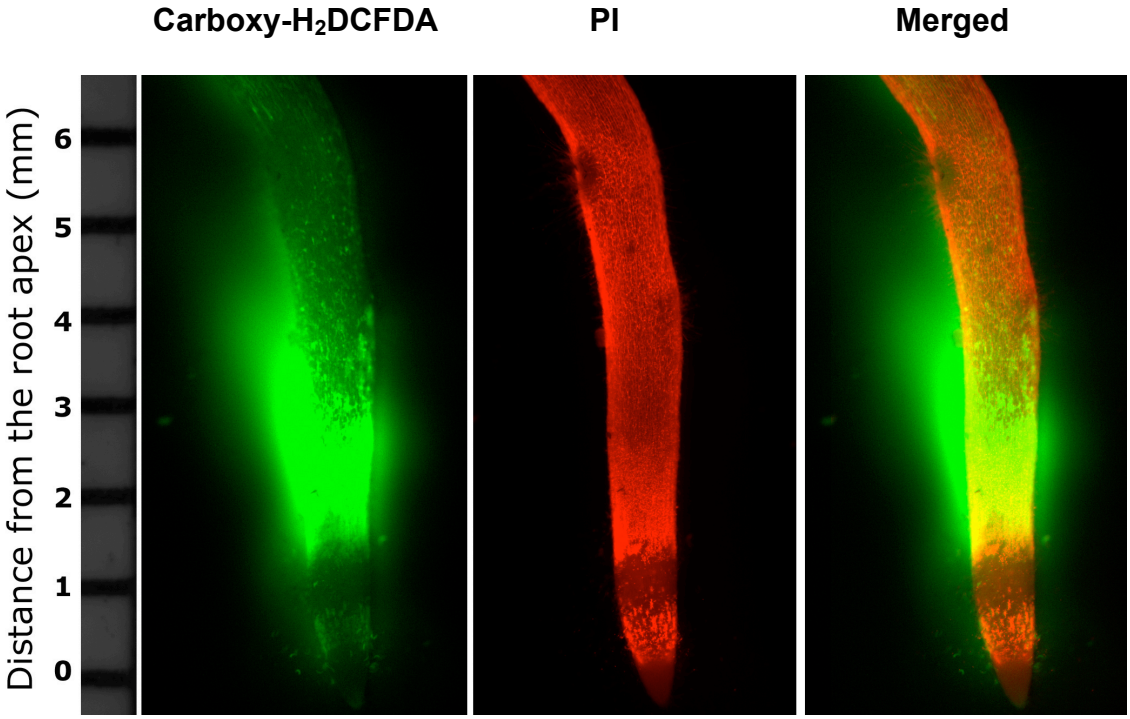
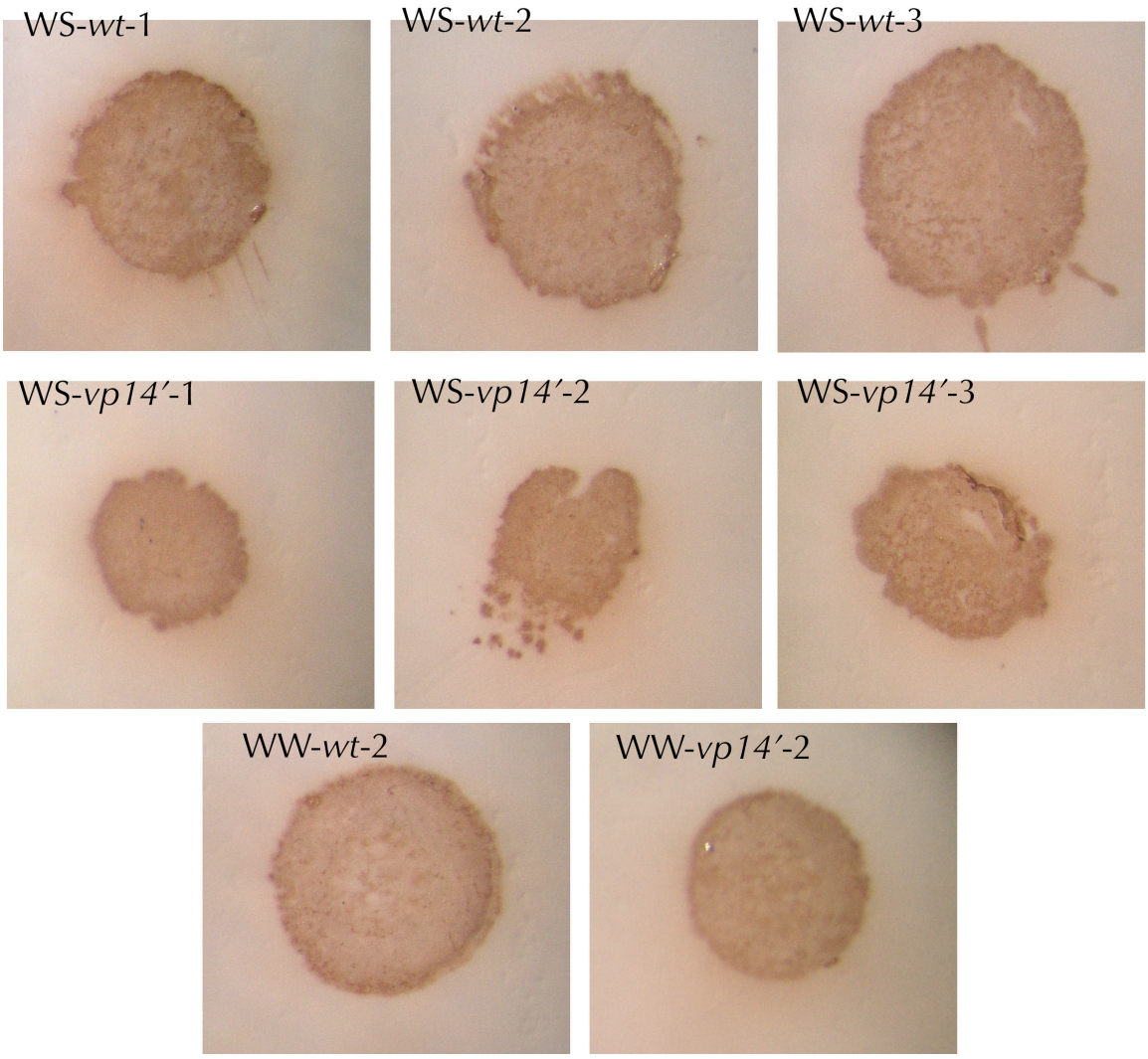


Figure 11. DAB staining patterns on nitrocellulose membranes from transverse sections at different distances from the apex of wild-type (*wt*) and *vp14'* root after 18 h of growth under well-watered (WW) and water stressed (WS) conditions. WS-*wt*-1, 0-1 mm; WS-*wt*-2, 1-2 mm; WS-*wt*-3, 2-3 mm; WS-*vp14'*-1, 0-1 mm; WS-*vp14'*-2, 1-2 mm; WS-*vp14'*-3, 2-3 mm, WW-*wt*-2; 1-2 mm, WW-*vp14'*-2; 1-2 mm section.



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Chapter 4-Supplement

Total catalase activity in the growth zone of wild-type and *vp14'* roots under well-watered and water-stressed conditions.

INTRODUCTION

Scandalios *et al*. (1994) suggested that ABA can regulate total catalase (CAT) activity to control ROS levels, and that CAT1 might function as a dominant ROS scavenger in maize roots (Scandalios, 1994). CAT exerts a major control on hydrogen peroxide metabolism by comparison with ascorbate peroxidase (APX) which scavenges very small quantities of hydrogen peroxide and is more sensitive to the substrate (Shigeoka *et al*., 2002).

Results from DCFDA staining presented in Chapter 4 indicate that ROS levels are increased in the growth zone of the maize primary root at low Ψ_w, especially in *vp14'*. Accordingly, modifications of CAT activity might play a dominant role in regulating ROS levels in the maize primary root at low Ψ_w and as affected by ABA accumulation.

MATERIALS AND METHODS

To conserve *vp14'* seed, a preliminary experiment (Experiment 1) was conducted using the hybrid FR27 X FRMo17 with or without treatment with fluridone to induce ABA deficiency. Growth conditions at high and low Ψ_w and fluridone treatment were the same as described in Chapter 2. The apical 10 mm of the root tips were harvested 18 h after transplanting and frozen in liquid nitrogen. Samples of 0.3 g fresh weight were homogenized in 700 µL of extraction buffer containing 0.05 M KPO_4 (pH 7.0), 0.0001M EDTA, 1% PVP, 0.1mM PFSF, and 0.005 M sodium ascorbate (Rao *et al*., 1996). The samples were centrifuged at 15,000 g for 20 min, and the supernatants were used for

measurements of catalase activity. 100 µL of each sample was added to 3 mL of reaction buffer containing 50 mM KPO_4 (pH 7.0) and 30% H_2O_2 in a quartz cuvette, and the mixture was incubated for 15 min. After incubation, the cuvette was placed in a spectrophotometer and the absorbance was read at 240 nm after 30 sec to monitor the reduction of H_2O_2. Total CAT activities were expressed as A_{240}/min/g total protein. Total protein of each root sample was quantified by the Bradford method (Bradford, 1976).

CAT activity measurements of *wt* and *vp14'* root segments

Seedlings of *wt* and *vp14'* were grown in vermiculite at either high and low Ψ_w as described in chapter 2. In a second preliminary experiment (Experiment 2) using *wt* and *vp14'*, roots were carefully removed from the vermiculite after 18 h, and cut into three serial segments at 0-1, 1-3, and 3-6 mm from the root tip. The samples were then immediately frozen in liquid nitrogen. In subsequence experiments (Experiment 3), roots were washed with distilled water for 15 sec and gently wiped with Kleenex tissue to remove mucilage before sectioning. Samples of 100 root segments were then ground in liquid nitrogen and extracted with the extraction buffer provided by the Amplex-Red kit (Molecular Probes, Invitrogen Corp.). After centrifuging at 10,000 g for 40 min, the supernatants were measured for CAT activity and protein quantification. Due to minute sample quantities, both CAT activity and protein quantification were measured using microplate methods. The Amplex-Red catalase kit and Bradford protein quantification kit (Bio-red) measured colorimetric changes by catalase at 650 nm and by protein at 695 nm.

RESULTS AND DISCUSSION

In experiment 1, total CAT activity was three-fold higher in water-stressed roots (10 mm root tip) compared to well-watered roots (Table 1). CAT activity of fluridone-treated roots under water deficit was 27% less than in the untreated roots under water deficit, although the activity remained substantially higher than in well-watered roots. The inhibition of CAT activity in the fluridone-treated roots correlated with a 30% inhibition of root elongation in comparison with the untreated roots (Table 1), as previously observed (Chapter 2).

These results indicated that ABA accumulation in the root tip under water stress up-regulates CAT activity in association with the maintenance of root elongation.

Because the 1-3 mm section showed the most severe phenotype of increase in ROS and membrane damage in *vp14'* (Chapter 4), it was expected that this region would show the greatest effect of ABA deficiency on CAT activity. Therefore, CAT activities in segments of *wt* and *vp14'* roots (0-1, 1-3, 3-6 mm from the apex) were quantified for the rest experiments. In the second experiment, the CAT activities of the 1-3 mm sections of *wt* and *vp14'* showed two-fold and 1.5-fold increases, respectively in water-stressed compared to well-watered conditions (Table 2). Moreover, the CAT activity in *wt* was 2.7 times higher than the activity in *vp14'* under water-stressed conditions.

In experiment 3, unexpectedly there was no increase in CAT activity in any region of water-stressed compared to well-watered roots in either *wt* or *vp14'*

seedlings, nor was there any indication of decreased CAT activity in any region of *vp14'* compared to *wt* roots under water-stressed conditions (Table 3). The contradictory results with those obtained for hybrid seedlings with and without FLU treatment (Table 1) and with the preliminary result of *wt* and *vp14'* seedlings (Table 2) may have been due to technical difficulties or may be genuine results. Possible technical limitations include 1) minute sample size, 2) numerous manipulations such as washing and wiping. Further investigation is required to clarify if CAT activity is regulated by ABA in water-stressed root tips.

Table 1. Total CAT activities in the apical 10 mm of the maize primary root under well-watered (WW, Ψ_w = -0.02 MPa) and water-stressed (WS, Ψ_w = -1.6 MPa) conditions. Water-stressed roots were grown with or without treatment with fluridone (FLU). Each sample was collected from 120-140 seedlings.

	Total CAT activity (mg^{-1} total protein)	Root length increase (mm)
WW	0.91	76.87 ± 0.81
WS	2.82	21.43 ± 0.41
WS+FLU	2.05	15.02 ± 0.35

Table 2. Preliminary measurements of total CAT activity in segments of *wt* and *vp14'* root tips that were not washed and not wiped. Well-watered (WW, Ψ_w = -0.02 MPa) or water-stressed (WS, Ψ_w = -1.6 MPa) *wt* and *vp14'* roots were sectioned at 0-1, 1-3, and 3-6 mm from the apex. 1 unit (U) represents the amount of enzyme that catalyzes 1 µmole of substrate per minute.

	U/mg protein			
	WW-*wt*	WW-*vp14*	WS-*wt*	WS-*vp14*
0-1 mm	5.25	3.45	5.59	4.07
1-3 mm	12.20	5.63	24.14	8.97
3-6 mm	20.07	19.71	36.64	Not available

Table 3. Total CAT activities in segments of *wt* and *vp14'* root tips. Well-watered (WW, Ψ_w = -0.02 MPa) or water-stressed (WS, Ψ_w = -1.6 MPa) *wt* and *vp14'* roots were sectioned at 0-1, 1-3, and 3-6 mm from the root tip (n = 3). 1 unit (U) represents the amount of enzyme that catalyzes 1 µmole of substrate per minute. Data are means ± SE. Analysis of variance was performed using Fisher's LSD test at the P = 0.05 level.

	U/mg protein			
	WW-*wt*	WW-*vp14*	WS-*wt*	WS-*vp14*
0-1 mm	8.39 ± 3.22[a]	7.98 ± 2.32[a]	11.14 ± 7.45[a]	7.53 ± 2.34[a]
1-3 mm	8.24 ± 1.17[a]	9.17 ± 1.79[a]	8.87 ± 1.95[a]	8.22 ± 1.32[a]
3-6 mm	16.59 ± 4.31[a]	21.51 ± 5.77[a]	13.26 ± 2.44[a]	16.93 ± 3.11[a]

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CHAPTER 5

In-situ visualization of apoplastic ROS in the growth zone of well-watered and water-stressed roots

This project is in collaboration with Dr. Mayandi Sivaguru (Molecular Cytology Core).

INTRODUCTION

As described in Chapter 1, previous work by Sharp and co-workers has shown that maintenance of cell elongation in water-stressed maize primary roots involves increased longitudinal cell wall extensibility (Wu *et al*., 1996). This idea was originally suggested because under severe water stress conditions (Ψ_w of -1.6 MPa), the local rate of elongation was fully maintained in the apical few mm of the growth zone (Chapter 1, Fig. 1; Sharp *et al*., 1988; Liang *et al*., 1997) despite the fact that turgor levels were less than 50% of well-watered values (Spollen and Sharp, 1991).

Recently, evidence has been published in support of the idea that hydroxyl radicals ($^{\bullet}OH$) can directly cause cell wall loosening by cleavage of the polysaccharide structure (Fry, 1998; Liszkay *et al*., 2004). Accordingly, it can be hypothesized that under water deficit, increased ROS levels in the apoplast might loosen the cell wall structure and thereby contribute to the maintenance of cell elongation rate in the root apical region. This hypothesis is supported by recent results in the Sharp laboratory on changes in cell wall protein composition in water-stressed root tips (Zhu *et al*., 2005). The proteomics results predicted that higher production of ROS, especially H_2O_2, occurs in the apical few mm of roots at low compared to high Ψ_w. This prediction was supported by biochemical measurements of H_2O_2 (Tarpey and Fridovich, 2001) in apoplastic fluid extracted from the root tips by vacuum infiltration and centrifugation (J Zhu and R Sharp, unpublished). However, ROS measurements can be variable under different conditions and using different methods (Tarpey and Fridovich, 2001). Therefore,

verification of this result requires confirmation by a method that is independent of tissue extraction. In this chapter, a method of visualizing apoplastic ROS *in situ* in the apical region of intact well-watered and water-stressed roots using a membrane-impermeable fluorescent indicator of ROS levels was developed and used to confirm the hypothesis that apoplastic ROS increases in order to maintain cell elongation under water-stressed conditions.

MATERIALS AND METHODS

A novel dye, 2', 7'-dichlorofluorescein (H_2DCF), was synthesized by Molecular Probes (Eugene, OR, Invitrogen Corp.) for these studies. The dye is a derivative of carboxy-H_2DCFDA in which the diester moiety, which allows the molecule to cross the plasma membrane, has been cleaved. Therefore, H_2DCF should be restricted within the cell wall and apoplastic space unless the plasma membrane is breached. Seedlings (cv FR697, previously used for the cell wall proteomics studies) were grown in either high (-0.02 MPa) or low Ψ_w (-1.6 MPa) vermiculite for 48 h, and then were removed from the vermiculite and the tips of intact roots were immersed in a staining agar solution containing either 15 μM H_2DCF or PI (50 μl/ml). The agar solution was prepared as a combination of 1% high- and 1% low-gelling temperature agarose (1:1) in 1 mM $CaSO_4$, for which the agar solidification starts around 30°C. The dye was added immediately before a root was embedded into the agar-staining solution at approximately 30°C. The mixed agar protocol provides several advantages; 1) solid material to handle due to the high-gelling temperature agar, 2) less friction between roots

and agar due to the low-gelling temperature agar, and 3) minimal diffusion of H_2DCF and ROS from the apoplast to the staining solution during immersion of the roots. After 30 min of staining, when the agar had solidified, an agar block around the 20 mm root tip was cut and placed on a cover glass; thus the root tip was not mechanically disturbed. Since the laser could penetrate through the agar, the agar around the root did not affect the imaging process. The epidermal cells were imaged using two-photon laser scanning confocal microscopy (Zeiss LSM NLO 510 combined with a Coherent, Chameleon 720-950 nm tunable two-photon laser) at 750 nm infrared excitation (this reduces heat induced physiological changes compared to visible excitation lines) and the emission was captured between 500-550 nm. The pinhole aperture was kept wide open because the excitation is two-photon. The laser power at attenuation (AOTF-accousto Optical Tunable Filter) was 2-4% of approximately 1500 mW. The images were scanned without scan averaging (fast acquisition) using a 10x (EC Plan-NeoFluar NA 0.3) objective together with a 1.9 digital zoom under 512 pixel resolution in XY. The power, gain, offset and detector gain levels were optimized and kept constant between different experimental samples during a given day. All images were processed by LSM 5 Image Examiner under identical conditions.

RESULTS AND DISCUSSION

Apoplastic ROS in well-watered and water-stressed roots of cv FR697

Staining with H_2DCF provided direct evidence that apoplastic ROS levels are substantially increased in region 1 (0-3 mm from the root tip) of water-

stressed compared to well-watered roots (Fig. 1C and D). Analyses of successive focal planes resulting from the confocal images indicated that H_2DCF staining was indeed localized to the apoplast in both the well-watered and water-stressed roots (Fig. 2). Staining was especially prominent in the longitudinal cell walls (Fig.1D), consistent with the hypothesized role of apoplastic ROS in cell elongation. A single plane (3 μm) comparison reveals staining of the radial as well as longitudinal walls with PI (Fig. 1E), but only the longitudinal walls with H_2DCF (Fig. 1F).

In region 2 (3-7 mm from the root tip), well-watered roots showed the same distinctive pattern of ROS staining in longitudinal walls as the water-stressed roots in region 1, although to a lesser degree, correlating with the zone of maximum cell elongation rate under well-watered conditions (Chapter 1, Fig. 1). On the other hand, region 2 of water-stressed roots showed no evidence of apoplastic ROS staining. However, there was an indication of plasma membrane breaching, which was suggested by nuclear staining with H_2DCF (arrow in Fig. 1 B). It is difficult to fully interpret the levels of apoplastic staining once intracellular staining occurs due to the staining intensity shift; the cytoplasmic volume is much larger than the apoplast volume. The large cytoplasm creates a sink for the dye and thereafter an intense staining. Accordingly, apoplastic ROS staining, if there was any, could have been masked by the intense intracellular staining. A ROS indicator which is physically trapped in the apoplast will be required to confirm the absence of apoplastic ROS staining in region 2 of water-stressed roots.

CONCLUSION

As suggested by the cell wall proteomics results, substantial increase of apoplast ROS in region 1 of water-stressed roots suggests that ROS generation may contribute to the maintenance of cell elongation under water deficit. The slight increase of apoplastic ROS in the zone of rapid elongation under well-watered conditions supports the idea that ROS production contributes to root cell expansion even under well-watered conditions (Liszkay *et al*., 2004).

Figure 1. A-D, F: representative images of apoplastic ROS as visualized by staining with H_2DCF in region 1 (1.5 mm from the apex) and region 2 (5 mm from the apex) of maize primary roots grown under well-watered (Ψ_w = -0.02 MPa) and water-stressed (Ψ_w = -1.6 MPa) conditions for 48 h after transplanting (n = 4-10, from eight independent experiments). A to D are projections of seven optical sections (each of 2-3 μm in thickness) obtained by a two-photon laser scanning confocal microscope; A, WW region 2; B, WS region 2; C, WW region 1; D, WS region 1. E, single optical section of WW region 1 with PI staining. F, single optical section of WS region 1 with H_2DCF. Scale bar = 50 μm.

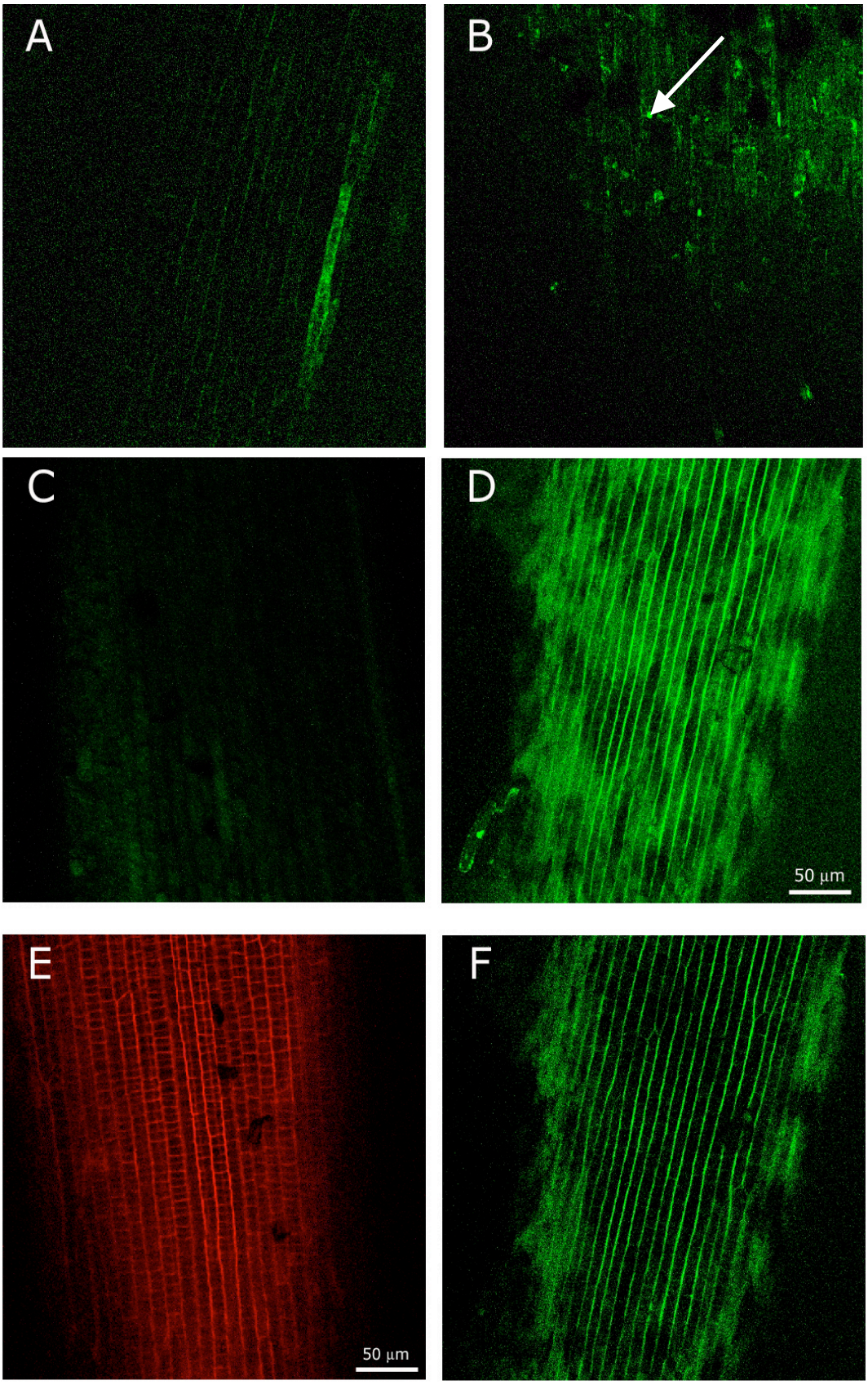
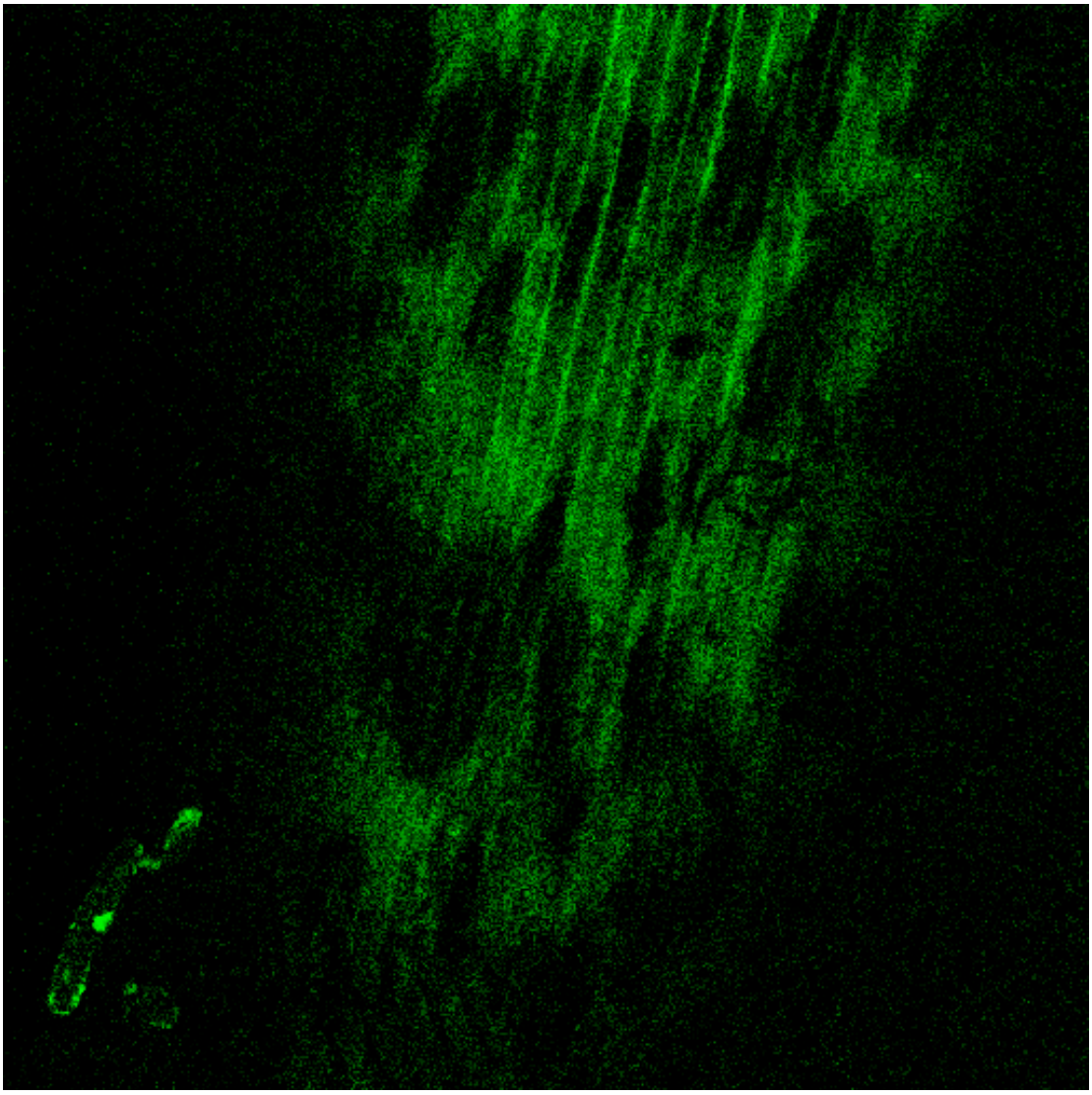

A
B
C
D
50 µm
E
50 µm
F

Figure 2. Sequential play of the confocal images of root region 1 (Fig. 1D) under water-stressed conditions.



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CHAPTER 5-Supplement

Preliminary experiments for *in-situ* visualization of apoplastic ROS in the growth zone of *wt* and *vp14'* roots under well-watered and water-stressed conditions

This project is in collaboration with Dr. Mayandi Sivaguru (Molecular Cytology Core).

INTRODUCTION

The results presented in Chapter 4 provided evidence that ABA functions to maintain ROS at non-toxic levels for intracellular protection under water deficit. In addition, the results in Chapter 5, Fig. 1 indicated that increased apoplastic ROS may substantially contribute to the maintenance of cell wall extension in region 1 under water deficit. Taken together, maintenance of ROS balance may be a key function of ABA not only in the cytoplasm but also in the apoplast under water stressed conditions. In fact, osmo-swelling experiments indicated that the cell walls in root tips of *vp14'* were weakened compared to the *wt* at low Ψ_w (Chapter 4, Fig. 7), which could have resulted from excess apoplastic ROS levels by ABA deficiency. To test this possibility, apoplastic ROS levels were investigated in region 1 of *vp14'* compared to *wt* roots.

MATERIALS AND METHODS

Seedlings of *wt* and *vp14'* were grown in either well-watered (Ψ_w = -0.2 MPa) or dry (Ψ_w = -1.6 MPa) vermiculite, as described in Chapter 2. After 18 h, the seedlings were removed from the vermiculite and then the roots were stained with H_2DCF and imaged especially in region 1 (1.5 mm from the apex) as described in Chapter 5. For direct comparison between intracellular and apoplastic ROS, 18 h after transplanting was chosen for this study.

RESULTS AND DISCUSSION

Apoplastic ROS in region 1 of *wt* and *vp14'* roots

H_2DCF staining of *wt* and *vp14'* roots showed two unexpected results. First, under well-watered conditions, both *wt* and *vp14'* roots already exhibited high levels of apoplastic ROS in region 1 (1.5 mm from the apex) (Fig. 1A and B)[1]. In contrast, FR697 roots demonstrated no detectable apoplastic ROS in region 1 under well-watered conditions (Chapter 5, Fig. 1C). Thus, the *wt* genotype and *vp14*' mutant might require high levels of ROS in order to achieve cell elongation in region 1 even under well-watered conditions. Second, water-stressed *wt* and *vp14'* roots showed the same pattern and degree of apoplastic ROS production as well-watered roots (Fig. 1C and D), contrasting with the large increase in apoplastic ROS in region 1 of water-stressed compared to well-watered FR697. In water-stressed *vp14'*, intracellular leakage was also observed due to plasma membrane breaching (Fig. 1D). The indication of impairment of membrane integrity in region 1 of *vp14'* under water-stressed conditions is consistent with the PI staining results presented in Chapter 4, Figs. 3 and 4.

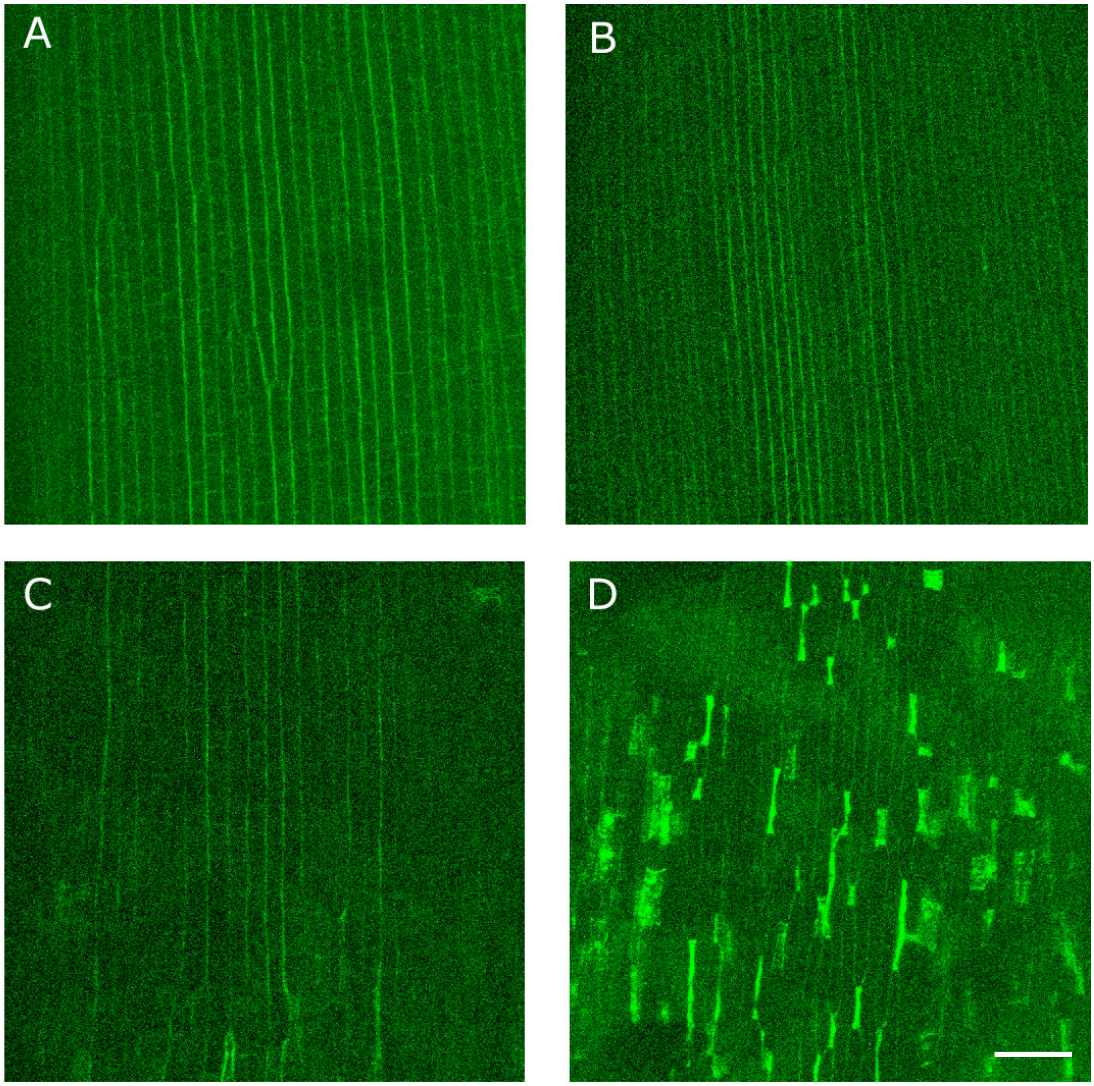
CONCLUSION

No differences in apoplastic ROS staining were observed between *wt* and *vp14'* roots under either well-watered or water-stressed conditions. Accordingly, the results suggest that ABA does not play a role in regulating apoplastic ROS levels in the apical region of water-stressed roots. It is possible, however, that

[1] In the same experiment, FR697 roots were also examined at 48 h after transplanting and their region 1 showed no apoplastic ROS under well-watered conditions, consistant with the results in Chapter 5 Fig. 1.

ROS staining was already saturated in the well-watered roots, which would have precluded visualization of further increase in ROS resulting from water stress and/or ABA deficiency. Therefore, further investigation is required to fully access whether ABA is involved in the control of apoplastic ROS levels in water-stressed roots.

Figure 1. Representative images of apoplastic ROS as indicated by staining with H_2DCF at 1.5 mm from the apex of *wt* and *vp14'* roots under well-watered and water-stressed conditions at 18 h after transplanting (n = 3-8, from two independent experiments). All images are projections of seven optical sections (each of 2-3 μm in thickness) obtained by a two-photon laser scanning confocal microscope. A; well-watered *wt*, B; well-watered *vp14'*, C; water-stressed *wt*, D; water-stressed *vp14'*. Scale bar = 50 μm.



APPENDIX I

vp14' GENOTYPIC CHARACTERIZATION

These studies are in collaboration with Dr. Georgia Davis.

Recent discoveries indicate, unexpectedly, that all of the *vp14* mutant seeds used for my experiments were obtained from segregating ears from heterozygous mother plants. The original *vp14* mutation was created by mutator (*Mu*) insertion (Tan *et al*., 1997). Original *vp14* seeds were provided by Dr. Don McCarty at the University of Florida-Gainesville. The *vp14* seeds were hand selected from segregating ears and were believed to be homozygous based on a judgment of a mildly viviparous phenotype. This hand-selected *vp14* was used for initial development of the *vp14* root growth system. Some of the *vp14* seed were grown in the greenhouse and the progenies were produced by self-pollination. The greenhouse-grown *vp14* seeds were used to generate the majority of the results on root growth, ABA content and ethylene production presented in Chapter 2 (Fig. 2 and 3). Because the mother plants were selfed, it was assumed that the *vp14* seeds used for these experiments were uniform homozygotes.

For large quantities of seeds, *vp14* and its wild type were grown in the field. The first field-planted *vp14* and wild-type seeds came from a second supply of seeds from Dr. McCarty's lab (Florida 2000 seed). The first Missouri field-grown seeds (2002) were obtained by sibbing because all of the *vp14* seeds were assumed to be homozygotes, and were used for the ROS imaging experiments presented in Chapter 4.

For the current NSF root genomics project (http://rootgenomics.missouri.edu/prgc/index.html), Dr. Georgia Davis generated field-grown *vp14* seeds in summer 2003, winter 2004, and summer 2004 by planting both the Florida 2000

seeds and the Missouri field-grown (2002) seeds. The seeds produced by Dr. Davis were genotyped using specific primers for *vp14* and wild type, and it was discovered that none of the ears were homozygous *vp14*. Therefore, 20 seeds from each heterozygous ear (summer 2003) were grown for two weeks and then leaf DNA was used for genotyping. The combined results of wild type and heterozygous *vp14* confirmed that all field-grown seeds were from segregating ears. Therefore, the Florida 2000 seeds were also genotyped, and the results showed that the seed were a mixture of wild-type and heterozygote; no homozygous *vp14* seedlings were found. These results suggest that a) homozygous *vp14* seeds do not readily germinate (segregating ears should have 25% homozygous seeds), and b) all of the seeds I used for my experiments which were presumed to be homozygous *vp14* were in fact a mixture of wild-type (approximately one-third) and heterozygotes (approximately two-thirds) obtained from segregating ears from heterozygous mother plants.

This finding was unexpected, because none of the physiological studies had indicated segregating behavior of the putative *vp14* seedlings. For example, all of the individual water-stressed roots imaged for ROS in the experiments presented in Chapter 4 exhibited consistently high ROS levels compared to homozygous wild-type plants. Accordingly, it was suspected that the inheritance of *vp14* might follow a maternal pattern with all kernels from a segregating ear on a heterozygous plant displaying the heterozygous genotype. To directly test this possibility, seed from four segregating ears were pooled and 550 seedlings were grown at low Ψ_w (-1.6 MPa) for 48 h, at which time the primary root length was

measured, the apical 10 mm of each root was sampled for ABA assay, and the rest of the root was harvested for DNA genotyping using PCR. The primer sequences were as followes: *vp14'* forward, CGTCGAATCCGCTTCTCT CTTCG ; reverse, CACGCGTTCCACAGGTGG; *wt* forward, GGCCGCTACGACTTCGAC GG; reverse, CCACGAAGTTCTCGGTGATGGCG. After denaturation at 94° for 2 mins, 10 cycles of condition-1[2] and 40 cycles of condition-2[3] were performed to multiply DNA. The results showed that out of 366 seedlings which were genotyped, 97 were wild-type, 260 were heterozygous, and 9 were homozygous *vp14*. After genotyping, root tip segments from seedlings of the same genotype were combined for ABA measurements (seven root tips per sample). The results (Table 1) showed that root length increases were not significantly different among seedlings grown from the segregating seed regardless of their genotype (Table 1), and in all cases were impaired compared to wild-type seedlings from homozygous wild-type mother plants (Chapter 2, Fig. 2). Similarly, root tip ABA contents were not significantly different between wild-type and heterozygous seedlings grown from the segregating seed (only one ABA measurement could be made for homozygous *vp14* because of the small sample size), and were substantially lower than the ABA levels in wild-type seedlings from homozygous mother plants (Chapter 2, Fig. 2). The results strongly suggest that maternal effects are involved in the inheritance of *vp14*.

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[2] 94° for 1min, 65° for 1min, and 72° for $1^{1/2}$ min.

[3] 94° for 1min, 55° for 1min, and 72° for $1^{1/2}$ min.

Possible mechanisms of maternal effects include paramutation and/or gene silencing due to the generation of the *vp14* mutation by mutator (*Mu*) insertion (Chandler and Stam, 2004; Della Vedova and Cone, 2004). As an initial step to further examine these possibilities, root tip segments were collected from seedlings grown at high and low Ψ_w from kernels obtained from wild-type and segregating ears, in order to examine *Vp14* expression by RT-PCR. The rest of the root (>6 mm) was collected for DNA genotyping. If paramutation or gene silencing is involved in the inheritance of *vp14*, then the results should show low *Vp14* gene expression under water stress regardless of the genotypes among the segregating seeds. Molecular analyses for these studies are in progress in Dr. Davis' lab.

Table 1. Primary root length increase and root tip ABA content at 48h after transplanting to water-stressed conditions (Ψ_w = -1.6 MPa) in seedlings of different genotypes grown from a segregating ear from a heterozygous plant. Data are means ± SE. For root lengths, n = 97, 260 and 9 for wild type (wt), heterozygotes, and *vp14*, respectively. For ABA contents, n = 14, 15, and 1 for wt, heterozygotes, and *vp14*. Data are combined from two independent experiments. Within each row, values with the same letters are not significantly different at the 0.05 level.

	wt	heterozygous	*vp14*
Root length increase (mm/48 h)	27.1 ± 0.5[a]	28.6 ± 0.8[a]	26.2 ± 2.8[a]
ABA content (ng/g water)	43.9 ± 3.7[a]	39.0 ± 3.0[a]	29.3[*]

[*] only one ABA measurement of *vp14* due to small sample size.

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VITA

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