REGULATION OF ALPHA-DIOXYGENASE EXPRESSION AND FUNCTIONAL ANALYSES IN SALT-STRESSED *ARABIDOPSIS THALIANA*

by

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ABSTRACT

Alpha-dioxygenase (α-DOX) catalyzes the oxygenation of fatty acids to produce a newly identified group of oxylipins. In Arabidopsis, *α-DOX* is represented by a small gene family comprised of two members, *Atα-DOX1* and *Atα-DOX2.* Both *α-DOX* genes were constitutively expressed but in distinct locations. *Atα-DOX1* was expressed in roots, stamens and *Atα-DOX2* was expressed in shoots, sepals, siliques and developing seeds. The expression of both *α-DOX* genes was responsive to salt in the roots and shoots and this salt-responsive expression was accompanied by increased α-DOX activity in both root and shoot tissues of salt stressed Arabidopsis. 2-Hydroxylinolenic acid (2HOT) and heptadecatrienal (C17 aldehyde) were the major products detected in in-vitro α-DOX assays using linolenic acid as a substrate.

The role of hormones in regulating salt-induced changes in *Atα-DOX* expression was explored using exogenous application of hormones and hormone mutants. Abscisic acid (ABA) and salicylic acid (SA) were major hormone signals that up-regulated *Atα-DOX* expression in roots, whereas ABA, SA and ethylene up-regulated *Atα-DOX* expression in shoots. The functional significance of α-DOX in salt-stressed Arabidopsis plants was explored using lines with altered *Atα-DOX1* and/or *Atα-DOX2* expression. Knockout lines lacking *Atα-DOX1* or *Atα-DOX2* expression were more sensitive to the damaging effects of salt than wild type suggesting that α-DOX products contribute to salt tolerance. In the same lines, increased levels of H_2O_2 were detected in the roots indicating that α-DOX may suppress the accumulation of reactive oxygen species or promote their removal. An unanticipated function for α-DOX in regulating root system architecture was discovered whereby *Atα-DOX1* and *Atα-DOX2* play a role in checking LR emergence under salt stressed conditions. Such a role is consistent with the spatial expression of *Atα-DOX* in roots, which occurred in the zone of cell differentiation within which LR primordia are known to develop. The expression of both *Atα-DOX* genes was ABA and salt-responsive; it is therefore possible that the resulting α-DOX products contribute to the known ability of ABA to check LR emergence in osmotically-stressed plants. The α-DOX products contribute to salt tolerance in Arabidopsis by protecting cells against oxidative stress and checking LR production.

Keywords: α-dioxygenase; Arabidopsis; roots; salt-stressed; abscisic acid; salicylic acid; ethylene

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GLOSSARY

LEA	Late embryogenesis abundant.
ABRE	ABA-responsive element
DRE	Dehydration responsive element
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
HKT	High-affinity potassium transporter
NHX	Na^+/H^+ antiporter
2HOT	2-hydroxylinolenic acid
C17 ald	Heptadecatrienal (C17 aldehyde)
α-DOX	α-dioxygenase
Wt	Wild type
D1K	*α-DOX 1* knock out mutant
D2K	*α-DOX 2* knock out mutant
DK	*α-DOX* double knock out mutant
RSA	root system architecture
RGR	root growth rate
LR	lateral root
LRD	lateral root density

1: LITERATURE REVIEW

1.1 Introduction

Plants require mineral nutrients, mined from the soil, to grow and develop. However, soil salinization, a condition in which there is an excessive level of minerals present as soluble salts (mainly Na^+, Cl^-, and SO_4^{2-}) in the soil, imposes serious problems for most plants (Flowers and Yeo, 1995). This process can occur naturally (primary salinization) or can be induced by human agricultural practices (secondary salinization). Naturally occurring saline soils can be found in all parts of the world such as coastal areas, salt marshes, and deserts. However, soil salinization resulting from irrigation is of concern, particularly in arid regions (Xiong and Zhu, 2002). The concern arises because water used for irrigation contains low levels of salts that accumulate in the soil over time following the removal of water by evaporation or by transpiration by the plants. Saline soils affect at least 20% of arable land and greater than 40% of irrigated land (Rhoades and Loveday, 1990). In extreme cases, the land can no longer support agricultural production and has to be abandoned. Irrigated farmland produces approximately one third of the world's food source and when this is considered with the growing human population, it is apparent that soil salinization is a threat to the world's food supply (Ghassemi et al., 1995). In Canada, dryland salinity is a major soil degradation problem on the Prairies (Acton, 1995). In Alberta, approximately 1.6 million acres (or 647 485 ha) of dryland are affected

by salinity with an average crop yield reduction of 25 per cent (Alberta Agriculture, 1991).

Although species and the developmental stage at which salinity is imposed contribute to the degree of salt tolerance, most plants are unable to grow in 0.1 M NaCl (Smith and McComb, 1981). High NaCl concentrations interfere with plant nutrient uptake, decrease the water potential in the soil, and cause toxicity when ions enter the plant. These occurrences are collectively referred to as salinity or salt stress. In addition to these stresses, NaCl also induces the production of reactive oxygen species (ROS) (see section 1.6). Salt stress, which is caused by higher than threshold level of ions such as Na^+, Cl^-, Ca^{2+} and SO_4^- in the soil (Bernstein and Kafkafi, 2002), can have drastic effect on an agricultural productivity. High salinity imposes two primary effects on plants: the first is osmotic stress, which results in a loss of cellular turgor, and the second is ionic toxicity leading to inhibition of cellular function due to an increase in the intercellular concentration of Na^+ or Cl^-. These primary effects of salinity stress can impose negative effects on various physiological processes including growth, photosynthesis and cytosolic metabolism. These dramatic changes in physiological processes lead to the reduced growth and productivity of crop plants. Plants cope with salt stress by employing a number of physiological and metabolic responses (Bohnert and Sheveleva, 1998; Hagasawa et al., 2000). However, these responses are energetically expensive and, when combined with decreased growth, the outcome is a reduction in crop productivity as a result of salt stress (Xiong and Zhu, 2002).

Good agricultural practices such as proper drainage after irrigation and the remediation of saline soils will help alleviate salinized land (Ghassemi et al., 1995). In addition, the production of plants with improved salt tolerance either by applying traditional breeding programmes or gene transfer technology will help to maintain crop yields. Identifying salt stress responsive genes and understanding the function of their encoded proteins is the first step to understanding salt stress signalling and tolerance mechanisms. With a better understanding we will find more effective ways to improve plant salt tolerance.

1.2 Glycophytes and Halophytes

Plants exhibit a range of tolerance to salinity and can be broadly categorized as glycophytes or halophytes. Glycophytes are sensitive to salts and are damaged by them (Flowers et al., 1977). They are poor at minimizing salt entry and compartmentalizing salt into the vacuole. Most plants, including crop plants, are glycophytes and the majority of glycophytes cannot survive on media containing 0.1 M NaCl. Halophytes are plants adapted to saline habitats and can grow and survive in medium containing up to 0.5 M NaCl (Smith and McComb, 1981, Hasegawa et al., 2000). The ability of the halophytes to grow in a saline medium is due in large part to osmotic adjustment through intracellular compartmentation that partitions sodium ions (Na^+) and chloride ions (Cl^-) away from the cytoplasm through energy-dependent transport into the vacuole (Apse et al., 1999; Binzel et al., 1988; Glenn et al., 1999, Zhu and Bohnert 2000). Salt that enters the leaves is stored in vacuoles or exuded into salt glands or bladders

away from the cytoplasm. This lowers the osmotic potential of the shoot, which in turn facilitates water uptake.

Most halophytes and glycophytes respond to salt stress similarly, but halophytes are better at minimizing the entry of salt into the plant cell than glycophytes (Hasegawa et al., 2000). In both cases, toxic ions are compartmentalized in the vacuole and used as osmotic solutes (Blumwald et al., 2000; Niu et al., 1995). Both halophytes and glycophytes accumulate organic solutes in the cytosol and thylakoid lumen, in the matrix of the mitochondria, and stroma of the chloroplast for osmotic adjustment (Hasegawa et al., 2000). Glycophytes restrict sodium ion movement to the shoot by minimizing ion influx into the root xylem; on the other hand, halophytes tend to more readily take up sodium ions (Hasegawa et al., 2000). It is possible that halophytes rely on sodium and chloride ions for osmotic adjustment that then supports cell expansion in growing tissues and turgor in differentiated organs (Adams et al., 1992; Glen et al., 1999; Yeo 1998)

The use of Arabidopsis (a glycophyte) and a close relative, the halophytic salt cress (*Thellungiella halophila*) (Taji et al., 2004) in a comparative genomics study provided a better understanding of the mechanisms involved in salt tolerance in halophytes and glycophytes. Arabidopsis contains most, or nearly all, of the salt tolerance genes that exist in *T.halophila* (Zhu, 2000; Shinozaki et al., 2003). The absence of morphological adaptations in *T.halophila*, such as salt glands, which are often present in halophytes led to the hypothesis that salt respnse mechanisms in *T.halophila* are similar to those in glycophytes (Bressan

et al., 2001; Zhu, 2001). It was determined that the ability of *T.halophila* to tolerate salt stress is likely due to pre-existing mechanisms that were present prior to stress. These include the constitutive expression of various abiotic or biotic stress-inducible genes. Gong et al. (2005) defined genes and pathways with shared and divergent responses to salinity stress in *T.halophila* and Arabidopsis by microarray transcript and metabolite profiling. The two species shared 40% of the salt regulated genes which are involved in ribosomal functions, photosynthesis and cell growth, as well as in activating osmolyte production, transport activities and abscisic acid (ABA)-dependent pathways. Analysis of the differences showed that Arabidopsis exhibited a global defense strategy that required bulk protein synthesis, whereas in *T. halophila*, salt stress induced genes functioning in protein folding, post-translational modification and protein redistribution.

1.3 Mechanisms of Salt Entry into Roots

Roots are the main point of salt entry to plants. Most plants do not have a specific transport system for sodium (Na^+) uptake. However, Na^+ ions can easily enter the plant via either the transmembrane or apoplastic routes. Since the concentration of Na^+ in the soil solution is greater than that in the cytosol of the root cells, Na^+ movement into the root is passive (Cheeseman, 1982).

1.3.1 Apoplastic Na^+ Influx

Sodium ions can enter roots via the extracellular transport or apoplastic pathway. This pathway is also known as bypass-flow and operates in

glycophytes and halophytes (Yeo and Flowers, 1987). The apoplastic bypass flow appears to be the main pathway for Na^+ entry in rice (Yeo and Flowers, 1985; Yeo et al., 1987). The casparian strip in the root endodermis plays an important role in preventing apoplastic Na^+ entry into the root stele. This barrier is not perfect and there may be apoplastic flow in the apical region before the endodermis differentiates fully (Peterson, Swanson, and Hall, 1986). In addition, apoplastic flow may occur where the endodermis is ruptured with the development of each lateral root (Yeo et al., 1987). Silica deposition and polymerization of silicate in the endodermis and rhizodermis decrease apoplastic Na^+ entry into the roots of rice (Fang and Ma, 2006). Some halophytes such as *T.halophila* have anatomical adaptations including the development of an extra endodermis and the thickening of the cortex cell layer (Stelzer and Lauchli, 1977; Inan et al., 2004). Moreover, salt-stressed maize seedlings increase the radial width of the casparian strip by 47% compared to non-stressed seedlings (Karahara et al., 2004). These adaptations help minimize Na^+ entry into the transpirational stream.

1.3.2 Transmembrane Na^+ Influx

Na^+ can enter root cells by crossing the cell membrane (transmembrane Na^+ influx) via various cation channels including voltage-dependent or -independent channels (Tester and Davenport, 2003). The non-selective voltage-dependent cation channels (NSCCs) are thought to be the dominant pathway for Na^+ entry into plant roots (Amtmann and Sanders, 1999, Tyerman and Skerrett, 1999; Schachtman and Liu 1999; Demidchik et al., 2002). The NSCCs are a

large and heterogenous group of channels, which have a high selectivity for cations over anions. There are many candidate genes that encode NSCCs; however, the molecular identity of these NSCCs is still unclear (Demidchik and Maathuis, 2007). The two proposed candidates for NSCC's are the cyclic nucleotide-gated channels (CNGCs) and the putative glutamate-activated channels (GLRs) (Maathuis and Sanders, 2001; Leng et al., 2002; Lacombe et al., 2001). The CNGCs are expressed in roots and they effect potassium (K^+) and calcium (Ca^{2+}) uptake (Sunkar et al., 2000; White et al., 2002). The possible involvement of the glutamate-activated channels (GLRs) in Na^+ influx needs further confirmation.

Since Na^+ and K^+ have similar properties, Na^+ can also enter root cells by the same means as K^+, for example by the K^+ voltage-dependent ion channels. High-affinity potassium transporters (HKTs) are considered to be the major route for Na^+-influx into plant cells (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; White, 1999). In addition, the HKT family of proteins function as Na^+/K^+ symporters (Rubio et al., 1995; Garciadeblás et al., 2003; Haro et al., 2005). In wheat (*Triticum spp.*), screening of a cDNA library of K^+-starved roots resulted in the isolation of *HKT1*, which belongs to the high affinity K^+ uptake system. The HKT transporter was subsequently isolated from various plant species including Arabidopsis (Uozomi et al., 2000), eucalyptus (*Eucalyptus globules*) (Fairbairn et al., 2000; Liu et al., 2001), the common ice plant (*Mesembryanthemum crystallinum*) and rice (*Oryza sativa*) (Golldack et al., 2002; Horie et al., 2001). In Arabidopsis, only one HKT transporter (*AtHKT1*),

was identified whereas in eucalyptus two genes with high sequence identity to *HKT1* were identified (Uozumi et al., 2000; Horie at al., 2001; Garcideblas et al., 2003). Expression analysis of *AtHKT1* in *Saccharomyces cerevisiae* and *Xenopus oocytes* showed that at low external Na^+ concentrations, HKT1 functions as an active K^+ transporter whereas at high external Na^+ concentrations, it can function as a low affinity Na^+ transporter (Rubio et al., 1995). Although AtHKT1 can act as a Na^+- transporter, it also plays an important role in controlling cytosolic Na^+ detoxification (Sunarpi et al., 2005). Sunarpi et al., (2005) suggested that AtHKT1 functions by unloading Na^+ from xylem vessels to xylem parenchyma cells and thus is involved in salt tolerance by reducing salt movement to the leaves.

1.4 Salt and osmotic stress

Osmotic stress occurs when the dissolved salts in soil water lower the solute potential, which then decreases the water potential, making it difficult for plants to obtain water. This condition resembles a physiological drought (Xiong and Zhu, 2002). Salt-induced osmotic stress reduces water availability for plants, that impacts plant growth, since water-generated turgor pressure is the driving force for cell expansion (Munns, 2002). The inhibition of shoot growth is greater than that of root growth and it is believed that reduced growth is an adaptive feature to save energy resources so as to cope with stress conditions and for later recovery (Zhu, 2001; Munns, 2002). Also the ability of roots to maintain some growth during salt stress allows plants to explore more soil volume in order to absorb water and minerals. The outcome is to restore the balance between

the size of the shoot system and the ability of the roots to supply the plant with H_2O.

Low water availability is also a major limiting factor for photosynthesis and therefore, plant productivity. One of the responses to osmotic stress is the closure of stomates, which is accomplished by the redistribution and accumulation of ABA. However, there has been a continuous debate with regards to whether the negative effect of salt stress on photosynthesis is caused as a result of stomatal closure or metabolic impairment. It was recently elucidated that salinity stress mainly limits CO_2 uptake into the leaves by inducing stomatal closure and not by the reduction of the biochemical capacity to assimilate CO_2 (Flexas et al., 2004).

1.4.1 Osmotic adjustment

At the cellular level, the plant's response to osmotic stress results in an accumulation of solutes to prevent water loss and to re-establish tugor so as to maintain growth (Rhodes, 1987). These accumulated solutes are often referred to as osmolytes. They are "compatible" solutes, which means that they are chemically benign and do not inhibit normal metabolic reactions. Metabolites with osmolyte function include simple sugars (mainly sucrose and fructose), sugar alcohols (glycerol, methylated inositols), and complex sugars (trehalose, rafinose, fructans) (Hasegawa et al., 2000). The accumulation of ions (K^+) or charged metabolites (glycine betaine, dimethyl sulfonium propionate (DMSP), proline and ectoine) is also induced (Hasegawa et al., 2000). Osmolytes facilitate osmotic adjustment by lowering the osmotic potential of plant cells

allowing the continued uptake of H_2O. The hydrophilic nature of the osmolytes allow them to act as osmoprotectants by replacing the water at the surface of proteins, protein complexes, or membranes. Osmolytes at high concentrations reduce the inhibitory effects of ions on enzyme activity, increase the thermal stability of enzymes and prevent dissociation of protein complexes, for example photosystem II (Bray et al., 2000). Aside from osmotic adjustment and osmoprotection, osmolytes may participate in the prevention of oxygen radical production or in the scavenging of reactive oxygen species (ROS), a by-product of a secondary oxidative stress (Noctor and Foyer, 1998). The synthesis of osmolytes or osmoprotectants is energetically costly to the plant (Raven, 1985).

1.5 Salt and Ionic Stress

Metabolic toxicity imposed by Na^+ is one of the primary effects of salt stress in plants. Plant cells use potassium ions as co-factors in the cytosol to activate more than 50 enzymes, which are susceptible to high Na^+ concentration as a result of Na^+ competing with K^+ for uptake by plant cells (Tester and Davenport, 2003; Munns et al., 2006). In addition, elevated levels of Na^+ can change the permeability of the plasma membrane by displacing Ca^{+2} and inducing K^+ leakage from the cell. Therefore, maintenance of intercellular K^+ levels and avoidance of Na^+ influx into the cytosol are vital for a variety of cellular functions such as enzyme activity, the maintenance of membrane potential and an appropriate osmotic-dependant regulation of cell volume. Metabolic toxicity imposed by Na^+ is caused by the replacement of K^+ with Na^+, which results in conformational changes and a loss of protein function as Na^+ and Cl^- ions

penetrate hydration shells and interfere with noncovalent interactions between the amino acid residues of proteins (Zhu, 2002). In serious cases, high salinity disrupts water potential homeostasis and, together with ion toxicity at the cellular and whole plant levels, can lead to molecular damage, growth arrest and even death (Zhu 2001, 2003).

1.5.1 Ion Homeostasis

It is important to maintain or re-establish ion homeostasis in plants suffering from salt stress. Plants have evolved several mechanisms to cope with toxic levels of sodium ions such as restricting sodium uptake, increasing sodium extrusion, compartmentalizing sodium, controlling the transport of sodium into the shoot, and the recirculation of sodium from the shoots to the roots (Zhu, 2002; Munns, 2002). Re-establishing ion homeostasis in a saline environment is dependent on transmembrane transport proteins that mediate ion fluxes, including H^+ translocating ATPases and pyrophosphatases, Ca^{2+}-ATPases, secondary active transporters, and channels (Binzel et al., 2000; Bressan et al., 1995). Plasma membrane and vacuolar H^+-ATPases create a proton-motive force for compartmentalization of Na^+ into the apoplast and vacuole through Na^+/H^+ antiporter during salinity stress (Janicka-Russak and Kłobus, 2007), and thus aid the plants re-establishment of ion homeostasis (Sibole et al., 2005). Following salt stress in the halophyte *Tamarix hispida*, an increased number of H^+-ATPase family genes were expressed, suggesting an important role in re-establishing ion homeostasis (Wang et al., 2009). Over-expression of the Arabidopsis antiporter gene, *AtNHX1* increased salt tolerance (Apse et al., 1999)

suggesting that the enhanced ability of plants to compartmentalize Na+ in vacuoles improves salt tolerance.

Screening for *Arabidopsis* mutants with hypersensitivity to salt uncovered several gene loci and a group of genes, salt overly sensitive (*SOS*), which are involved in ion transport (Wu et al., 1996; Liu and Zhu, 1997; Zhu et al., 1998). Among the *SOS* genes, *SOS1* encodes a plasma membrane-localized Na^+/H^+ antiporter (Shi et al., 2000). SOS1 transports sodium ions out of the cell by coupling their transport to the downhill movement of H^+ from the external medium into the cell. Other SOS proteins include SOS2, a serine/threonine protein kinase and SOS3, a myristoylated calcium-binding protein, which shows sequence similarity with the B-subunit of calcineurin (type 2B protein phosphate) and with animal neuronal calcium sensors (Liu and Zhu, 1998; Guo et al., 2001). SOS1, SOS2, and SOS3 constitute the SOS pathway that is activated by salt stress. Salt stress elicits a cytosolic calcium signal (Knight et al., 1997) that is perceived by SOS3 (Ishitani M et al., 2000; Liu and Zhu, 1998). SOS3 interacts with and activates SOS2. The SOS2/SOS3 complex regulates the expression of *SOS1* (Shi et al., 2000) which transport Na^+ out of the cell. Mutations in *AtHKT1* suppressed the salt sensitive phenotype of *sos3* mutant. *AtHKT1* mediates sodium uptake (Rodriguez-Navarro, 2000; Rubio et al., 1999; Uozumi et al., 2000) and together, SOS2 and SOS3 may down regulate AtHKT1 activity to reduce Na^+ influx into the cell. SOS3 has also been shown to interact with non-membrane bound SOS2-like proteins (e.g. protein kinase, PKS), which mediate the induction of ABA biosynthesis. SOS2 and SOS3 may also participate in

controlling sodium uptake into the vacuole by activating vacuolar Na^+/H^+ antiporters (Shi et al., 2000).

1.6 Salt and Oxidative Stress

A consequence of salt-induced osmotic and ionic stress is an oxidative stress (Chinnusamy et al., 2005). Reactive oxygen species (ROS) such as superoxide ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and singlet oxygen are unavoidably generated in mitochondria, chloroplasts and peroxisomes as by-products of aerobic metabolic processes such as respiration and photosynthesis. The main sites of ROS production in leaves during salt stress are peroxisomes and chloroplasts. In the roots, which lack chloroplasts, the main sites of ROS production are plastids and mitochondria (Skutrik and Rychter, 2009). ROS are highly reactive and they inactivate enzymes, and damage cellular components. In the absence of a protective system, ROS can have damaging effects on proteins, DNA and lipids (Gechev and Hille 5005; Apel and Hirt 2004). However, increasing evidence indicates that the ROS generated during stress can also act as signalling molecules in the stress-response signal transduction pathway. ROS regulates signal transduction pathways by modulating the activity of ion channels (Neill et al., 2002).

ROS-scavenging mechanisms prevent or reduce the cytotoxic properties of ROS. ROS scavenging systems are classified as nonenzymatic or enzymatic antioxidants. The main nonezymatic antioxidants include ascorbate (APX) and glutathione (GSH), which directly or through the ascorbate-glutathione cycle (Halliwel-Asada cycle) scavenge ROS from the cell. Enzymatic ROS scavenging

systems include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) (Apel and Hirt, 2004). SODs are considered to be the first line of defense against ROS. SODs reduce superoxide to form H_2O_2 and O_2 (Apel and Hirt 2004). Catalases (CAT) in turn convert H_2O_2 to water and oxygen. Three classes of SODs have been identified according to the metal co-factor they utilize: iron SOD (Fe SOD), manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD) (Scandalios, 1993). Typically, Fe SODs are in the chloroplast, Mn SODs are in the mitochondrion and the peroxisome, and Cu-Zn SODs are located in the chloroplast and the cytosol (Alscher et al., 2002; Apel and Hirt, 2004).

Hydrogen peroxide is also converted into water by GPX and the ascorbate-glutathione cycle. In contrast to CAT, detoxification of ROS by the ascrobate-glutathione cycle and GPX necessitates the presence of ascorbate and the GSH regeneration system (Apel and Hirt, 2004). Components of the ascorbate-glutathione cycle are extensively in the cellular compartments where ROS detoxification is necessary, such as the chloroplasts, mitochondria and cytosol. This indicates that the cycle functions as a key factor to keep ROS generation under control (Yabuta et al., 2004).

1.6.1 Salt-induced ROS in chloroplasts

In the leaves of stressed plants, chloroplasts are associated with the production of ROS. ROS in the chloroplast arise due to decreases in intercellular CO_2 concentrations as a result of stomatal closure. The decreased CO_2 concentration reduces the efficiency of the Calvin cycle and results in insufficient

regeneration of $NADP^+$ (Miyake et al., 2005). A low level of the electron accepter $NADP^+$ for light driven electron flow initiates the reduction of O_2, which results in the formation of ROS. Hernandez et al. (1995) showed that salinity decreases chlorophyll content and PSII activity and at the same time increases the concentration of H_2O_2 in chloroplasts.

Antioxidant systems of the chloroplast keep the levels of ROS under control (Rios-Gonzalez et al., 2002). The ascorbate-glutathione cycle is the major ROS scavenging system in the chloroplast and its activity was increased under salinity stress (Møller 2001). Hernandez et al. (2001) observed an increase in the activity of cytosolic and chloroplastic Cu-Zn-SODII in pea plants exposed to 110-130 mM NaCl. The expression of Fe-*SOD* increased in the chloroplast of salt-treated *Lycopersicon pennellii* (Lpa) plants and this was accompanied by increased activity of ascorbate peroxidase and GST (glutathione-S-transferase) in the chloroplasts of salt-treated Lpa plants (Hernández et al., 2001; Mittova et al., 2004).

1.6.2 Salt-Induced ROS in Mitochondria

The electron transport chain (ETC) is the major site of ROS production in mitochondria. The ETC contains complexes I-IV, an alternate oxidase (AOX) and four NAD(P)H dehydrogenases, which are potential sites of ROS generation (Møller 2001). Damage to mitochondria is an early event under high saline conditions. Hamilton and Heckathorn (2001) showed that salt stress causes damage to mitochondrial electron transport in *Zea mays*. Salinity affects the mitochondrial ETC by damaging Complex I (NADH dehydrogenase) and II

(succinate dehydrogenase). Oxygen, as an electron acceptor, directly interacts with complex I to oxidize NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane (Møller 2001). The leakage of electrons from complex I under salt stress leads to the formation of ROS (Mittova et al., 2004). The accumulation of H_2O_2 in mitochondria coincides with the disintegration of the mitochondrial matrix and with the appearance of the first symptoms of leaf damage (Pellinen et al., 1999). Elevated levels of leaf mitochondrial H_2O_2 under salt stress was reported for pea and tomato plants (Dixit et al., 2002). Under salt stress, plants protect the mitochondrial electron transport chain with osmoprotectants (proline, betaine, and sucrose), antioxidants and small heat shock proteins (sHsps). Antioxidants and sHsps protect complex I, whereas complex II is protected by osmoprotectants. The osmoprotectants protect complex II either through balancing osmotic potential or by direct stabilization of membranes and/or proteins (Hamilton 2001).

The protective role of the root mitochondrial antioxidant system against oxidative stress has been studied less frequently than for the leaves (Mittova et al., 2004). The adaptation of salt tolerant *Lycopersicon pennellii* roots to oxidative stress resulted from salt-induction of mitochondrial SOD and APX (Mittova et al., 2004). Furthermore, the levels of ASC and GSH increased in the mitochondria of *L. pennellii* under salinity, which indicate that non-enzymatic mechanisms of H_2O_2 detoxification also contribute to decrease salt-induced H_2O_2 (Mittova et al., 2004).

1.6.3 Salt-Induced ROS in the Apoplast

Little is known about the generation of apoplastic ROS under salinity. Likely, the main enzyme generator of apoplastic ROS is the NADPH oxidase enzyme similar to that present in mammalian phagocytes (Apel and Hirt, 2004). The NADPH oxidase in tobacco cells is activated by cations (Cross et al., 1999; Kawano et al., 2001), which might account for how Na^+ could generate apoplastic ROS in salt stressed plants. Furthermore, application of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase significantly reduced the accumulation of superoxide and H_2O_2 under salt stress (Dixit et al., 2002) which provides additional evidence that NADPH oxidases generate ROS during salt stress.

ROS generated in the apoplast can induce necrotic lesions on plant leaves (Hernández et al., 2002). Salt-induced necrotic lesions (SINLs) are localized initially on the minor veins and are observed during the first stages of injury (Hernández et al.,2002). SINLs are reminiscent of the leaf “microbursts” observed in response to pathogens where H_2O_2 accumulates. Similar lesions appears in the vicinity of veins together with cell death and ROS accumulation has been observed in ozone exposed plants (Langebartels et al., 2002). Nitroblue tetrasolium (NBT) staining of SINLs of minor veins demonstrated that both O_2^- and H_2O_2 accumulated in the necrotic lesions. Thus, SINLs are a result of apoplastic ROS (Hernández et al., 2002). The presence of SOD, APX and catalase activities have been reported in the apoplast of both barley (*Hordeum vulgare*) and oat (*Avena sativa*) leaves. Leshem et al. (2006) demonstrated the intracellular production of ROS in salt-stressed roots that was mediated by

NADPH oxidase and shown to be important for salt tolerance. Further studies are needed to determine the mode of action of NADPH oxidase and ROS scavenging mechanisms in the apoplast during salt stress (Hernández et al., 2002).

1.7 **Salt stress-induced changes in gene expression**

Upon exposure to salt stress, plants undergo global changes in gene expression (Ozturk et al., 2002; Kreps et al., 2002). A number of genes have been reported to respond to salt stress and the proteins they encode play a role not only in protecting cells from salinity but also in the regulation of genes that are involved in signal transduction. Thus, these gene products can be classified into two groups. The first group includes proteins that function in stress tolerance: proteins involved in transport (e.g, aquaporins), proteins involved in ion homeostasis (e.g, ion transporters), enzymes required for the biosynthesis of various osmoprotectants (sugars, proline, and Gly-betaine), proteins that may protect macromolecules and membranes (late embryogenesis abundant or LEA proteins, osmotin, antifreeze poteins), proteases for protein turnover (thiol proteases, Clp protease, and ubiquitin) and the detoxification enzymes (catalase, superoxide dismutase, and ascorbate peroxidase) (Xiong and Zhu, 2002). For example, genes encoding LEA proteins are up-regulated by salt in roots of Arabidopsis (Kreps et al., 2002). Salt treatment induced genes encoding S-adenosylmethionine decarboxylase (SAMDC), phenylalanine ammonia-lyase (PAL) and mono-oxygenase in tomato roots (Ouyang et al., 2007). SAMDC is an essential enzyme for the biosynthesis of polyamines, which were reported to

have specific roles in salt tolerance (Roy and Wu, 2002). Increased expression of SAMDC under salt stress suggests that polyamine accumulation might help tomato plants to tolerate salt stress. PAL and mono-oxygenase may alleviate the damage from oxidative stress caused by salt stress (Lee et al., 2003).

The second group of genes encodes proteins involved in the regulation of signal transduction and gene expression: protein kinases, transcription factors, phospholipase and signaling molecules are included. For example, genes that encode protein phosphatase 2C and proteins involve in the MAP kinase signalling pathway were upregulated at the later stages of severe salt stress in tomato roots (Ouyang et al., 2007). In Arabidopsis, a MAP kinase signalling cascade regulating salt stress tolerance has been identified. Teige et al. (2004) demonstrated that MAP kinase kinase 2 (MKK2) was activated in reponse to salt stress and overexpressing *MKK2* resulted in elevated MAPK kinase activity and enhanced salt tolerance. The rice Ca^{2+}-dependent protein kinase (*OsCDPK7*) was induced in salt stressed roots and over-expression of *OsCDPK7* in transgenic rice conveyed an increased tolerance to salinity stress (Saijo et al., 2000).

1.7.1 ABA dependent and independent gene expression

ABA regulates many aspects of plant growth and development such as stomatal closure, embryo maturation, seed dormancy, germination, cell division and elongation, and responses to environmental stress including drought, hypoxia, heat, salt, cold, pathogen attack, and UV radiation (Finkelstein and Rock, 2002). Expression profiling in rice and Arabidopsis under salt stress

revealed that a number of genes induced by salt are also responsive to abscisic acid (ABA) (Kawasaki et al., 2001; Seki et al., 2001). Furthermore, the expression of some of the salt stress responsive genes were severely reduced in the ABA deficient mutant (*los5-1*), suggesting that salt induction of these genes is almost completely dependent on ABA (Xiong et al., 2001). However, less is understood about ABA and its involvement in the signaling cascades activated upon these stresses than is known regarding ABA-induced stomatal closure. There is also an overlap of genes induced by salt stress and other abiotic stresses (Kreps et al., 2002). For example, genes encoding Ca^{2+}-ATPase and peroxidase are induced by salt, drought and cold stresses.

Other studies using Arabidopsis ABA deficient (*aba1-1*) and insensitive (*abi1-1* and *abi2-1*) mutants have shown that the regulation of cold stress responsive genes is relatively independent of ABA whereas osmotic and salt stress regulated genes are activated by ABA-dependent and/or -independent pathways (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Drought-responsive genes contain several characterized *cis*-elements that coordinate their expression. The dehydration responsive element (DRE) mediates ABA-independent expression. The ABA-responsive element (ABRE) and MYC recognition sequence and/or MYB recognition motifs mediate ABA-dependent expression (Zhu, 2002).

The basic leucine zipper transcription factors referred to as ABRE-binding proteins (AREB) or ABRE-binding factors (ABF) can bind to the conserved ABRE (ACGTGG/TC) present in the promoters of many ABA-inducible genes to induce

ABA-dependent stress responsive gene expression (Uno et al., 2000; Choi et al., 2000; Fujita et al., 2005). MYC (*AtMYC2*) and MYB (*AtMYB2*) transcription factors bind to the *cis*-elements in the promoter of the ABA-respsonsive gene, *RD22* (*RESPONSIVE TO DESSICATION*), to activate gene expression (Abe at al., 1997; 2003). Studies on ABA-induced gene expression show an "early" transient change in gene expression that peaks around 3 hours and a "later" sustained response from 10 hours onward (Xiong et al., 2002). The "early" genes include those encoding members of the mitogen activated-protein kinase (MAPK) cascade, transcription factors, and others termed *EARLY RESPONSIVE TO DEHYDRATION* (*ERD*) genes. For example, *ERD10* is upregulated in response to exogenous ABA in *Brassica napus* (Deng et al., 2005). The "late" genes include those that encode proteins that are similar to the LEA proteins that accumulate during seed desiccation (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong and Zhu, 2001). LEA proteins are assumed to protect cellular or molecular structures from the damaging effects of dehydration stress such as hydration buffering, ion sequestration, protection of other proteins or membranes, or renaturation of unfolded proteins (Goyal et al., 2005). Other "late" genes encode proteases, chaperonins, enzymes of sugar or compatible solute metabolism, ion and water channel proteins, and enzymes responsible for ROS detoxification (Ingram and Bartels, 1996).

As mentioned before, ABA-independent, stress responsive gene expression is regulated through DRE *cis*-elements. AP2-type transcription factors, DREB2A and DREB2B bind to the DRE *cis*-element of stress responsive

genes (Liu et al., 1998). Many drought and cold responsive genes (e.g. *RESPONSIVE TO DESSICATION 29A, RD29A*) contain both DRE and ABRE elements in their promoters and are regulated in an ABA-dependent and ABA-independent manner (Narusaka et al., 2003). Salt stress responsive genes that are regulated in an ABA-independent manner include *KIN1*, *COR6.6*, and *COR47*, which all encode polypeptides related to the LEAs (Shinozaki and Yamaguchi-Shinozaki, 1997). Among them, the drought and cold inducible gene, *RD29A* from Arabidopsis was extensively analyzed by Yamaguchi-Shinozaki and Shinozaki, (1994). At least two separate regulatory systems function to control *RD29A* expression in response to drought, one is ABA independent and the other is ABA dependent. The 9-bp DRE is involved in the regulation of this gene by an ABA independent pathway induced by water deficit. Many drought and cold responsive genes contain both DRE and ABRE elements in their promoter and are regulated in an ABA-dependent and ABA-independent manner (Narusaka et al., 2003).

1.8 Research Background

In an attempt to characterize salt-induced changes in gene expression in tomato roots, Wei et al. (2000) examined salt stressed tomato (*Solanum lycopersicum* L.) root mRNA profiles by differential display-polymerase chain reaction (DD-PCR). One of the salt responsive cDNAs detected in tomato roots showed nucleotide sequence identity with an α-*DIOXYGENASE* (α-*DOX*), previously reported in tobacco as a pathogen-induced oxygenase (PIOX) (Sanz et al., 1998). In tobacco leaves, *PIOX* or α-*DOX1* expression is induced by

bacterial infection. Alpha-DOX catalyses the first step of α-oxidation of fatty acids in plants (Hamberg et al., 1999). To date, *α-DOX* genes have been identified in many plant species, including Arabidopsis, pea, rice and cucumber.

1.8.1 Fatty acid α-dioxygenases

Lipoxygenases (LOX) were the first enzymes known to catalyze the primary oxygenation of polyunsaturated fatty acids in plant tissues and the resulting reactive hydroperoxides are further metabolized into an array of oxygenated fatty acids (oxylipins) (Hamberg et al., 2002). It was reported in 1998, that tobacco leaves infected with the bacterium *Pseudomonas syringae* accumulate a 75 kDa oxygenase named PIOX, later known as "α-dioxygenase", showing identity to mammalian prostaglandin endoperoxide synthases-1 and -2, which are also referred to as cyclooxygenase (COX). COX is a key enzyme that mediates the conversion of polyunsaturated substrates, such as arachidonic acid, to prostaglandins or leukotrienes in mammals (Nelson and Seitz, 1994). Prostaglandins serve as intracellular and/or extracellular lipid-derived signals and mediate many cellular responses in mammalian species, such as the immune response, fever, pain and inflammation. The similarity between α-DOX and COX suggested that tobacco α-DOX and a very similar oxygenase from Arabidopsis (α-DOX1) catalyzed a non-lipoxygenase type of fatty acid dioxygenation, specifically, stereospecific oxygenation at the α-carbon of the fatty acid chain to provide unstable 2-hydroperoxy fatty acids, a new class of oxylipins. In mammals, this type of fatty acid α-oxidation is of critical importance for the degradation of phytanic acid (3, 7, 11, 15-tetramethyl hexadecanoic acid) and

other β-methyl branched fatty acids (Verhoeven et al., 1998); however, the function of the corresponding pathway in plants is not fully understood.

Database searches have identified several plant proteins showing significant similarity to tobacco α-DOX1. The amino acid sequence of tobacco α-DOX1 shares identity with the proteins encoded by α-DOX cDNAs from *Nicotiana attenuata*, *Capsicum annuum*, *Solanum lycopersicum*, *Arabidopsis thaliana*, *Pisum sativum* and *Oryza sativa*. The amino acid identity between tobacco α-DOX1 protein and α-DOX from the above mentioned plants is between 73%-95% (Hamberg et al., 2005).

1.8.1.1 Catalytic properties of α-dioxygenase

α-DOX are heme proteins with conserved catalytic domains among plants. Amino acid residues involved in heme-binding (His-167 and His-397) and initiation of the oxygenation reaction in tobacco (Tyr-389) are conserved in all plant α-DOX (Sanz et al., 1998). The results from the study of rice α-DOX catalytic activity have shown that Arg-559 is required for high affinity binding of the fatty acid substrate to α-DOX and His-311 is involved in optimally aligning carbon-2 of the fatty acid below Tyr-379 for catalysis (Koszelack-Rosenblum, 2008). Incubation of linolenic acid with the α-DOX1 enzyme from tobacco or Arabidopsis led to the formation of 2(R)-hydroperoxylinolenic acid (hydroperoxide), which is unstable and decarboxylates to provide 8(Z),11(Z),14(Z)-heptadecatrienal (fatty aldehyde) as a major product (Hamberg et al., 1999). In addition, small amounts of 2(R)-hydroxylinolenic acid (2-hydroxy fatty acid) and 8(Z),11(Z),14(Z)-heptadecatrienoic acid (chain shortened fatty

acid) were produced (Hamberg et al., 1999, Figure 1-1). Other fatty acids, including linoleic, oleic and palmitic acid can act as substrates for α-DOX1 in Arabidopsis. This resembles the profile encountered previously in studies of α-oxidation in plant tissues (Hamberg et al., 1999). α-oxidation was first characterized by Stumpf (1956), who found that a preparation from peanut cotelydons catalyzed the oxidation of palmitic acid into a shorter chain fatty aldehyde with the liberation of CO_2. Subsequent studies in preparations of pea leaves (Hitchock and James, 1966), cucumber (Galliard and Mattew, 1976; Baardseth et al., 1987; Anderson et al., 1997), potato (Laties and Hoelle, 1967) and the green alga *Ulva pertusa* (Kajiwara et al., 1988) have identified the α-oxidation of various C_n fatty acids into C_{n-1} aldehydes together with varying amounts of of C_n-hydroxy acids and C_{n-1} fatty acids. It has been suggested that the α- oxidation enzymes operate together with aldehyde dehydrogenase and NAD^+ to provide a pathway for the stepwise degradation of fatty acids into shorter chain fatty acids (Galliard, 1980). The presence of two subunits in a purified α-DOX enzyme was reported in germinating pea, *Pisum sativum* (Saffert, et al., 2000). The purified enzyme with fatty acid α-oxidation activity had a molecular mass of 230 kDa, and analysis of the denatured enzyme by SDS-PAGE revealed the presence of two subunits, a 50-kDa enzyme identical to a turgor-responsive NAD+ aldehyde dehydrogenase (ALD) from pea and a 70-kDa enzyme showing similarity to α-DOX1 from tobacco and Arabidopsis. This suggests that α-DOX works along with ALD in the stepwise degradation of fatty acids in plants.

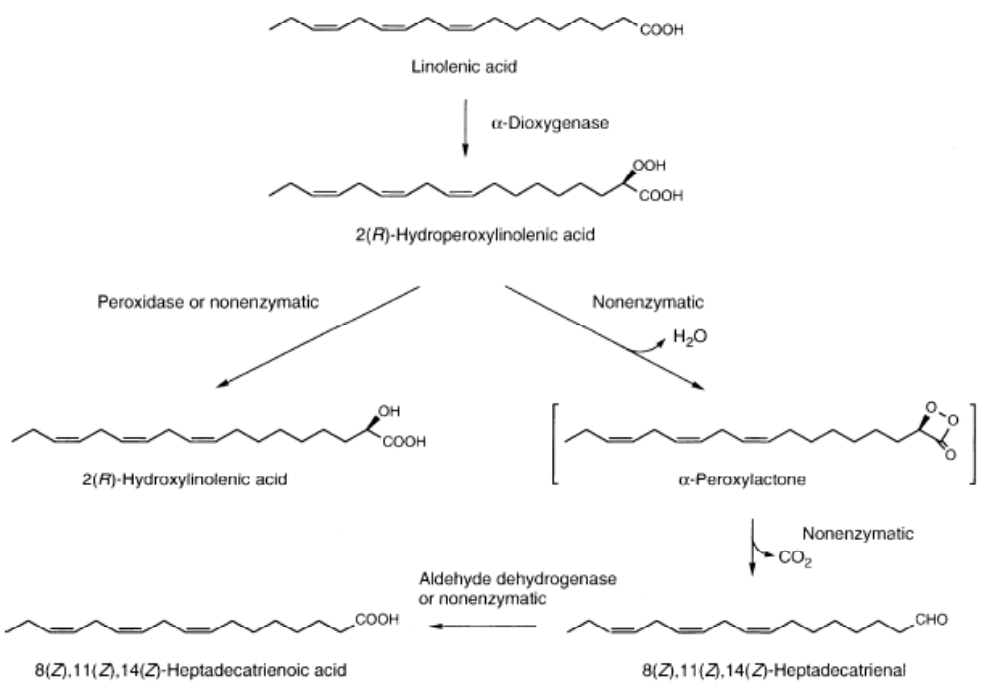


Figure 1-1 α-Dioxygenase catalyzed oxygenation of linolenic acid.

From Hamberg et al., (2002). Fatty acid α-dioxygenases, Prostaglandins & other lipid mediators, 68-69, 363-374.

1.8.1.2 Biological roles of α-dioxygenase

Studies with tobacco and Arabidopsis α-DOX1 demonstrated the participation of this enzyme in plant defense against microbial infection (Hamberg et al., 2003; Ponce de León, 2002). In both plant species, *α-DOX1* expression was activated in leaves exhibiting the hypersensitive reaction (HR) as a result of bacterial pathogens. The HR involves programmed cell death at the point of pathogen invasion that results, in part, from an oxidative burst. Studies on the cellular signals mediating the regulation of *α-DOX1* in Arabidopsis revealed that gene expression is induced by salicylic acid (SA), intracellular superoxide (O_2^-) or singlet oxygen and nitric oxide (NO). Ion leakage studies with transgenic Arabidopsis lines with altered α-DOX1 activity suggested a protective role for α-DOX against oxidative stress (Ponce de León et al., 2002). In addition to plant defence against microbial infection, results with *Nicotiana attenuata* indicated that the enzyme might play a role in the response of plants to insect attack (Hermsmeier et al., 2001). In a manner similar to COXs and LOXs, α-DOX1 may be involved in the synthesis of lipid-derived signal molecules that mediate the plant response to pathogen infection. In addition, a role in the metabolism of polyunsaturated fatty acids released during membrane damage is also consistent with the expression of *α-DOX1* in pathogen infected and wounded tissues.

A second *α-DOX*-like sequence is present in the Arabidopsis genome. An Amino acid alignment analyses revealed that the second α-DOX-like protein is more similar to the tomato FEEBLY protein than to either Arabidopsis α-DOX1 or to Tomato α-DOX1 (Hamberg et al., 2005). This suggests the presence of a

distinct α-DOX isoform that has been designated α-DOX2. The expression of *α-DOX2* differs significantly from that described for *α-DOX1*. *α-DOX2* expression was not induced in response to microbial infection but was weakly activated by mechanical damage (Hamberg et al., 2005). A high level of the *α-DOX2* transcript was detected in leaves subjected to artificial senescence by leaf detachment (Hamberg et al., 2005). The increased level of *α-DOX2* expression in detached leaves might signify participation in protection mechanisms activated during senescence to control cell disruption, a critical process to allow appropriate remobilization and redistribution of nutrients to the growing parts of the plant (Hamberg et al., 2005). Mutations at the *α-DOX2* locus in tomato resulted in the *feebly* mutant, which showed a strong phenotypic alteration of seedlings that developed into small fragile plants (Van der Biezen et al., 1996). However, characterization of an *α-DOX2* T-DNA insertional mutant revealed that the absence of this enzyme does not produce any apparent developmental alterations in Arabidopsis (Hamberg et al., 2005). Therefore, there may be significant differences between the functions of tomato and Arabidopsis α-DOX2 enzymes (Hamberg et al., 2005).

1.8.1.3 α-DOX in salt-stressed tomatoes

Almost all of the studies on α-DOX have focussed on leaves or seedlings responding to biotic stress. The only report of *α-DOX* that has concerned roots has been its constitutive expression in roots (Ponce de León et al., 2002; Meisner et al., 2008). In tomato, α-DOX was isolated as a salt-responsive gene that is expressed in roots (Wei et al., 2000). Tirajoh et al. (2005) confirmed the

salt-responsive nature of *α-DOX1* expression in tomato roots and went on to demonstrate that the expression of *α-DOX1* in roots is also up-regulated by ABA and ethylene (Tirajoh et al., 2005). Roots are important organs for carrying water and mineral nutrients to the rest of the plant and are the primary site of perception and injury for damage caused by salinity stress (Jiang and Deyholos, 2006). Therefore, studies of gene expression in roots are important to understand the molecular mechanism that confers the ability to tolerate salt stress in plants.

1.9 Research objectives

When I initiated my research, very little was known regarding α-DOX in tomato and nothing was known about the function of α-DOX in plants responding to salt stress. Therefore, my first objective was to determine whether *α-DOX1* was responsive to biotic stresses, especially pathogen and wounding in tomato roots. My second and major objective was to investigate the role played by α-DOX in salt stressed plants. Arabidopsis was used to accomplish the second objective because of the availability of genome information, hormone mutants, T-DNA insertional mutants and ease of genetic transformation. However, to use Arabidopsis it was necessary to examine the expression of *α-DOX* in roots and shoots of salt stressed plants to determine whether *α-DOX* was regulated by salt in Arabidopsis. In tomato, α-DOX is regulated by ABA and ethylene and these hormones interact to regulate α-DOX expression in roots. Therefore, I explored the hormone signals that regulate *α-DOX* expression in salt stressed Arabidopsis. Finally, using forward and reverse genetic approaches, I assessed the role of α-

DOX in roots and shoots of salt stressed plants. My research was guided by the following four hypotheses:

1. **Pathogen challenge and wounding up-regulate *α-DOX1* expression in tomato roots.**
2. **Salt stress alters the expression *α-DOX1* and *α-DOX2* in Arabidopsis.**
3. **More than one hormone regulates *α-DOX* expression in Arabidopsis.**
4. **α-DOX plays a role in salt tolerance.**

The objectives of the research to test the above hypotheses were to examine:

1. The expression of *α-DOX1* in tomato roots challenged with *Pythium aphanidermatum* (fungal pathogen) or wounding.
2. The spatial and temporal expression of *α-DOX1* and *α-DOX2* in Arabidopsis.
3. *α-DOX* expression and enzyme activity in salt-stressed roots and shoots of Arabidopsis.
4. The expression of *α-DOX1* and *α-DOX2* in hormone treated Arabidopsis plants and hormone deficient mutants.
5. The salt tolerance of transgenic lines over-expressing *α-DOX1* or *α-DOX2* and in knockout *α-DOX* mutants.
6. The extent of ROS production and scavenging in wild-type and in transgenic lines over-expressing *α-DOX1* or *α-DOX2* and in knockout *α-DOX* mutants.

7. The effect of salt on the growth and production of lateral roots in Arabidopsis over-expressing α-*DOX1* or α-*DOX2* and in α-*DOX* knockout.

2: EXPRESSION OF ALPHA-DOX IN TOMATO AND ARABIDOPSIS RESPONDING TO ENVIRONMENTAL STRESS

2.1 Introduction

Roots play a number of important roles during plant growth and development and are the first organs to encounter soil salinity, a major stress that reduces crop productivity. Salt affected roots undergo physiological and metabolic changes, which are driven by changes in gene expression. These genes encode products involved in co-ordinating changes in plant metabolism, re-establishing ion homeostasis, protection against the damaging effects of excess ions, dehydration and ROS, repair of cellular components, as well as signal transduction and gene regulation (Zhu, 2001). In tomato roots, salt treatment resulted in the altered synthesis and accumulation of a number of prominent polypeptides and subsequently, using differential display-polymerase chain reaction (DD-PCR), a number of cDNA clones corresponding to salt-responsive genes were identified (Chen and Plant, 1999; Jin et al., 2000; Wei et al., 2000). These salt-responsive genes encoded a laccase, a potential helicase, a protein with homology to a mitotic control protein of yeast and α-dioxygenase (α-DOX) (Wei et al., 2000). Prior to this research, α-DOX encoding genes had been identified in tobacco (*Nicotiana tabacum*) leaves responding to bacterial infection (Sanz et al., 1998). Later studies revealed that *α-DOX* expression is up-regulated by caterpillar herbivory, wounding, UV-B radiation, and the application

of jasmonic acid (JA), salicylic acid (SA), and chemicals that elicit the production of reactive oxygen species (ROS) (Hermsmeier et al., 2001; Izaguirre et al., 2003).

To date, *α-DOX* genes have been identified in several plant species including *Arabidopsis thaliana*, *Pisum sativum*, *Capsicum annum*, *Nicotiana attenuata* and *Oryza sativa* (Hamberg et al., 2005). The amino acid sequence of the polypeptide encoded by tomato *α-DOX* (*Leα-DOX1*) shares high similarity with α-DOX from *Nicotiana attenuata* (85% identity) and *N. tabacum* (84% identity). There are two *α-DOX* genes in Arabidopsis, *Atα-DOX1* and *Atα-DOX2* (Tirajoh et al., 2005), whereas in tomato, α-DOX related ESTs define a small gene family comprised of at least three members (Tirajoh et al., 2005). Of the three members only one, *Leα-DOX1,* was responsive to salt (Tirajoh et al., 2005). *Atα-DOX1* expression is induced by bacterial infection, SA, ROS and nitric oxide (NO). The expression of the *AtαDOX2* gene differs significantly from that of *Atα-DOX1* since its expression was not induced in response to microbial infection but was weakly activated by mechanical damage (Hamberg et al., 2005). In terms of its amino acid sequence, *Atα-DOX1* is more similar to *Leα-DOX1* and -2 whereas *Atα-DOX2* is more similar to *Leα-DOX3* and *Leα-DOX3* is more similar to *Atα-DOX2* than to *Leα-DOX1* and *Leα-DOX2* (Tirajoh et al., 2005). Thus, the *Atα-DOX1* and *Atα-DOX2* encoded proteins may represent two distinct isoforms possibly possessing different or overlapping functions.

In this chapter, research is presented that explores whether α-DOX expression in tomato was regulated by both biotic and abiotic stresses.

Inaddition, *Leα-DOX1* is salt responsive, the possible roles of α-DOX in salt stressed plants is unknown and no other report for salt-responsive α-DOX expression is known. Functional analyses of α-DOX was undertaken in Arabidopsis, which has two isoforms rather than the three or more in tomato. In addition, Arabidopsis was an ideal candidate to study the role of α-DOX in salt stressed plants due to the availability of resources such as a complete genome sequence and mutants. However, in order to use Arabidopsis for functional analyses of α-DOX in salt stressed plants, I first had to establish that *Atα-DOX* genes are regulated by salt.

Hypothesis 1. Wounding and pathogen challenge up-regulate *Leα-DOX* expression in tomato roots.

To test this hypothesis I undertook the following experiments:

1. Northern blot analysis of *Leα-DOX* expression in wounded and pathogen challenged roots of tomato.

Hypothesis 2. Salt up-regulates *Atα-DOX1* and *Atα-DOX2* expression in Arabidopsis roots and shoots

To test this hypothesis I undertook the following experiment:

1. Northern blot analysis of *Atα-DOX1* and *Atα-DOX2* in roots and shoots of salt stressed Arabidopsis plants.

Hypothesis 3. *Atα-DOX1* and *Atα-DOX2* are expressed in different organs during plant development.

To test this hypothesis I undertook the following experiments:

1. Generation of transgenic Arabidopsis plants harbouring *Atα-DOX1* or *Atα-DOX2* promoter::GUS fusion proteins.
2. Histochemical staining for GUS activity in Arabidopsis plants harbouring *Atα-DOX1* or *Atα-DOX2* promoter::GUS fusion proteins.

2.2 Materials and Methods

2.2.1 Experimental treatments

2.2.1.1 Tomato

Tomato seeds (*Solanum lycopersicum* L. cv Ailsa Craig [AC], wild type) were surface sterilized by wrapping the seeds in a plastic mesh, securing with string, and then rinsing in 70% ethanol for 30 seconds under vacuum. This was followed by a one-minute rinse in sterile double-distilled water under vacuum. Afterwards the seeds were transferred to 10% commercial Javex bleach for 3 minutes under vacuum. Thereafter, the seeds were transferred to sterile double-distilled water for one minute under vacuum. The seeds were then placed in sterile double-distilled water for five minutes and this last step was repeated.

Approximately 35 sterilized tomato seeds were individually sown into a plastic grid lined with a plastic mesh containing sterilized moistened vermiculite. There were 35 (1 x 1 cm) compartments in the grid and one seed was placed in each compartment. Up to six grids were placed in a 7 L plastic tray filled with double-distilled water. Once the roots emerged, aeration was provided and the water was replaced with a solution containing 2/3-strength Murashige and Skoog

(MS) solution (Murashig and Skoog, 1962). The MS solution was changed weekly until the plants were ready to be treated at six weeks. The plants were kept in a growth chamber (Conviron Basic Model 125L Incubator) in the light (120 µmol/s/m^2) for 16 hours at 25 ^oC and in the dark for 8 hours at 21 ^oC with 70% relative humidity.

Six-week-old plants were used for all experiments. MS solutions were changed 24 hours prior to the experimental treatments. Salt treatment was imposed by the addition of NaCl to the nutrient solution to a final concentration of 170 mM. Wounding was conducted by crushing along the whole length of the roots with forceps. Pathogen challenge was carried out by adding mycelia fragments of *Pythium aphanidermatum* (Edson) Fitzp. (400,000 L^{-1}) to the nutrient solution. Root and shoot tissue was collected after 24 h, frozen and stored at -80 ^oC.

2.2.1.2 Arabidopsis

Seeds of wild type *Arabidopsis thaliana* (Col 4) were gas-sterilized with NaClO and HCl in an airtight container for 2 hr. Approximately 80 seeds were individually sown on sterilized 3 MM paper set in a magenta box with 50 mL of 1/2 MS solution supplemented with 1.5% (w/v) sucrose (Figure 2-1). The boxes were incubated at 4 °C for 3 days for stratification and then transferred to a growth chamber and incubated at 23 °C under a 16-h/8-h light/dark regime. Once the roots emerged, the magenta boxes were placed on an orbital shaker so that the media was constantly agitated (80 rpm) for aeration. The plants were grown for 3 weeks.

Figure 2-1 The experimental system used in this study.

Arabidopsis seeds were individually sowed onto sterilized 3MM paper set up in a magenta box containing ½ MS+1.5% sucrose. Upon germination, the roots grew along the paper (arrows) towards the media.

To impose a salt stress, the ½ MS solution was replaced with ½ MS media with salt. Varying concentrations (50, 100, 150 and 250 mM) of NaCl were applied to identify the concentration of salt that induced *α-DOX* expression. Time course experiments were carried out by applying 250 mM NaCl to the growing media and harvesting after 0.5 h, 2 h, 8 h and 24 h. For control treatments, the ½ MS solution was replaced with fresh ½ MS solution without salt. After harvesting, root and shoot tissues were collected, frozen and stored at -80 ^{o}C.

2.2.2 RNA isolation

2.2.2.1 Tomato

Total RNA was extracted using a LiCl-phenol method (Prescott and Martin, 1987). All procedures were carried out in an RNAse free environment by using glassware or ceramic ware that was baked overnight at 180 ^{o}C, sterile RNAse-free plastic, or solutions that were treated with 0.1% diethyl pyrocarbonate (DEPC) [Sigma-Aldrich, Oakville, Ontario, Canada].

Frozen tomato roots were ground to a fine powder with liquid nitrogen using a ceramic mortar and pestle. Approximately 10 mL of RNA extraction buffer (50 mM Tris-HCl pH 9, 150 mM lithium chloride [LiCl], 5 mM ethylenediamine tetraacetic acid pH 8 [EDTA], and 5% sodium dodecyl sulfate [SDS]) was added per 5 grams (FW) root tissue and ground until an ice-cream-like consistency was obtained. Liquid nitrogen was also added to the mixture to ensure that the sample remained frozen. Afterwards, an equal volume of phenol/chloroform (50:50) was added and the mixture was stirred. The homogenate was transferred to an RNAse-free 50 mL conical Falcon^{TM} tube.

The sample was vortexed, placed on ice for 5 minutes, and centrifuged at 4500 rpm (Sorvall P11788 bucket, SH3000X rotor) for 30 minutes at 4 ^{o}C (Sorvall RC-6 centrifuge).

The top aqueous layer was removed and transferred to a new 50 mL conical Falcon^{TM} tube. An equal volume of phenol/chloroform was added to the sample, vortexed, and centrifuged at 4500 rpm for 30 minutes at 4 ^{o}C. The top aqueous layer was removed and transferred to a new 50 mL conical Falcon^{TM} tube. An equal volume of chloroform isoamyl alcohol (24:1) was added to the sample, vortexed, and placed on ice for 5 minutes. Thereafter, the sample was centrifuged at 4500 rpm for 30 minutes at 4.0 ^{o}C. The top layer was removed and transferred to an RNase-free 15 mL conical Falcon^{TM} tube. An appropriate amount of 8 M LiCl was added to the solution to obtain a final concentration of 2 M LiCl. The sample was then placed at -20 ^{o}C overnight.

The following day, the samples were thawed on ice, vortexed gently, and centrifuged at 4500 rpm for 45 minutes. One hundred microlitres (1/20 volume) of 3 M sodium acetate (NaAc) pH 5.2 was added to the supernatant solution and mixed gently. Five millilitres or 2.5 volumes of 99% ethanol was then added, mixed and the solution was placed at -80 ^{o}C for 2 hours. Thereafter, the sample was centrifuged at 4500 rpm for 45 minutes at 4 ^{o}C. The solution was decanted and the RNA pellet was washed with 2 mL 70% ethanol and centrifuged at 4500 rpm for 20 minutes at 4 ^{o} C. The supernatant was decanted and the RNA pellet was air-dried.

The RNA pellet was dissolved in 100 µL DEPC-treated water and transferred to an RNAse free 1.5 mL microcentrifuge tube and stored at -80 ^{o}C. To measure the concentration of RNA, a 1:100 dilution of RNA was prepared in 1X TE (10 mM Tris, 1 mM EDTA; pH 7) and RNA concentration was quantified with a spectrophotometer (Bio-Rad SmartSpec^{TM} 3000, Ontario, Canada) at 260 nm and 280 nm.

2.2.2.2 Arabidopsis

Total RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON). Frozen tissues (300 mg) were ground to a fine powder in liquid nitrogen. One millilitre of Trizol was added per 100 mg (FW) of sample, and ground until an ice-cream-like consistency was obtained. The ground tissue was transferred to a 15 mL tube and mixed thoroughly by vortexing. The mixture was incubated at 65 °C for 5 minutes followed by centrifugation at 5000 rpm in a SS34 SORVALL rotor for 40 min at 4 °C. Following centrifugation, the top aqueous layer was removed and transferred to a new 15 mL tube. Chloroform (0.2 mL $CHCl_3$/mL Trizol) was added to the supernatant, mixed thoroughly, and then centrifuged as before. Following the second centrifugation, the top layer was removed and transferred to a new 15 mL tube. A half volume of cold isopropanol and a half volume of 0.8 M Na_3-citrate was added, mixed thoroughly, followed by a 10 min incubation at room temperature. The sample was centrifuged as before for 40 min at 4 °C. Following centrifugation, the supernatant was discarded, and 70% ethanol was added to the pellet and the sample was centrifuged at the same speed as before for 30 min. The pellet was briefly air-dried and dissolved in 100 µL DEPC-ddH_2O

and transferred to a 1.5 mL microfuge tube. The concentration of RNA was measured using a 1:100 dilution of the RNA in ddH_2O in a spectrophotometer (BIO-RAD SmartSpec™ 3000, Mississauga, Ontario) at 260 nm and 280 nm.

2.2.3 Northern hybridization analyses

Fifteen micrograms total RNA was separated by gel electrophoresis in a formaldehyde denaturing 1.2% (w/v) agarose gel containing 2.2 M formaldehyde and 1X MOP (20 mM 3-(N-morphino) propanesulphonic acid (MOPS), 5 mM sodium acetate pH 7, 1 mM EDTA). RNA was added to 1X MOPS buffer, 2.2 M de-ionized formaldehyde and 50% de-ionized formamide and heated at 65 ^{o}C for 15 min and transferred to ice. Two µL RNA loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol, 1.0 mM EDTA pH 8 and 0.5 µg/mL ethidium bromide) was added to the RNA solution. The gel was run at 65 V for 2 hours in 1X MOPS running buffer.

Following electrophoresis, the RNA gel was photographed under UV illumination rinsed in DEPC-treated double-distilled water prior to soaking for 5 min in 20X SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7). A positively charged nylon membrane (Amersham Hybond™-N+, Oakville. Ontario) was soaked briefly in DEPC-treated double-distilled water, followed by a 5 minute soak in 20X SSC. RNA was capillary blotted overnight in 20X SSC as described by Sambrook et al. (1989). Thereafter, the nylon membrane was soaked in 6X SSC for 5 min and air-dried. RNA was fixed to the membrane by exposing to UV light using the auto crosslink setting on the UV Stratalinker^{TM} 2400 (Statagene) for 30 seconds followed by baking at 80 ^{o}C for 30 min. The membrane was pre-

hybridized at 65 ^{o}C for 2.5 hours in a rotary hybridization oven (Techne Hybridiser HB-2D, Mandel, Ontario, Canada) in 10 mL of pre-hybridization buffer (0.25 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA (bovine serum albumin)) containing denatured salmon sperm DNA (100 µg/mL). The radiolabelled probe (section 2.2.3.1) was added directly to the pre-hybridization buffer and hybridization proceeded overnight at 65^{o}C.

Following hybridization, the membrane was washed in 10 mL 2X SSC, 0.1% SDS at room temperature for 5 min and this process was repeated twice. The membrane was washed with 1X SSC, 0.1% SDS at 65 ^{o}C for 45 min after which this step was repeated at 68 ^{o}C. Lastly, the membrane was washed in 0.5% SSC, 0.1%SDS at 65 ^{o}C for 30 min. The washed membrane was air dried briefly and wrapped with a layer of Saran^{TM} wrap before exposure to X-ray film (Kodak Scientific Imaging Film X-Omat Blue XB1, NEN^{TM} Life Science, Boston, Massachusetts, USA) with an intensifying screen at -80 ^{o}C for varying time periods (between 24 hours to 2 weeks). For *Leα-DOX* expression, band intensity from autoradiography films was determined with Scion Image Beta 4.0.3 software using macrofunction gel plot 2 (Scion Corporation, MD, USA). Expression data from Scion Image was normalized by dividing the hybridization signal for the cDNA probe by that of the 18S rRNA probe.

2.2.3.1 Preparation of hybridization probes

The *Leα-DOX1* full-length cDNA was prepared by PCR using a clone containing the *Leα-DOX1* cDNA, cLEW8G12 (Clemson University Genomic Institute), as a template and the T3 and T7 universal primers. The 5' probes of

Leα-DOX1 and Leα-DOX2 were generated utilizing the following forward and reverse primers: 5'-TAT CTT GAA GCA CGG CGG AG-3' and 5'-CTA AAG GAC TTG AGT GGG-3' or 5'-CAA AAT GAA TCT CCG CGA CA-3' and 5'-TCC GGT AGG AGT TTC TTT TGA T-3', respectively. Thermal cycling was carried out in a Thermo Hybaid MBS 0.2G thermal cycler programmed to complete one cycle of 95 °C for 2 min, then 35 cycles of 95 °C for 30 sec, 55 °C for 30 seconds, 72 °C for 2 min followed by one cycle of 72 °C for 5 min.

Total RNA extracted from 3-week-old Arabidopsis roots exposed to 250 mM salt for 24 h was used for RT-PCR to isolate the cDNA corresponding to *Atα-DOX1*. RNA (2.5 µg) was reverse-transcribed by M-MuLV reverse transcriptase (Fermentas, Burlington, ON) from an anchored oligo-dT primer using standard methods in a reaction volume of 20 µL. Each reverse transcription (RT) reaction contained 2.5 µg RNA, 20 U RNase inhibitor (Fermentas), 20 U reverse transcriptase (Fermentas), 10 mM reaction buffer, 10 µM oligo-dT and 20 µM each dNTP and was incubated at 70 °C for 5 min followed by 42 °C for 90 min. The 5' region of the *Atα-DOX1* cDNA was amplified using specific (5'-GAATGTCCCTCCTCGATGCC-3') forward and (5'-GGAGCTTAAGGGACACTTG-3') reverse primers. The cDNA clone U16142, containing the *α-DOX2* ORF (Gen Bank accession AY128743) was obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus, OH. The 5' region of the *Atα-DOX2* ORF was amplified using forward (5'-ATGTCGGTCCGATCACGGTC-3') and reverse (5'-CAAGGGGTTCTAGTGTTGAC-3') primes. Each PCR reaction contained 0.2 µM

of each primer, 10 µM dNTP mixture, 1.25 mM $MgCl_2$, 2 U Taq DNA polymerase, supplied with its own buffer (Fermentas) and 20 ng template cDNA. The PCR conditions were identical to conditions described above with the exception of an annealing temperature of 58 °C. After PCR the products were separated in a 1% agarose gel in 1X TAE (0.04 M Tris-Acetate, 0.01 M EDTA (pH 8.0)) and bands of the expected size were excised and purified using the QIAquick PCR purification kit (QIAGEN, Mississauga, ON) according to the manufacturer's instructions.

Atα-DOX1 and *Atα-DOX2* specific probes were prepared by mixing 20 ng DNA in approximately 2.0 µL double-distilled water, 4.5 µL 10X TE, and sterile water to a total volume of 45 µL in a 1.5 mL microcentrifuge tube. DNA was denatured by placing in boiling water for 3 min and transferred to ice afterwards. The DNA mixture was then transferred to a reaction mix (Rediprime II™ DNA Labeling System or Ready-To-Go™ DNA Labeling Beads (-dCTP), GE Healthcare, Quebec, Canada) and 5 µL α^{-32}P dCTP (3000 Ci/mmol, NEN, Perkin Elmer Life Sciences, Markham, Ontario, Canada) was added and incubated at 37 ^{o}C for at least 15 min. The mixture was transferred to a pre-spun micro spin column (MicroSpin™ S-200 HR Columns, GE Healthcare, Quebec, Canada) and then centrifuged at 3000 rpm for 3 min. This step removes any unincorporated α^{-32}P dCTP. The specific activity of the sample was measured using a scintillation counter and probes with an activity of 10^9 cpm/µg were used for hybridization. The probe was denatured by placing on a heat block set to 95 ^{o}C for 4 min and

then transferred to ice for 4 min. The probe was then added to the pre-hybridization buffer (1 x 10^6 cpm probe/mL buffer).

2.2.4 Amino acid sequence alignment of α-DOX polypeptide from tomato and Arabidopsis

Amino acid sequences for Leα-DOX1 (AAR05646), Leα-DOX2 (AAR05647), Leα-DOX3 (DAA01542), Atα-DOX1 (NP_186791) and Atα-DOX2 (NP_186791) were acquired from the National Centre for Biotechnology Information (NCBI). Multiple alignments were performed using CLUSTALW (http://www2.ebi.ac.uk/clustalw/) and Mac Vector 7.1.1 using a 10.0 open gap penalty, 40% delay divergent and Blosum similarity matrix.

2.2.5 Extraction of genomic DNA from Arabidopsis plants

Genomic DNA was extracted from rosette leaves of three week old plants. Leaves were ground in microfuge tubes with plastic pestles in 400 µL DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The mixture was centrifuged at room temperature for 5 min at 13000 rpm. The supernatant was mixed with an equal volume of isopropanol and incubated at room temperature for 5 min. The mixture was centrifuged as above to pellet genomic DNA. The supernatant was discarded, the pellet air-dried and then resuspended in 100 µL of 1XTE (10 mM Tris HCl, 1 mM EDTA) buffer (pH 8.0).

2.2.6 Generation of constructs carrying the promoter of *Atα-DOX1* and *Atα-DOX2*

The 2 Kb upstream sequences of the *Atα-DOX1* and *Atα-DOX2* genes (5'-flanking sequences that govern gene expression or promoter regions) were amplified by PCR with the following primers:

5'-CACCTGGTGCGTTTGGTTGGATCG-3' (DOX1 pro forward),

5'-CATTCTGTTAAATTTTTTCTTGGGG-3' (DOX1 pro reverse),

5'-CACCATTCCTTACAATTTGATACG-3' (DOX2 pro forward),

5'-GCCTTGATTTTTTTTTGTCTCC-3' (DOX2 pro reverse) using genomic DNA as a template.

PCR conditions were similar to that described in Section 2.2.3.1 with the exception of the annealing temperature of 60 °C. The amplified promoter regions were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen) following the manufacturer's instructions. *Escherichia coli* TOP10 (Invitrogen) was then transformed with the plasmid DNA by the heat-shock method whereby the reaction was incubated on ice for 30 min and subsequently heat-shocked in a water bath for 30 seconds at 42 °C without shaking. The heat shocked cells were immediately transferred to ice, followed by addition of 250 µL LB medium. The cultures were incubated at 37 °C for 2 h with shaking (250 rpm). Aliquots of bacterial culture (50-100 µL) were spread on pre-warmed agar plates containing 50 µg/mL kanamycin, and incubated at 37 °C overnight. Plasmid DNA was isolated using the Qiagen plasmid purification kit following the manufacturer's instructions (Qiagen, Missisauga, Ontario, Canada). Positive clones were

confirmed by PCR using the same primers used to amplify the original promoters. Confirmed positive clones were sent for sequencing to make sure that the nucleotide sequence exactly matched the published 5'-upstream sequences.

The (5'-upstream) promoter sequences from pENTR clones were recombined into the Gateway pKGWFS7 destination vector (Karimi et al., 2002) upstream of the UidA::GFP gene fusion using the LR clonase reaction (Invitrogen) to generate the *At*α*-DOX1*-promoter::UIDA(Gus)::GFP and *At*α*-DOX2*-promoter::UIDA(GUS)::GFP constructs. The ligation reaction comprised 2 µL LRClonase II enzyme mix, 1 µL entry clone (50-150 ng DNA), 1 µL (150 ng DNA) destination vector (pKGWFS7). The mixture was brought to 10 µL with 1XTE buffer (pH 8.0) and incubated at 25 °C for 1 hour. The ligation reaction was terminated by adding 2 µL Proteinase K solution to each sample, followed by incubation at 37 °C for 10 min.

Destination vectors carrying promoter sequences were introduced into chemically competent *E.coli* (DH5α^{TM}) was carried out as described previously. Aliquots of bacterial culture (50-100 µL) were spread on pre-warmed agar plates containing 100 µg/mL spectinomycin, and incubated at 37°C overnight. Positive clones were confirmed using PCR with the same primers used to amplify the original promoters. The orientation of the transgenes within the pKGWFS7 destination vectors was determined using PCR with forward primers that annealed to the *nptII* (5'-CTGCGTGCAATCCATCTTGT-3') in the destination

vector and the reverse primers used to amply the promoter region (see section 1.1.6).

2.2.6.1 Transformation of *Agrobacterium tumefaciens* via electroporation

Aliquots (100 µL) of *Agrobacterium tumefaciens* (GV3101) were chilled on ice and approximately 5-10 ng of expression vectors (*Atα-DOX1*-promoter::UIDA(Gus)::GFP) (*AtαDOX1*::GUS) and *Atα-DOX2*-promoter::UIDA(Gus)::GFP) (*AtαDOX2*::GUS) were added to the *Agrobacterium* cells and carefully mixed in a pre-cooled electroporation cuvette and left for 30 min. The cuvettes were then subjected to electroporation at 2500 V, 400 Ω and 25 µF in an Electro Cell Manipulator (BTX, San Diego, California). One mL LB medium was added to the transformed cells and they were incubated for 2 hr at 28 ºC and shaking at 250 rpm. 200 µL aliquots of the cells were plated on LB media containing 100 µg/mL spectinomycin (100 µg/mL) for two days at 28 ºC. Positive clones were confirmed using PCR with the same primers used to amplify the original primers.

2.2.6.2 *Agrobaterium*-mediated transformation of Arabidopsis plants

Arabidopsis thaliana was transformed via *Agrobacterium tumefaciens*-mediated transformation with *AtαDOX1*::GUS and *AtαDOX2*::GUS using the floral dip method (Clough and Bent, 1998). Briefly, the plants were allowed to grow to the stage of inflorescence. *Agrobacteria* carrying the constructs were cultured at 28 ºC in 100 mL LB/Spectinomycin until they reached to OD_{600}= 0.6. The culture was centrifuged at 4 °C for 5 min at 13000 rpm. Bacterial pellets were

resuspended in 50 mL inoculation medium (5% sucrose and 250 µL Triton X-100) in magenta boxes. The plants were inverted and immersed in the solution with gentle shaking for one min. The plants were then placed back in the trays and covered with plastic bags for three days. After three days the covers were removed and plants were allowed to grow to harvest the first generation of seeds (T1).

2.2.6.3 Screening for transformed plants

After transformation, T1 seeds were collected, gas-sterilized and sown on 0.8% agar, ½ MS nutrient medium containing 50 mg/L kanamycin. After two weeks of growth, transgenic seeds were able to germinate and produce green leaves in the presence of kanamycin while the non-transformed plants failed to grow and turned yellow. The presence of *At*α*DOX1*::GUS and the *At*α*DOX2*::GUS construct in host plants was checked by PCR amplification using NPTII primers (forward: 5'-CGTCAAGAAGGCGATAGAAG-3' and reverse: 5'-TATGACTGGGCACAACAGAC-3'). Positive plants were transferred to soil and allowed to grow separately to collect T3 seeds.

2.2.7 Histochemical detection of GUS activity

Un-opened flowers (buds), open flowers, siliques and seedlings were collected from *At*α*DOX1*::GUS and *At*α*DOX2*::GUS T3 transgenic plants for GUS staining. The samples were incubated on ice in fixing solution (0.5% formaldehyde, 10 mM MES (pH 5.6) and 0.3 M mannitol) for 30 min. The samples were washed in 100 mM sodium phosphate (pH 7.0) and then

submerged in 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-gluc, Inalco Pharmaceuticals, San Luis Obispo, CA) solution (1 mM X-gluc, 50 mM sodium phosphate (pH 7.0) and 0.5% (v/v) Triton X-100). After the addition of the X-gluc, the samples were vacuum infiltrated for 30 sec and then incubated at 37 °C overnight to allow the blue stain to develop. After staining, the samples were washed in sterile distilled water, followed by 70% ethanol to remove chlorophyll. This experiment was replicated 3 times. Plants were photographed using a digital camera.

2.2.8 Data mining of publicly available microarray data for *Atα-DOX1* and *Atα-DOX2* expression

Genevestigator (http://www.genevestigator.ethz.ch/gv/index.jsp) was utilized to assess *Atα-DOX1* and *Atα-DOX2* expression in different organs of the plant. Array selection "*Arabidopsis thaliana*" and array type "ATH1:22K array" were selected. The gene probes used were AGI: At3G01420 (*Atα-DOX1*) and AGI: At1G73680 (*Atα-DOX2*). Microarray data for spatial expression of *Atα-DOX1* and *Atα-DOX2* was acquired using the "anatomy" tool.

2.3 Results

Some of the data presented in Figures 2-2 and 2-3 were generated by Anachanok Tirajoh (Simon Fraser University). These include the generation of *Leα-DOX1*-5', *Leα-DOX2*-5' and *Leα-DOX3* probes and hybridization experiments that assessed the expression of *Leα-DOX2* and *Leα-DOX3* genes in salt treated roots. These figures were published in the Journal of Experimental Botany (Tirajoh et al., 2005) and are shown here with permission.

2.3.1 *Leα-DOX1* expression in salt stressed, wounded and pathogen challenged roots

In tomato, α-DOX is represented by a small gene family comprising at least three members: *Leα-DOX1, -2* and *-3*. All three *Leα-DOX* genes were constitutively expressed in roots (Figure 2-2). Salt treatment up-regulated *Leα-DOX1* expression in roots at 8 h and 24 h (Figure 2-2). *Leα-DOX2* expression was not affected by salt, whereas the expression of *Leα-DOX3* was marginally up-regulated at 8 h, but not 24 h, after the application of salt. Salt had a negative effect on the *Leα-DOX* transcript level for all isoforms at 0.5 h following salt exposure (Figure 2-2). *Leα-DOX2* and *Leα-DOX3* were down-regulated 2 h after the salt treatment. Due to the high degree of nucleotide sequence indentity between *Leα-DOX1* and *Leα-DOX2*, gene specific probes were prepared from the 5' end of the cDNA. Northern analyses with either the *Leα-DOX1* full length or -5' cDNA probe detected a similar pattern of transcription accumulation that was distinct from that observed using the *Leα-DOX2* 5' probe or the *Leα-DOX3* probe (Tirajoh et al., 2005).

To determine whether *Leα-DOX1* expression was responsive to mechanical wounding, roots were wounded by pinching with forceps. Wounding elicited up-regulation of *Leα-DOX1* and *Leα-DOX2* expression at 8 h and 24 h after wounding (Figure 2-3). *Leα-DOX3* expression was not wound responsive. Due to the salt and wound responsive nature of *Leα-DOX1*, this gene was chosen to examine its expression in response to a pathogen challenge. Exposure of roots to a fungal pathogen *Pythium aphanidermatum* elicited an increase in *Leα-DOX1*

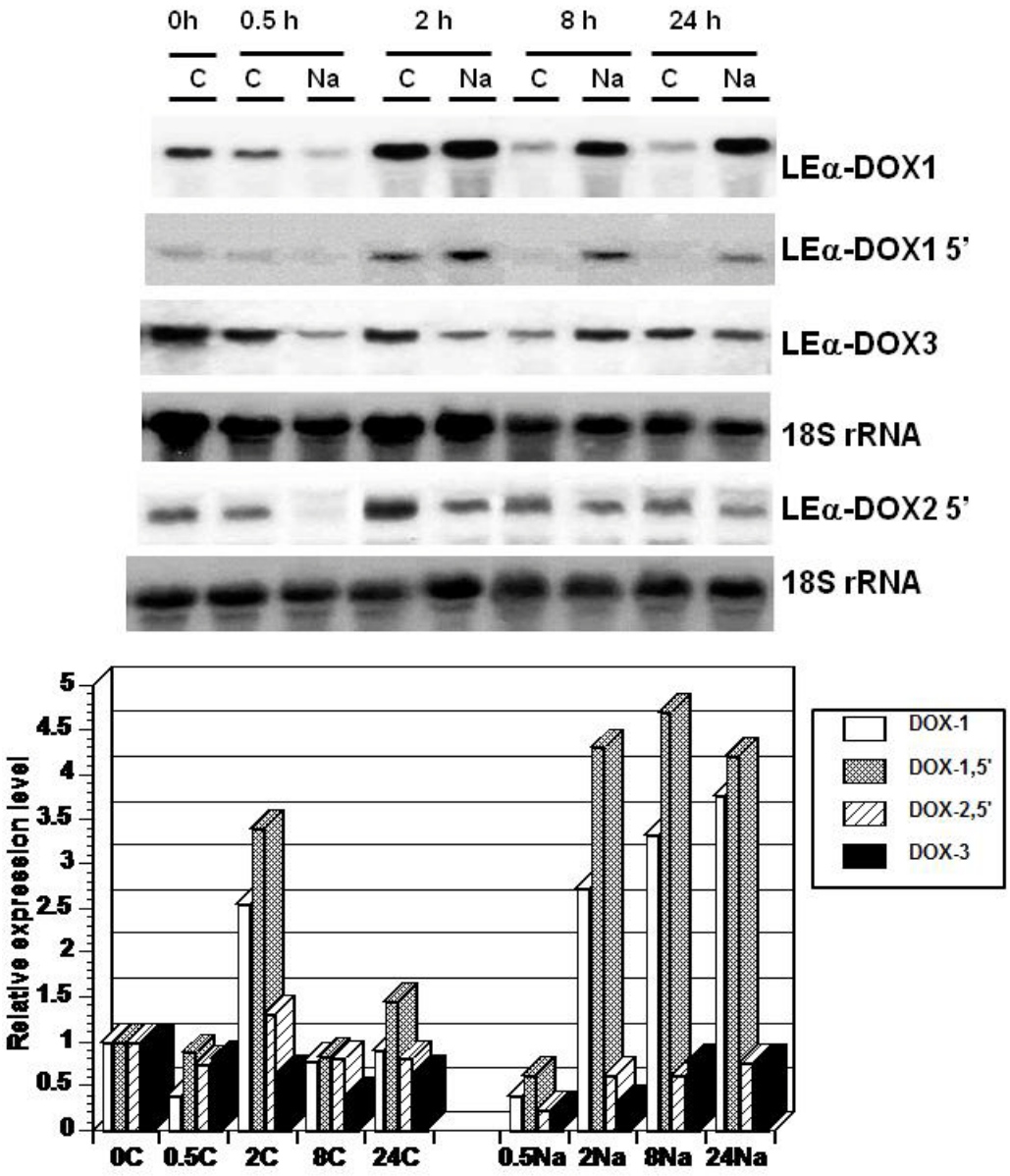


Figure 2-2 *Leα-DOX* expression in salt treated roots.

RNA was isolated 0, 0.5, 2, 8, and 24 h after transfer to salt (Na) or MS media (C). Blots were hybridized with *Leα-DOX1*, a partial probe derived from the 5' end of *Leα-DOX1* (Leα-DOX1 5') or *Leα-DOX2* (Leα-DOX2 5'), *Leα-DOX3* and with an 18S rRNA probe that served as a loading control to obtain normalized expression values. The plot shows the relative expression level obtained relative to the 0 h control sample.

The hybridization experiments with Leα-DOX1 5', Leα-DOX2 5' (AY344540), Leα-DOX3 (BK001477) and quantification of expression data were carried out by Ananchanok Tirajoh.

From Tirajoh et al., 2005. Stress-responsive α-dioxygenase expression in tomato roots, Journal of Experimental Botany, 56, 713-723.

expression in tomato roots (Figure 2-3). These results have been published in Tirajoh et al., (2005).

2.3.2 Amino acid sequence alignment of Leα-DOX1, Leα-DOX2, Leα-DOX3 from tomato with Atα-DOX1 and Atα-DOX2

There was a high degree of identity between the amino acid sequences of the ORFs encoded by *Leα-DOX* and those encoded by *Atα-DOX*. The percent of amino acid sequence identity between tomato Leα-DOX1 and Leα-DOX2 is 84% whereas the amino acid sequence identity between Leα-DOX1 and Leα-DOX3 is 63%. The polypeptides encoded by *Leα-DOX1* and *Leα-DOX2* share high similarity with Atα-DOX1from *Arabidopsis thaliana* (73% and 71% identity, respectively) (Figure 2-4). Leα-DOX3 (FEEBLY) is more similar to the Arabidopsis feebly-like (Atα-DOX2) protein than to Leα-DOX1 or 2 (71% identity vs. 63% and 61% identity respectively) (Figure 2-4).

Alpha-dioxygenases are heme enzymes that incorporate O_2 into fatty acids and share structural similarity with mammalian prostaglandin-H synthase (PGHS). The three amino acid residues (His-207, Tyr-385, and His-388), which are essential for enzyme activity in PGHS are also conserved in all tomato and Arabidopsis α-DOX amino acid sequences at similar positions (for example, His-165, Tyr-389, His-392 in Leα-DOX1; His-163, Tyr-387, His-390 in Atα-DOX1) (Figure 2-4). Amino acid residues involved in heme binding (His-165 and His-

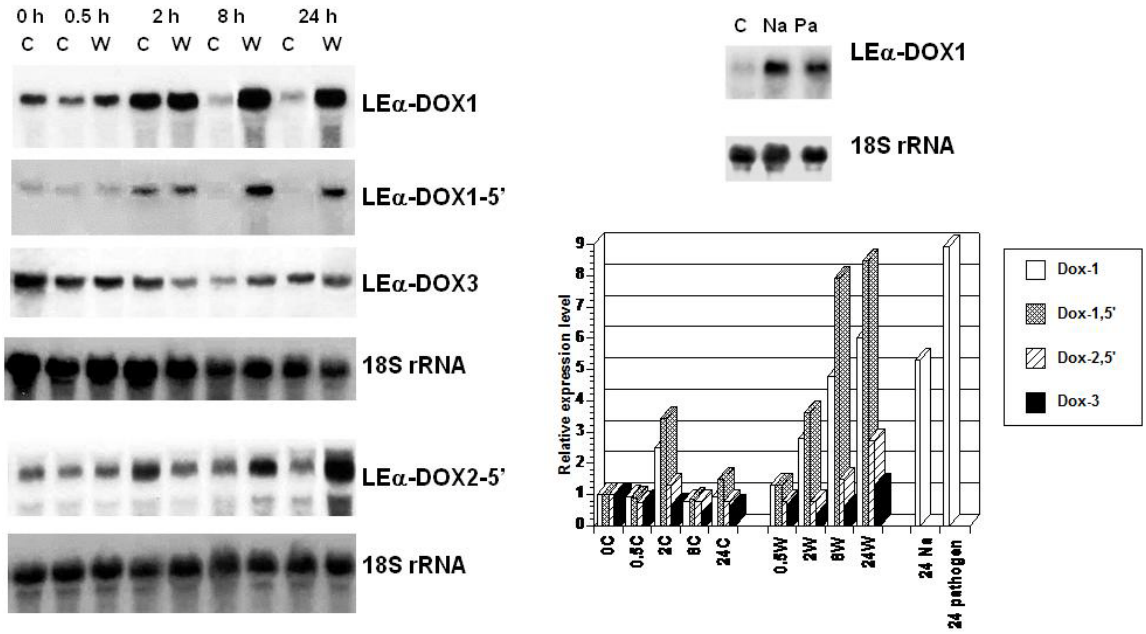


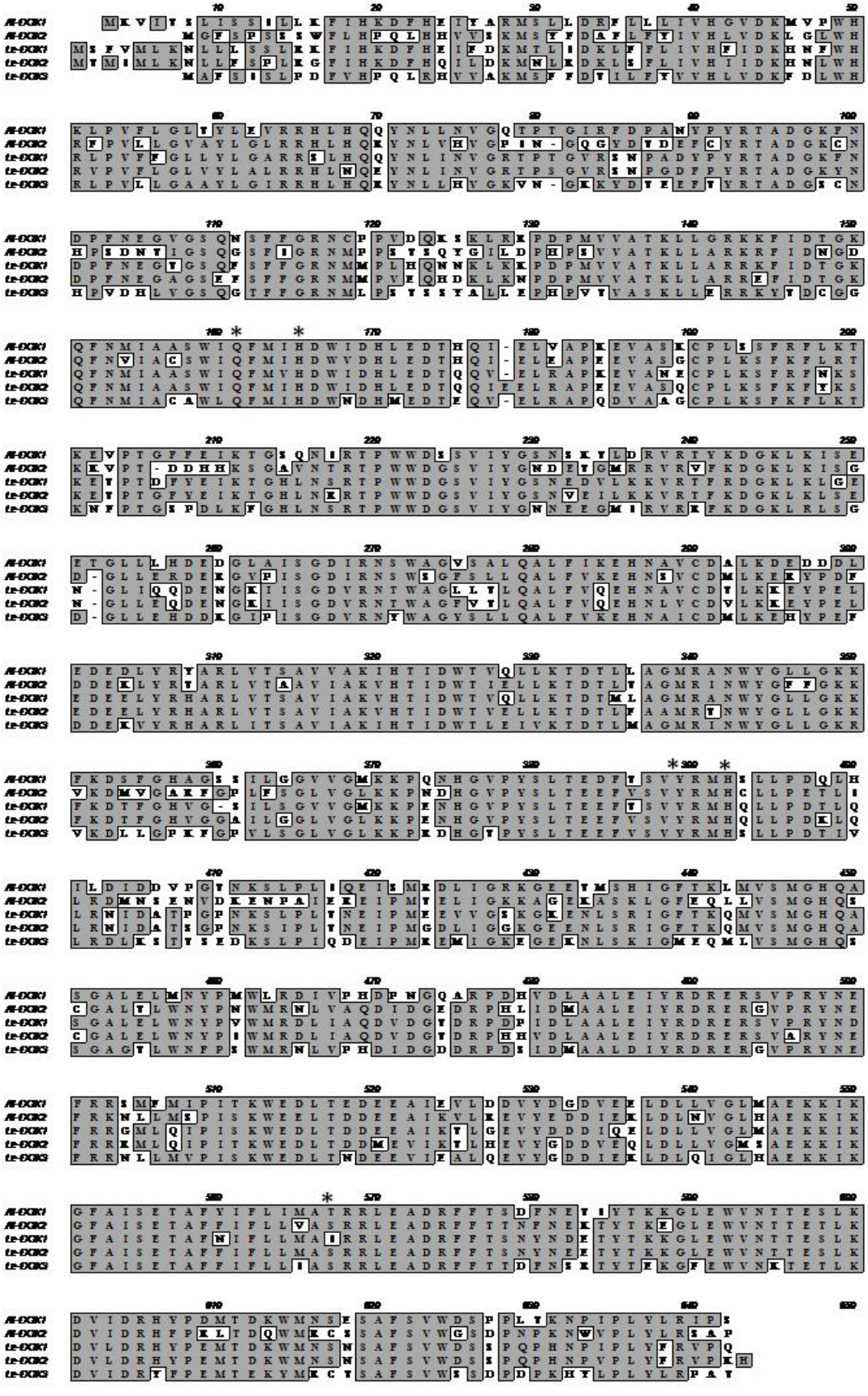
Figure 2-3 *Leα-DOX* expression in wounded and pathogen challenged roots.

RNA was isolated 0, 0.5, 2, 8, and 24 h after wounding (W) or transfer to MS media (C). RNA was also isolated from tissue harvested 24 h after treatment with *P. aphanidermatum* (Pa) or salt (Na). Blots were hybridized with *Leα-DOX1*, a partial probe derived from the 5' end of *Leα-DOX1* (Leα-DOX1 5') or *Leα-DOX2* (Leα-DOX2 5'), *Leα-DOX3* and finally with an 18S rRNA probe. The plot shows the relative expression level obtained relative to the 0 h control sample. The experiments concerning *Leα-DOX2, Leα-DOX3* and quantification of expression data were carried out by Annachanok Tirajoh.

From Tirajoh et al., 2005. Stress-responsive α-dioxygenase expression in tomato roots, Journal of Experimental Botany, 56, 713-723.

Figure 2-4 Deduced amino acid alignment of *Leα-DOX1, -2, -3* from *Solanum lycopersicum* (Le-DOX1, Le-DOX2, Le-DOX3) with α-DOX1 and -2 from *Arabidopsis thaliana* (*At-DOX1, At-DOX2*).

The shaded region indicate identical or conserved amino acid residues. Amino acids residues that are essential for α-DOX enzyme activity are indicated by an asterisk.



392 of Leα-DOX1), and initiation of the oxygenation reaction (Tyr-389 of Leα-DOX1) are also conserved (Figure 2-4) (Tirajoh et al., 2005). The putative charge-stabilizing residue, Gln-161 of Leα-DOX1is conserved in Arabidopis and tomato α-DOX amino acid sequences (Figure 2-4); however, a Ser residue involved in substrate binding (Ser-567) is not conserved in the Leα-DOX1 or Atα-DOX1 polypeptides but is in Leα-DOX2, -3 and in Atα-DOX2 (Figure 2-4).

2.3.3 Spatial expression analyses of Arabidopsis α-DOX expression

To determine the spatial and temporal expression pattern of *Atα-DOX1* and *Atα-DOX2*, their 5'-upstream (promoter) sequences (2 Kb of the genomic sequence upstream of the *Atα-DOX1* and *Atα-DOX2* translation start site) were fused to the *GUS* reporter gene. The resulting constructs were introduced into Arabidopsis to generate *Atα-DOX1*::*GUS* and *Atα-DOX2*::*GUS* lines. No GUS staining was observed in the siliques of *Atα-DOX1* promoter::*GUS* plants (data not shown). GUS expression was observed in the anthers of flower buds and its expression shifted to the filaments of open flowers of *Atα-DOX1*::*GUS* plants (Figure 2-5 A, B). The *Atα-DOX1* promoter directed GUS expression in root tips and the regions (approximately 0.8 mm) behind the root tip (Figure 2-5 C). In one-week old seedlings, GUS expression was observed in roots of *Atα-DOX1*::*GUS* plants but not in cotyledons or young leaves (Figure 2-5 E). GUS staining was shown in the epidermis and root hairs but not in lateral root primordia (Figure 2-5 C, D). The *Atα-DOX2* promoter directed *GUS* expression in the leaves but not in roots (Figure 2-6 C). The *Atα-DOX2* promoter directed

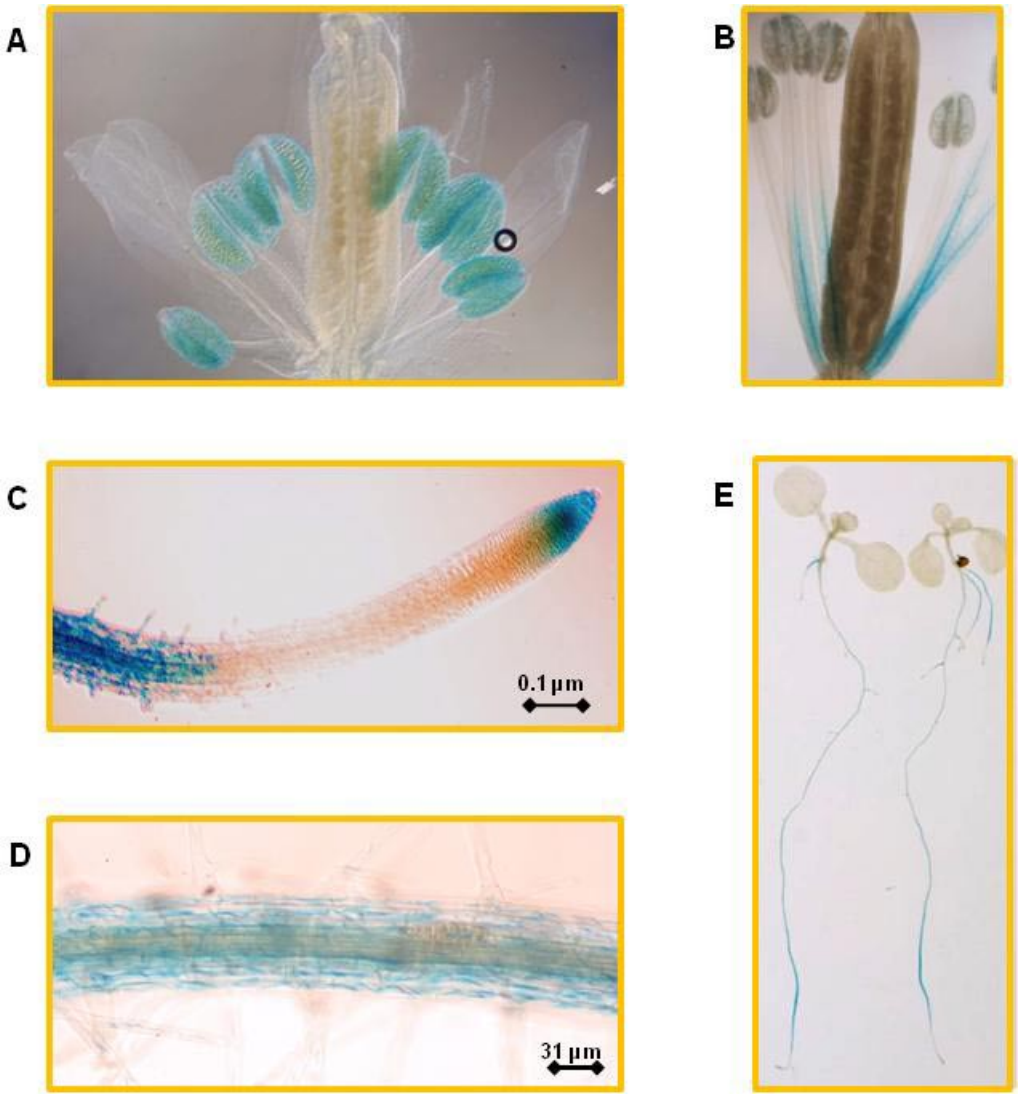


Figure 2-5 Spatial localization of *Atα-DOX1* expression in *Atα-DOX1* promoter::GUS plants.

Histochemical GUS staining of transgenic Arabidopsis plants containing the *Atα-DOX 1* promoter::GUS fusion protein. A , Flower bud. B, Flower. C, Root of one-week old plant D, 20X magnification of root with lateral root primordia E, One-week old plants.

GUS expression in the sepals of flower buds, in the sepals and developing seeds in flowers (Figure 2-6 A, B). *GUS* expression was also observed in the base and tip of young siliques (Figure 2-6 D). Microarray data indicate the highest levels of *Atα-DOX1* expression in radicles and roots whereas the highest levels of *Atα-DOX2* expression were reported in seed coats and senescent leaves (Table 2-1). *Atα-DOX2* expression levels were lower than that of *Atα-DOX1* in most anatomical parts listed. A moderate level of *Atα-DOX1* expression in root tip and senescent leaf was reported; however, *Atα-DOX1* expression in rosette and cauline leaf was very low (Table 2-1). Microarray data also indicate moderate expression of both *Atα-DOX1* and *-2* in the root hair zone and elongation zone. A very low level of *Atα-DOX1* expression in the endodermis and cortex was reported in the microarray data (Table 2-1).

2.3.4 Selecting specific *Atα-DOX1* and *Atα-DOX2* probes for Northern analyses

The *Atα-DOX1* and *Atα-DOX2* ORFs are very similar at the nucleotide level, sharing 67% sequence identity. To diminish the possibility of cross-hybridization between the two cDNA probes a nucleotide alignment of the *Atα-DOX1* and *Atα-DOX2* cDNA sequences was carried out to identify region(s) of low similarity. The nucleotide sequence alignment showed the lowest sequence similarity in the 5' region of the Arabidopsis *α-DOX* genes (Figure 2-7). Thus, gene-specific *Atα-DOX1* and *Atα-DOX2* probes were prepared from the 5'-end of the cDNA for northern hybridization analyses.

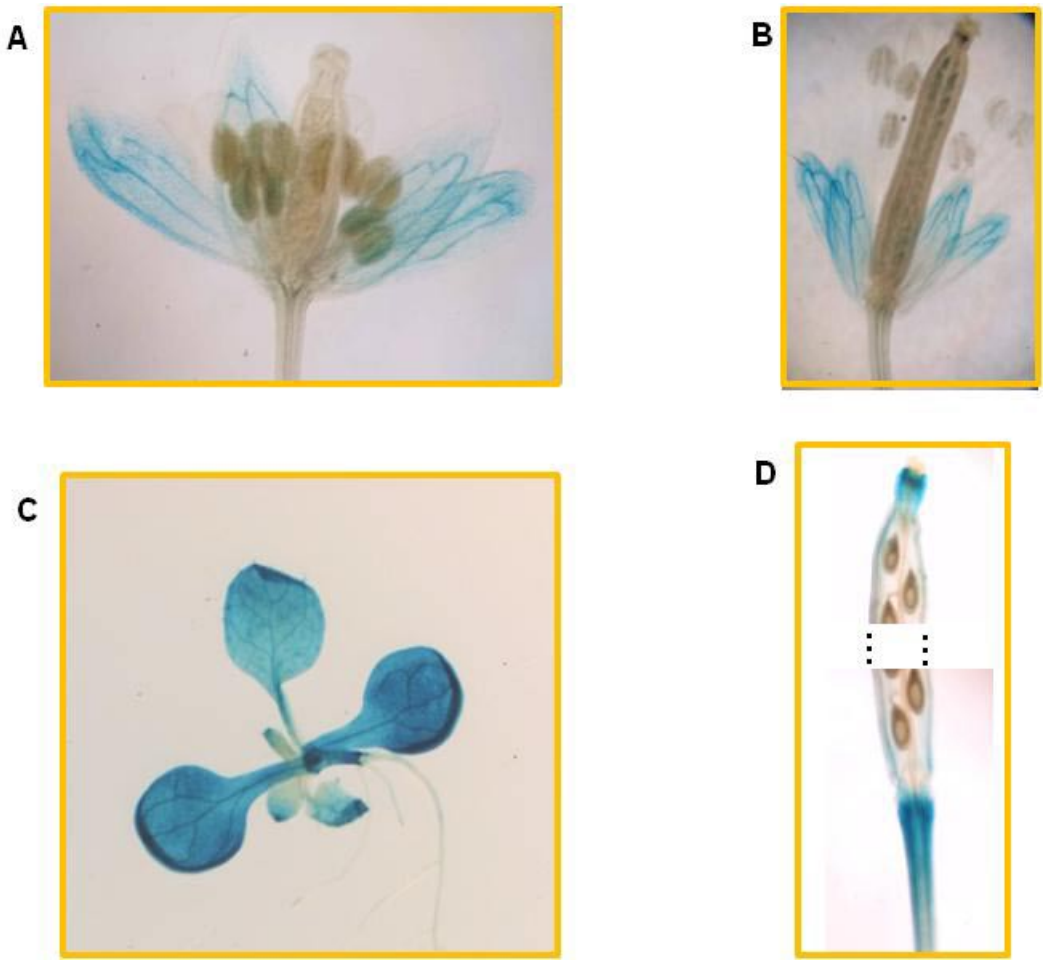


Figure 2-6 Spatial localization of *Atα-DOX2* expression in *Atα-DOX2* :GUS plants.

Histochemical GUS staining of transgenic Arabidopsis plants containing the *Atα-DOX2* promoter::GUS fusion protein. A, Bud. B, Flower. C, cotyledons and leaves of 1-week old plant. D, Silique.

Table 2-1 Microarray data for *At α-DOX1* and *At α-DOX2* expression in Arabidopsis plants (Genevestigator V3, Anatomy tool).

The mean expression level for different anatomical parts ±SE is listed from the highest expression value to the lowest. (For example, the expression of *At α-DOX1* in the radicle was the highest and the expression in the senescent leaves was the lowest in the list).

At α-DOX1 expression		At α-DOX2 expression	
1. Radicle	16810±225	1. Seed coats	3095±350
2. Roots	11719±324	2. Senescent leaves	2704±129
3. Lateral roots	5332±2616	3. Silique	1822±99
4. Seedlings	5047±153	4. Endosperm	1770±422
5. Sepals	3141±1226	5. Sepals	1575±66
6. Root tip	2474±896	6. Pedicels	1477±108
7. Senescent leaves	2199±138	7. Cauline leaf	1327±48
8. Root hair zone	1856±617	8. Rosette	823±227
9. Elongation zone	1367±581	9. Root hair zone	673±96
10. Stele	1168±227	10. Elongation zone	591±107
11. Epidermis atrichoblasts	935±445	11. Stele	515±227
12. Rosette	388±33	12. Endodermis+Cortex	364±104
13. Cauline leaf	158±24	13. Roots	320±10
14. Endodermis+Cortex	133±34	14. Epidermis atrichoblasts	233±227

Figure 2-7 Alignment of the nucleotide sequences of *Atα-DOX1* and *At-DOX2*.

Identical nucleotides are indicated by asterisks.
Multiple sequence alignment was generated by CLUSTALW (1.81)
(http://align.genome.jp)

```
AtDox1          ATGAAAGTAATTACTTCCCTAATCTCTTCTATTTTGCTTAAATTCATCCACAAAGACTTC
AtDox2          ---------ATGGGTTTCTCTCCATCCTCTTCATGGTTTC--TTCATCCTCAG----CTT
                          **   ** *      ** ***   * * **   ******* **      *

AtDox1          CATGAGATTTATGCAAGAATGTCCCTCCTCGATCGGTTTCTTCTTCTTATCGTGCATGGA
AtDox2          CATCATGTTGTTTCCAAGATGTCTTACTTTGATGCTTTTTTGTTCTATATTGTGCACTTA
                *** *  **  * * *  *****   * * ***   *** *  *   *** *****   *

AtDox1          GTGGATAAGATGGTTCCATGGCATAAGCTTCCAGTATTCTTGGGGTTAACCTATCTTGAA
AtDox2          GTGGACAAGCTAGGATTGTGGCATAGATTTCCAGTGTTATTAGGAGTGGCTTACTTGGGG
                ***** *** * *     *******   ******* ** ** **  *  * **  * *

AtDox1          GTACGAAGACATCTTCACCAACAATACAATCTTCTCAACGTCGGTCAAACTC-CGACTGG
AtDox2          TTACGAAGACATCTACATCAACGTTACAATCTGGTACATGTCGGTCCGATCAACGGTCAG
                 ************* ** ****  ********  *  * *******  *    **    *

AtDox1          GATCCGGTTTGATCCTGCTAATTATCCGTACCGGACTGCTGACGGAAAATTCAATGATCC
AtDox2          GGT----TACGACACCGATGAGTTCTGTTATCGTACGGCTGACGGCAAGTGTAACCATCC
                * *    *  **  * * * * *     ** ** ** ******** ** *  **  ****

AtDox1          CTTTAATGAAGGCGTCGGCAGTCAAAATAGTTTTTTCGGGAGAAAT-TGTCCCCCCGTCG
AtDox2          CTCCGATAACACCATCGGCAGCCAAGGCAGCTTCATCGGCCGGAATATGCCTCCCTCTAC
                **   ** *   * ******* ***   ** **  **** * *** ** * ***  *

AtDox1          ATCAGAAATCAAAGTTACGGAGGCCAGACCCCATGGTGGTGGCGACAAAACTATTAGGAA
AtDox2          TTCTCAGTACGGCATTTTGGAT-CCACATCCAAGTGTAGTGGCGACAAAGTTGTTAGCGA
                 **  *   *    **  ***  *** * ** *  ** ***********  * ****  *

AtDox1          GGAAAAAGTTTATCGACACGGGAAAACAATTCAACATGATTGCAGCTTCTTGGATTCAGT
AtDox2          GAAAAAGATTCATAGACAATGGGGACCAATTCAACGTGATAGCTTGTTCTTGGATCCAGT
                * ****  ** ** ****  **  * ********* **** **   ********* ****

AtDox1          TCATGATCCATGATTGGATTGATCATCTTGAAGACACTCACCAAATCGAACTTGTCGCTC
AtDox2          TCATGATCCATGATTGGGTTGATCACTTAGAAGACACCCACCAGATTGAGCTTGAAGCTC
                ***************** *******  * ******** ***** ** ** ****  ****

AtDox1          CAAAAGAAGTAGCGAGCAAGTGTCCCTTAAGCTCCTTTAGGTTCTTAAAGACCAAGGAAG
AtDox2          CAGAAGAAGTAGCAAGTGGATGTCCATTGAAGTCATTCAAGTTCCTCAGAACGAAGAAAG
                ** ********** **    ***** ** *  ** ** * **** * *  ** *** ***

AtDox1          TCCCTACCGGTTTCTTCGAAATCAAGACTGGCTCGCAAAATATCCGTACACCTTGGTGGG
AtDox2          TTCCCACCGATGATCACCA---CAAATCTGGCGCTGTCAACACTAGAACCCCTTGGTGGG
                * ** **** *     * *   ***  ***** *    ** *   * ** **********

AtDox1          ATTCGAGCGTCATCTATGGAAGCAACTCGAAAACATTGGATAGAGTAAGAACTTACAAAG
AtDox2          ATGGGAGTGTAATATATGGAAATGACGAGACTGGAATGAGAAGAGTTAGGGTTTTCAAGG
                **  *** ** ** *******   **  **    * **   ***** **   ** *** *

AtDox1          ACGGGAAACTAAAGATATCGGAGGAGACGGGTCTCCTTCTCCATGACGAAGACGGTTTAG
AtDox2          ACGGAAAGCTAAAAATCTCTGGGGAT---GGCTTGTTGGAGAGGGACGAAAGAGGTGTTC
                **** ** ***** ** ** * ***    **  *  *       ******   *** *

AtDox1          CTATCTCCGGCGACATTCGTAACAGTTGGGCTGGTGTCTCCGCTTTGCAAGCTCTCTTCA
AtDox2          CGATCTCCGGTGACATAAGAAACAGCTGGTCAGGTTTCTCTCTGTTGCAAGCTCTCTTTG
                * ******** *****  * ***** *** * *** ****    **************

AtDox1          TCAAAGAGCACAACGCCGTATGCGACGCCCTCAAGGATGAGGATGATGATTTGGAAGACG
AtDox2          TCAAAGAACACAACTCCGTCTGTGATATGCTCAAAGAACGGTATCCAGATTTTGATGATG
                ******* ****** **** ** **    ***** **   * **   ***** ** ** *
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AtDox1          AAGATTTGTACCGGTACGCTAGGCTAGTGACCTCAGCCGTGGTAGCCAAGATTCACACCA
AtDox2          AGAAACTCTACCGGACTGCTAGACTTGTGACAGCAGCGGTTATCGCTAAGGTTCATACGA
                *  *  * ******   ***** ** *****  **** **  * ** *** **** ** *

AtDox1          TAGATTGGACAGTCCAGCTTCTCAAAACCGACACTTTACTTGCTGGGATGCGAGCAAATT
AtDox2          TCGATTGGACAATAGAACTCTTAAAGACAGACACACTCACTGCTGGAATGAGGATCAACT
                * ********* *  * **  * ** ** *****  *   ****** *** *    ** *

AtDox1          GGTACGGACTACTAGGAAAGAAGTTTAAAGATTCTTTCGGACATGCAGGCAGTTCGATCT
AtDox2          GGTATGGGTTTTTTGGGAAGAAAGTGAAGGACATGGTAGGAGCAAGATTTGGTCCTCTAT
                **** **  *  * ** *****  * ** **     * ***     *    ** *  * *

AtDox1          TGGGAGGTGTCGTGGGAATGAAGAAACCGCAAAATCATGGGGTCCCTTACTCTCTAACTG
AtDox2          TTAGCGGATTAGTTGGTCTGAAGAAACCGAATGATCATGGAGTTCCTTATTCCCTTACCG
                *  * **  * ** **  ********** *  ******* ** ***** ** ** ** *

AtDox1          AAGATTTCACCAGCGTCTATCGAATGCATTCTCTCTTACCTGATCAACTCCATATACTTG
AtDox2          AAGAGTTCGTTAGTGTTTACAGGATGCATTGTCTTCTACCAGAGACACTTATACTCCGAG
                **** ***   ** ** **  * ******* ***  **** **   ***     * *  *

AtDox1          ACATTGATGATGT-ACCAGGAACTAATAAATCACTACCGTTGATTCAAGAGATTTCTATG
AtDox2          ATATGAACTCTGAGAATGTAGACAAAGAAAACCCTGCAATAGAA-CGAGAGATACCGATG
                * **  *   **  *      ** ** *** * ** *  * **  * ******  * ***

AtDox1          AGAGATTTGATTGGTCGCAAGGGAGAAGAAACCATGTCTCACATTGGATTCACTAAGCTA
AtDox2          ACGGAACTGATCGGGAAAAAAGCAGGAGAAAAGGCTTCGAAACTTGGGTTTGAGCAGTTA
                *  **  **** **    ** * ** *****     **  *  **** **     ** **

AtDox1          ATGGTCTCAATGGGTCACCAAGCAAGTGGTGCCCTTGAACTGATGAATTATCCGATGTGG
AtDox2          CTTGTTTCAATGGGACACCAATCTTGTGGCGCATTGACATTGTGGAACTACCCTAATTGG
                 * ** ******** ****** *  **** **  *   * **  *** ** ** *  ***

AtDox1          TTAAGAGACATTGTTCCCCACGACCCCAACGGCCAAGCTCGTCCAGACCACGTCGACTTA
AtDox2          ATGAGGAACCTTGTGGCTCAAGATATCGACGGAGAAGATAGACCTCACCTTATAGACATG
                 * **  ** ****  * ** **   * ****  *** * * **  ***   * *** *

AtDox1          GCTGCTTTAGAGATCTACAGGGACAGGGAGAGGAGCGTCCCACGGTACAACGAGTTCAGG
AtDox2          GCTGCCTTGGAGATTTATAGAGACCGGGAGAGAGGAGTTCCTCGGTACAACGAATTCAGA
                ***** ** ***** ** ** *** *******  * ** ** ********** *****

AtDox1          AGATCTATGTTTATGATTCCGATAACCAAGTGGGAAGATCTAACGGAGGATGAGGAAGCT
AtDox2          AAGAATCTGTTGATGAGTCCGATCAGCAAATGGGAAGAGTTGACAGATGATGAAGAAGCT
                *    * **** **** ****** * *** ********  * ** ** ***** ******

AtDox1          ATTGAAGTGTTGGATGACGTGTACGATGGTGATGTGGAGGAGCTTGATCTTCTCGTGGGA
AtDox2          ATCAAGGTTTTAAGAGAAGTGTACGAAGACGATATAGAGAAGCTTGATCTAAACGTGGGG
                **  * ** **    ** ******** *  *** * *** **********   ******

AtDox1          CTTATGGCAGAGAAGAAAATCAAAGGTTTTGCTATCAGTGAGACTGCTTTTTACATTTTC
AtDox2          TTGCACGCAGAGAAAAAGATCAAAGGATTCGCCATTAGCGAAACTGCTTTCTTCATCTTC
                 *    ******** ** ******** ** ** ** ** ** ******** * *** ***

AtDox1          CTCATCATGGCCACAAGGCGATTAGAAGCGGATAGGTTTTTCACGAGTGATTTCAATGAA
AtDox2          CTCCTTGTCGCCTCCAGGAGGCTAGAAGCAGATAGGTTTTTCACTACGAACTTCAATGAG
                *** *  * *** * *** *  ******* ************** *   * ********

AtDox1          ACGATTTACACGAAGAAGGGGCTTGAATGGGTGAATACTACAGAGAGTCTCAAGGATGTG
AtDox2          AAGACTTATACTAAGGAAGGGTTAGAGTGGGTCAATACTACAGAGACTTTAAAAGATGTG
                * ** *** ** *** * *** * ** ***** ************** * * ** ******

AtDox1          ATTGATCGTCATTATCCTGATATGACAGACAAATGGATGAACTCTGAAAGTGCATTTTCA
AtDox2          ATAGACCGACACTTCCCGAGGTTAACCGATCAATGGATGCGATGTTCGAGCGCCTTCTCT
                ** ** ** ** *  **     * ** **  ********   * *   ** ** ** **
```

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AtDox1          GTATGGGATTCACCTCCACTTACCAAAAATCCAATCCCTCTCTATCTCCGAATTCCCTCT
AtDox2          GTCTGGGGCTCGGATCCTAACCCAAAGAATTGGGTTCCTTTGTACCTCCGATCCGCTCCA
                ** ****  **   ***     * ** ***    * *** * ** ******    *  *
AtDox1          TAATTT------GATCATTTTAATGTGAT-CTATGGCACTTCGAATGATT--TTTTTTCT
AtDox2          TAGGCCATCATAGGCCATAGCCGTCGGATACTACGTGGCACATGTTCATTAGTGTCACTG
                **          *  ***     *  *** *** *   *      * ***  * *
AtDox1          TCCTTTTTTGGCATTTGTTGGCTGTGAGCTG---TGTTTCGACATTGTAA----------
AtDox2          TGTTTCCTTGGTATATTTTTGGGGGGCACTGGTATGGTGTAAAATCGTACATATGAATAA
                *  **  **** ** * ** *  * *  ***   ** *   * ** ***
AtDox1          ------------------------------------------------------------
AtDox2          TCATATTAGAAGGAGAGGTTTAGACATTAGTATCTTTGGTTAAATAAACGATTACCTCAA

AtDox1          ----------------------------------------
AtDox2          TTTCTCTTAACCGTTTGTTCTCAACTTTCCCCATTTTCA
```

To assess the extent of cross-hybridization between the *Atα-DOX1* and *Atα-DOX2* probes, the *Atα-DOX1* and *Atα-DOX2* cDNAs were blotted and hybridized against either the 5'-*Atα-DOX1* gene specific probe or the 5'-*Atα-DOX2* gene specific probe. This revealed no substantial cross- hybridization between these two probes (Figure 2-8). These probes were used for all hybridizations.

2.3.5 *Atα-DOX1* and *Atα-DOX2* expression is responsive to salt stress

In order to determine whether salt induced or up-regulated *Atα-DOX* expression in Arabidopsis, plants were treated with varying concentrations of salt. Constitutive expression of *Atα-DOX1* was observed in the roots but not in the shoots (Figure 2-9). *Atα-DOX1* transcript level in roots increased in response to a 100, 150 and 250 mM salt treatment. The concentration of 50 mM NaCl did not increase *Atα-DOX1* transcript accumulation in roots. Expression of *Atα-DOX1* was much lower in shoots compared to roots, which supports the microarray data (Table 2-1). *Atα-DOX1* expression in shoot tissues was induced by 250 mM NaCl (Figure 2-9). No induction of *Atα-DOX1* expression was observed in shoot tissues of plants treated with 50 mM, 100 mM or 150 mM NaCl.

Atα-DOX2 was expressed constitutively in the shoot but not in the root (Figure 2-9). *Atα-DOX2* expression in roots was induced by 150 mM and 250 mM NaCl. A salt concentration of 100, 150 and 250 mM up-regulated *Atα-DOX2* expression in shoots (Figure 2-9). For future experiments, NaCl at a concentration of 250 mM was applied. To elucidate whether *Atα-DOX1* or *Atα-*

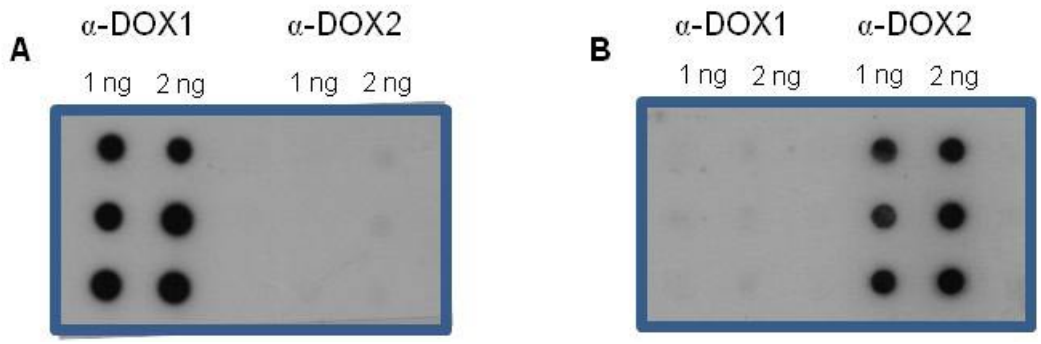


Figure 2-8 Cross-hybridization between *Atα-DOX1* and *Atα-DOX2* probes used for northern analysis.

***Atα-DOX1* and *Atα-DOX2* cDNA (1 ng and 2 ng) was blotted in triplicate onto duplicate nylon membranes. A, Membrane hybridized with A*tα-DOX1* probe. B, Membrane hybridized with *Atα-DOX2* probe.**

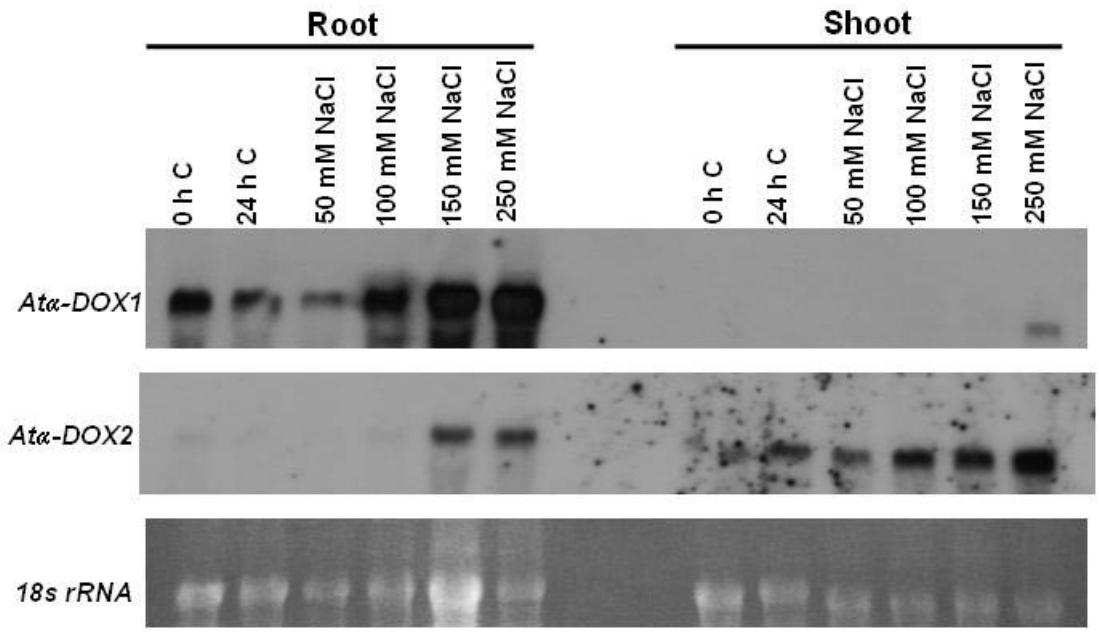


Figure 2-9 Transcript level of *Atα-DOX1* and *-2* in roots and shoots of salt stressed Arabidopsis plants.

RNA was isolated from root and shoot tissues 24 h after plants were transferred to salt (50 mM, 100 mM, 150 mM or 250 mM NaCl) or to MS media (C). Blots were hybridized with *Atα-DOX1* or *Atα-DOX2* probes. A representative northern blot and corresponding eithidium bromide stained gel is shown. The experiment was repeated two times with similar results.

DOX2 expression responds early or late to salt stress, root and shoot tissues were collected at different time points after a salt treatment. Salt treatment up-regulated *At*α*-DOX1* expression in the roots at 2 h, 8 h and 24 h (Figure 2-10). *At*α*-DOX2* expression was up-regulated in the shoot at 8 h and 24 h, after the application of salt (Figure 2-10). Since the highest expression of *At*α*-DOX1* was observed at the 24 h time point, this time point was selected for further experiments.

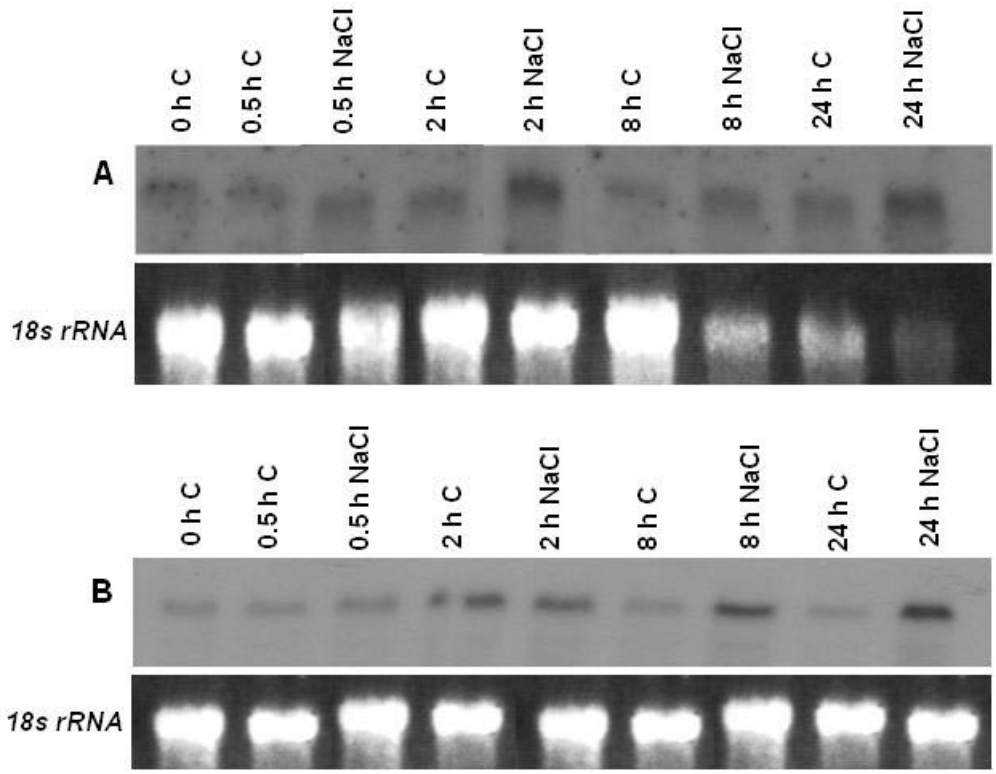


Figure 2-10 *Atα-DOX1* and *-2* expression in salt treated roots and shoots.

A, *Atα-DOX1* expression in roots. B, *Atα-DOX2* expression in shoots. RNA was isolated from 0, 0.5, 2, 8, and 24 h after transfer to salt (NaCl) or MS media (C). Blots were hybridized with the *Atα-DOX1* probe or the *Atα-DOX2* probe. A representative northen blot and corresponding eithidium bromide stained gel is shown. The experiment was repeated three times

2.4 Discussion

Alpha-DOX are heme proteins that catalyze the stereospecific introduction of molecular oxygen at the C-2 (α-) position of saturated and unsaturated fatty acids to form unstable 2(*R*)-hydroperoxide derivatives (Hamberg et al., 1999). The *α-DOX* genes have been identified in a number of plant species including tobacco, pepper, pea and rice (Hamberg et al., 2002). The amino acid residues His-165, Tyr-387, His-390 in pea α-DOX which are essential for enzyme activity as well as the distal Gln-203 are conserved among plant α-DOX (Saffert et al., 2000; Kim et al., 2002; Sanz et al., 1998), indicating that these heme proteins are capable of oxygenation and transformation of fatty acids into hydroperoxides. Amino acid sequence analysis of the plant α-DOX enzyme showed significant similarities with animal cyclooxygenase (COX) (Sanz et a., 1998) and amino acid residues of the catalytic domain (for example, His-165, His-390 and Tyr-387 of Arabidopsis) are also present in COX. COX enzymes, also known as prostaglandin endoperoxide synthases (PGHS), are key enzymes for the synthesis of prostaglandins and other eicosanoids in vertebrates (DeWitt and Smith, 1995). The presence of the same conserved catalytic domains in animal COX and plant α-DOX suggests similarities between COX and α-DOX at the level of biological function. The expression of *COX-2* is induced in response to the generation of O_2^- that takes place during macrophage activation, and production of active COX-2 enzyme plays a role in protecting cells against this toxic radical

(Kawamotoa et al., 2000). Like prostaglandins, the lipid-derived compounds synthesized through the activity of α-DOX enzymes could act as signal molecules to protect cells against reactive oxygen species induced by biotic (Ponce de León, et al., 2002) or abiotic stresses.

2.4.1 *Leα-DOX1* expression is responsive to salt stress, wounding and pathogen challenge of roots

In tomato, α-DOX enzymes are encoded by a small gene family comprised of at least three members *Leα-DOX1, -2* and *-3* (Tirajoh et al.,2005). Leα-DOX1 and Leα-DOX2 share a high degree of amino acid sequence identity, whereas Leα-DOX3 is less similar and may represent a distinct isoform. Of the three *α-DOX* genes in tomato, only *Leα-DOX1* was responsive to salt (Tirajoh et al., 2005) and this gene was constitutively expressed in roots but not in shoots (Tirajoh et al., 2005). In Arabidopsis leaves, *Atα-DOX1* expression is induced by pathogen attack (Ponce de León, 2002). Increased *α-DOX* expression has also been associated with biotic stress arising from mechanical or caterpillar-induced wounding to leaves (Sanz et al., 1998; Hermsmeier et al., 2001; Ponce de Leon et al., 2002). Ponce de León et al. (2002) demonstrated that Arabidopsis with reduced α-DOX activity was more susceptible to pathogen infection and suggested that α-DOX products protect plants from damage caused by oxidative stress. Tirajoh et al. (2005) and Wei et al. (2000) were the first to report salt-responsive *α-DOX1* expression and the first to focus on α-DOX expression in the roots of tomato. Here I have demonstrated that *Leα-DOX1* expression is increased by wounding and challenge with the necrotrophic fungal pathogen

Pythium aphanidermatum. This is consistent with reports in Arabidopsis leaves in which *Atα-DOX1* was induced by pathogen infection (Ponce de León et al., 2002). Thus, it is possible that α-DOX products in tomato play a role in protecting root cells against oxidative stress generated as a result of pathogen challenge. This suggests a general role for α-DOX in the protection of roots against a range of stresses.

2.4.2 *Atα-DOX* expression is responsive to salt stress

Arabidopsis has two α-DOX genes, *Atα-DOX1* and *Atα-DOX2*. The expression of *Atα-DOX1* and *Atα-DOX2* were analyzed to explore the possible functions of α-DOX in plants, especially under salt stress. Northern analyses revealed that *Atα-DOX1,* like *Leα-DOX1,* was constitutively expressed in root tissues and that *Atα-DOX2* was constitutively expressed in the shoots. Both *Atα-DOX1* and *Atα-DOX2* are up-regulated and/or induced by salt in both root and shoot tissues, thus adding *Atα-DOX* to the list of known salt-responsive genes. Genes that are up-regulated by salt stress have been assigned to several groups based on their possible function. These groups of genes include those that encode the late embryogenis abundant (LEA) proteins (osmoprotective proteins), enzymes (involved in the biosynthesis of osmolytes or hormones, detoxification, and general metabolism), transporters (ion transporters, aquaporins), and regulatory molecules such as transcription factors, protein kinases and phosphatases (Xiong and Zhu, 2002). Most of the encoded products of these genes protect cells against the damaging effects of salt stress. Ponce de León (2001) suggested a possible role for Atα-DOX1 in protecting cells from oxidative

stress during the hypersensitive reaction (HR). Oxidative stress is a secondary stress in salt-stressed plants that can arise due to a disruption in photosynthesis following stomatal closure and/ or damage to the chloroplasts and mitochondria. During salt stress, Atα-DOX1 may produce novel oxylipins that regulate the defense mechanisms induced to protect cells from oxidative stress. For example, 2-hydroxy fatty acids produced from the reaction catalyzed by α-DOX can act as signalling molecules (Hamberg et al., 2003). Alternatively, α-DOX could function in the degradation of damaged fatty acids released from disruptions of cell membranes during salinity stress.

2.4.3 *Atα-DOX* genes are expressed in different organs during development

In addition to a function in salt stressed plants, the constitutive expression of *Atα-DOX* in healthy plants is consistent with a role in defense where expression in specific plant organs (roots, stamens and leaves) might represent a permanent system of protection against oxidative stress. *Atα-DOX1* and *Atα-DOX2* expression was observed in different organs at different growth stages. *Atα-DOX1* expression in differentiating cells in roots suggests a possible role for *Atα-DOX1* in root development. Microarray analyses of *Atα-DOX1* expression revealed that *Atα-DOX1* expression was highest in roots followed by sepals and senescent leaves (Table 2-1). During their differentiation the cells of roots, particularly xylem, undergo cell death and oxidative stress. Also, senescence is a result of programmed of cell death and oxidative stress in plants. The expression of *Atα-DOX1* in differentiating cells of roots and in senescent leaves

suggests that Atα-DOX1 may protect cells from oxidative stress. A role in plant development has been implied for *Leα-DOX3* since insertional mutagenesis of *Leα-DOX3* resulted in a pronounced mutant phenotype termed *feebly* (van der Biezen et al., 1996; Meissner et al., 2000). *Feebly* contains high anthocyanin levels during seedling development and subsequently develops into weak plants with pale green leaves and deformed fruit. Van der Biezen et al. (1996) speculated that FEEBLY is involved in a metabolic pathway giving rise to physiologically disturbed plants when absent. This implies an α-DOX function in normal development in tomato. As presented in this chapter, there is a high level of identity between *Leα-DOX3* and *Atα-DOX2* at the amino acid sequence level. Thus, Atα-DOX2 might play a role during development. *Atα-DOX2* expression was detected in the sepals of buds, flowers, and in leaves but not in the roots or anthers and filaments of flowers where *Atα-DOX1* expression was observed. Microarray analyses of *Atα-DOX2* expression revealed the accumulation of the *Atα-DOX2* transcript in seed coats, senescent leaves, siliques, the endosperm, sepals and pedicels (Table 2-1). The temporal and spatial expression of pea α-DOX during germination and development suggests that the α-oxidation pathway for fatty acid degradation plays a role during developmental processes (Meisner et al., 2008). Meisner et al. (2008) suggested that different isoforms of α-DOX coexist in peas and that the expression of pea α-DOX1 shifts to other isoform(s) during the early stages of plant development. The constitutive expression of *Atα-DOX1* in roots and *Atα-DOX2* in shoots and the differences in spatial expression of *Atα-DOX1* and *Atα-DOX2* during development indicate that α-DOX may play a

role during development. In this regard, it would be interesting to know whether α-DOX1 and α-DOX2 provide the same functions in distinct locations where they were expressed.

3: REGULATION OF AT-ALPHA-DOX EXPRESSION IN SALT STRESSED ARABIDOPSIS

3.1 Introduction

Many important agricultural areas of our planet are affected by the accumulation of salt in soils due to centuries of irrigation and extensive use for agriculture. Salinity affects 7% of the world's land area, which amounts to 930 million ha and the area is increasing (Tester and Davenport, 2003). The majority of crop plants are sensitive to high salinity, which results in reduced productivity. Plants have evolved a wide spectrum of mechanisms to cope with salt stress. Examples of such mechanisms include changes in developmental processes (e.g., variations in flowering time), modifications of physiological processes (eg., ion partitioning to vacuoles) and metabolic responses (Quesada et al., 2000). Some metabolic responses include the synthesis and accumulation of compatible solutes, the detoxification of reative oxygen species (ROS), regulation of potassium acquisition and transcriptional activation of stress responsive genes (Quesada et al., 2000). Increased tolerance to salt stress conditions can be achieved by over expressing salt stress responsive genes or by manipulating transcription activators such as the hormone ABA (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). For future progress in the genetic manipulation of salt tolerance, it is critical to acquire a better understanding of the mechanisms responsible for the activation of salt stress-responsive genes.

Many studies of stress responsive genes have focussed on leaves, but studies on roots have received less attention to date. *α-DOX* was isolated as part of an effort to identify novel salt-responsive genes that are expressed in tomato roots (Wei et al., 2000). Two genes encoding α-DOX have been identified in Arabidopsis and the expression of both genes, *Atα-DOX1* and *Atα-DOX2* is responsive to salt stress in roots (Chapter 2). Thus, genes encoding α-DOX are regulated by salt stress in two different plant species, tomato and Arabidopsis. α-DOX catalyzes the conversion of linolenic acid and other fatty acids into their 2(*R*)-hydroperoxy derivatives, long chain aldehydes, 2(*R*)-hydroxy fatty acids (2HOT) and shorter chain fatty acids (Hamberg et al., 1999). In Arabidopsis, *Atα-DOX1* is expressed in leaves during the hypersensitive reaction (HR) induced by the bacterium *Pseudomonas syringae*.

Plants employ a variety of signalling pathways to activate defense-related genes including those mediated by hormones such as ABA, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). An increased transcript level for *α-DOX1* in tomato roots was observed in response to an exogenous application of ABA or ACC (ET), suggesting that these hormones may regulate *α-DOX* expression *in vivo* (Tirajoh et al., 2005). Cellular signals such as SA, intracellular superoxide or singlet oxygen and nitric oxide (NO) induced *Atα-DOX1* expression in Arabidopsis leaves (Ponce de León et al., 2005). Research presented in Chapter 2 demonstrated that *α-DOX* expression in Arabidopsis was responsive to salt stress in leaves and roots. This chapter describes experiments that were carried out to ascertain whether increased *α-DOX* expression corresponds to

increased α-DOX enzyme activity in salt-stressed Arabidopsis. In addition, the nature of the hormone signals that regulate salt-responsive *α-DOX* expression was explored.

Hypothesis 1. Increased Atα-DOX activity correlates with increased *Atα-DOX* expression in salt stressed Arabidopsis.

To test this hypothesis, I undertook the following experiment:

1. Enzyme assays for Atα-DOX were carried out in control and salt treated Arabidopsis.

Hypothesis 2. Hormone signals regulate the expression of *Atα-DOX1* and *Atα-DOX2* in both root and shoot tissues of salt stressed Arabidopsis.

To test this hypothesis I undertook the following experiments:

1. Northern blot analysis of *Atα-DOX1* and *Atα-DOX2* expression in the root and shoot tissues of Arabidopsis plants following treatment with various hormones.
2. Northern blot analysis of *Atα-DOX1* and *Atα-DOX2* expression in the root and the shoot tissues of hormone mutant genotypes.
3. Histochemical staining for GUS activity in Arabidopsis plants harbouring *Atα-DOX1* or *Atα-DOX2* promoter::GUS fusion proteins following treatment with salt or various hormones.

3.2 Material and methods

3.2.1 Materials

Seeds of wild type *Arabidopsis thaliana* (Col 4) were used in this study. Hormone mutants *aba1-3*, an ABA biosynthetic mutant; *abi1-1*, an ABA resistant mutant; *etr1-1* and *ein2*, ET resistant mutants; *sid2-1*, an SA resistant mutant; *eds5-1*, an SA biosynthetic mutant and *npr1-1*, a mutant defective in SA mediated systemic acquired resistance were obtained from the Arabidopsis Biological Resource Center (ABRC). Dr. J. Glazebrook (Torrey Mesa Research Institute, Syngenta Research and Technology, San Diego, USA) kindly provided NahG seeds (transgenic line expressing a bacterial SA hydroxylase that degrades SA).

3.2.2 Experimental treatments

Growth conditions for Arabidopsis were described previously in section 2.2.1.2. To impose a salt stress, ½ MS was replaced with ½ MS + 250 mM NaCl. For the control treatment, ½ MS was replaced with fresh solution of ½ MS with or without salt. After 24 h of salt stress or control conditions, root and shoot tissue were collected, frozen and stored at -80 ^{o}C.

Hormone treatments were imposed by replacing the ½ MS with ½ MS supplemented with 30 µM or 60 µM ABA (mixed isomers, +/- cis/trans ABA; Sigma-Aldrich, Oakville, Ontario) or 10 µM, 20 µM or 50 µM 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma-Aldrich, Oakville, Ontario, Canada) or with 0.1 mM, 0.5 mM or 1 mM salicylic acid (SA; Sigma-Aldrich,

Oakville, Ontario, Canada) or 30 μM jasmonic acid (JA; Sigma-Aldrich, Oakville, Ontario, Canada) or 10 μM auxin (IAA: Sigma-Aldrich, Oakville, Ontario, Canada). The plants were exposed to the respective hormone for 24 h after which root and shoot tissues were collected, frozen and stored at -80 ^{o}C.

3.2.3 RNA Extraction

Total RNA was extracted following the steps described in section 2.2.2.2.

3.2.3.1 Preparation of radiolabelled DNA probes

The preparation of *Atα-DOX1*-5' and *Atα-DOX1*-5' probes was described previously in section 2.2.3.1.

3.2.3.2 Northern Hybridization Analyses

Northern analyses of Arabidopsis root and shoot RNA was carried out as described in section 2.2.3. The membranes were exposed to either an X-ray autoradiography film (Kodak Scientific Imaging Film X-Omat Blue XB1, NEN™ Life Science, Boston, MA, USA) at –80 ^{o}C with an intensifying screen for 24 h, or to a phosphoscreen for a minimum of 1 h. The bound radioactive probe corresponding to the transcript of interest on the phosphoscreen was detected with a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Inc., Sunnyvale, CA).

Band intensity from the phosphoscreen was quantified using Image Quant 5.2 (Molecular Dynamics, Piscataway, NJ). For Atα-DOX1, the transcript level was normalized by dividing the signal obtained for hybridization with the 5'-*Atα-DOX1* probe by that obtained with an *Ubiquitin* (*UBQ5*) probe. This was done for

root samples due to the high level of constitutive expression of Atα-DOX1 in root tissues (See Figure 2-9 A). Experiments were carried out at least 3 times.

3.2.4 Generation of Atα-DOX1::GUS and Atα-DOX2::GUS

The generation of these transgenic lines was described in sections 2.2.5.1, 2.2.5.2, 2.2.5.3, and 2.2.5.4.

3.2.5 Histochemical detection of GUS activity in salt and hormone treated Atα-DOX1::GUS and Atα-DOX2::GUS lines

Seven-day-old seedlings were transferred to ½ MS liquid media containing 250 mM NaCl, 30 µM ABA, 0.5 mM SA or 5 µM ACC for 24 h after which the seedlings were incubated on ice in fixing solution (0.5% formaldehyde, 10 mM MES (pH 5.6) and 0.3 M mannitol) for 30 min. The seedlings were rinsed in 100 mM sodium phosphate (pH 7.0) and submerged in X-gluc. GUS staining was performed as described in section 2.2.6. The experiment was repeated 3 times each time with a minimum of two replicates containing four seedlings each.

3.2.6 Data mining of publically available microarray data for *Atα-DOX1* and *Atα-DOX2* expression

Genevestigator (https://www.genevestigator.com/gv/index.jsp) was utilized to assess *Atα-DOX1* and *Atα-DOX2* expression in response to various hormone treatments and in hormone mutants as described in section 2.2.7. Microarray data for *Atα-DOX1* and *Atα-DOX2* expression in salt and hormone treated plants was acquired using the “Stimulus” tool. Gene expression was presented as an expression ratio (expression in treated plants divided by expression in the control). The “Mutation” tool was used to obtain expression data for *Atα-DOX1*

and *Atα-DOX2* in hormone mutants. Gene expression was presented as an expression ratio that was obtained by dividing the transcript level in the mutant plant by that in the wild type.

3.2.7 α-Dioxygenase enzyme assays

Wild type Arabidopsis plants were grown in magenta boxes as previously described (section 2.2.1.2) containing 50 mL ½ MS+1.5% sucrose for 3 weeks (12 h light, 8 h dark cycle). The growing media was replaced with ½ MS solution (control treatment) or ½ MS +250 mM NaCl (salt treatment) and plants were left for 24 h. Root and shoot tissues were harvested, frozen in liquid N_2 and stored in -80°C. 1 g of frozen root or shoot tissue was homogenized in 10 mL of 100 mM Tris-HCl (pH 7.4) with an Utra-Turrax homogenizer at 0° C. The homogenized mixture was filtered through four layers of cheesecloth and the filtrate was collected in a 15 mL falcon tube. The protein content in the filtrate was measured using the Bradford method. Forty microlitres of filtrate was diluted with 760 μL sterile ddH_2O (1:20 dilution) and 200 μL of the Bradford reagent (Bio-Rad, Mississauga, Ontario, Canada) was added to bring the final volume to 1mL. The protein content was quantified with a spectrophotometer at 595 nm (Bio Rad SmartSpec^{TM} 3000, Ontario, Canada). α-DOX enzyme activity was assayed by adding 10 μL of 50 mM linolenic acid to 2 mL root filtrate and 4 µL of 50 mM linolenic acid to 2 mL of shoot filtrate and incubating for 30 min at 23°C. Four millilitres of 30 mM methoxyamine hydrochloride in methanol were added to the mixture and incubated at room temperature for 30 min. Thereafter, 0.5 mL of a deuterium standard for 2(R)-Hydroxylinolenic acid (2 Hot) and 8(Z),11(Z),14(Z)-

Heptadecatrienal (C17 ald) (kindly provided by Dr. M. Hamberg) was added. The final assays were packaged in dry ice and sent to Dr. Mats Hamberg (Department of Medical Biochemistry and Biophysic, Karolinska Institute, Sweden). Two products arising from α-DOX activity: 2(R)-Hydroxylinolenic acid (2 HOT) and 8(Z),11(Z),14(Z)-Heptadecatrienal (C17 ald) were measured with gas chromatography-mass spectrometry (GC-MS) by Dr. Mats Hamberg. GC-MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with an SPB-1701 capillary column (Length, 15 m; film thickness, 0.25 µm; Supelco, Bellefonte, PA). Helium at a flow rate of 32 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120 °C. The temperature was raised at 7 °C/min until 260 °C. For quantitative determination of 2 HOT and C17 ald, the instruments was operated in the selected ion monitoring mode, and the corresponding deuterated compounds were used as internal standards.

3.3 Results

3.3.1 α-DOX activity in salt stressed Arabidopsis

Salt increased *Atα-DOX* expression in Arabidopsis (Chapter 2; Figure 3-1). To determine whether the increased *Atα-DOX* transcript level in salt-stressed plants resulted in increased enzyme activity, α-DOX enzyme assays were carried out. Linolenic acid was used as a substrate and total α-DOX products (Heptadecatrienal=C17ald and 2-Hydroxylinolenic acid= 2 HOT) were measured by GC-MS. Total α-DOX products (C17 ald and 2 HOT) resulted from enzyme assay using extract from control roots with linolenic acid was 43.3 nmoles/mg of protein. The α-DOX products as results of enzyme assay using extract from salt stressed roots with linolenic acid was increased to 83 nmoles/mg protein (Figure 3-1). Thus, salt stress in Arabidopsis resulted in a two-fold increase of α-DOX activity in roots. There was less α-DOX activity in shoots than in roots and α-DOX activity in the shoot was approximately one tenth (1/10) of the activity in the root. When plants were salt stressed, there was an approximate two-fold increase of α-DOX activity in the shoots (Figure 3-1).

3.3.2 Hormonal regulation of *Atα-DOX1* and *Atα-DOX2* expression

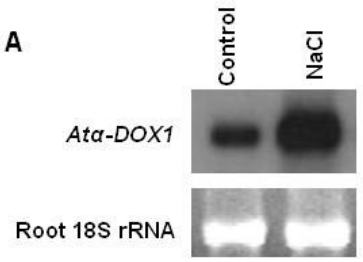
The accumulation of the *Atα-DOX1* transcript in roots and shoots and the *Atα-DOX2* transcript in shoots was investigated in Arabidopsis following exposure for 24 h to ABA, ACC, jasmonic acid (JA), auxin and SA. ACC was used to mimic an ethylene treatment. Root RNA blot analyses revealed that the transcript level of *Atα-DOX1* increased in response to ABA and SA (Figure 3-2).

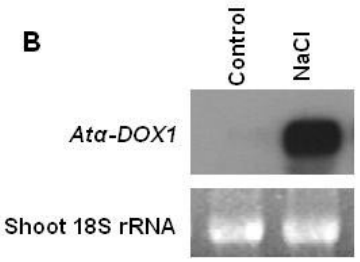
Figure 3-1 α-DOX enzyme activity in Arabidopsis roots and shoots responding to a salt treatment.

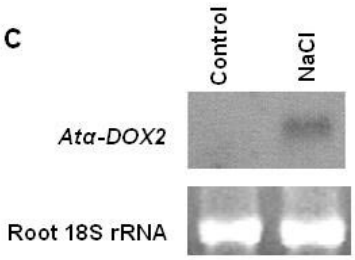
Northern blot analysis of *Atα-DOX1* expression in the root (A), the shoot (B). Northern blot analysis of *Atα-DOX2* expression in the root (C), the shoot (D). α-DOX products from root (E), α-DOX products from shoot (F).

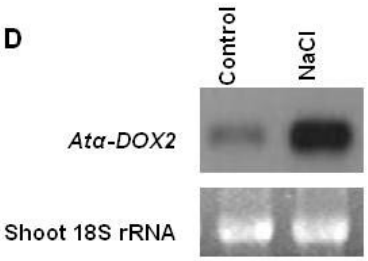
RNA was extracted from the roots and shoot of three-week-old Arabidopsis plants following exposure to ½ MS media (Control) or 250 mM salt (NaCl) for 24 h. A representative northern blot and corresponding ethidium bromide stained gel is shown. Each treatment was carried out more than 3 times.

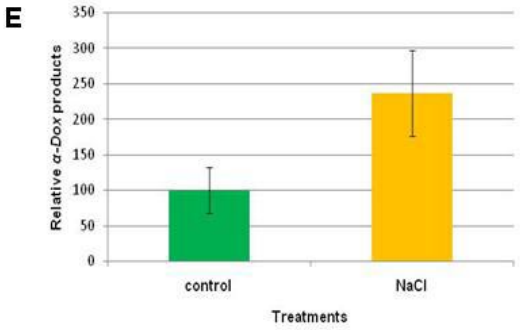
Crude extract was prepared from the roots and shoot of three-week-old Arabidopsis plants following exposure to ½ MS media (Control) or 250 mM salt (NaCl) for 24 h. Enzyme assays were carried out by incubating 2mL of crude extract for root or for shoot with linolenic acid. The α-DOX products (Heptadecatrienal and 2-Hydroxylinolenic acid) were measured by GC-MS. Total α-DOX products per mg of protein were calculated for each sample. The pooled data from 3 different experiments was normalized by setting the control values as 100 and total α-DOX products in salt stressed roots and shoots were presented relative to their control. The green bars represent normalized data for total α-DOX products in control±SE. The yellow bars represent α-DOX products in roots and shoots of salt stressed plants relative to its respective control±SE.

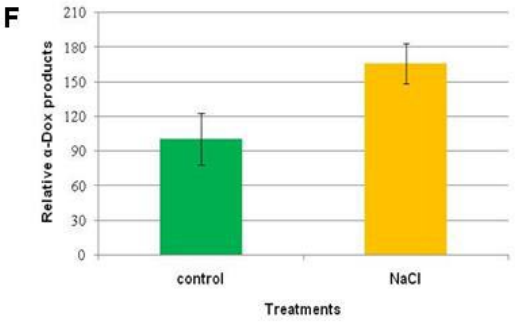

A
Control
NaCl
Ata-DOX1
Root 18S rRNA


B
Control
NaCl
Ata-DOX1
Shoot 18S rRNA


C
Control
NaCl
Ata-DOX2
Root 18S rRNA


D
Control
NaCl
Ata-DOX2
Shoot 18S rRNA


E
Relative α-Dox products
350
300
250
200
150
100
50
0
control
NaCl
Treatments


F
Relative α-Dox products
210
180
150
120
90
60
30
0
control
NaCl
Treatments

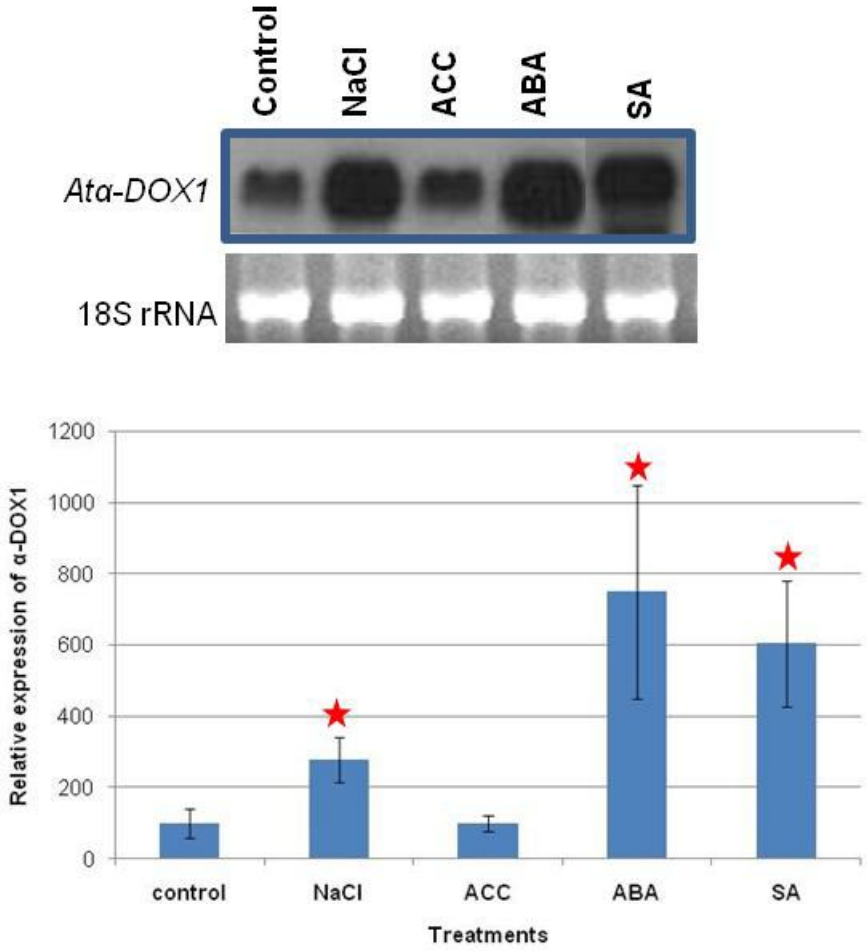


Figure 3-2 *Atα-DOX1* expression in roots of plants treated with salt, ABA, ACC or SA.

RNA was isolated from the roots of plants exposed for 24 h to ½ MS media (control), NaCl (250 mM), ACC (10 µM), ABA (30 µM) or SA (0.5 mM). Each experiment was carried out at least 3 times. A representative northern blot and corresponding ethidium bromide stained gel are shown. Plot represents mean expression level obtained by dividing the hybridization signal intensity by that obtained for an *UBQ5* probe and normalizing by the expression in the control sample. Bars represent the mean expression level +/- SE. Bars denoted with an asterisk are significantly different from the control (Student's t-test P<0.05)

ABA and SA elicited a nine and six-fold increase in the *Atα-DOX1* transcript level whereas, salt caused an almost three-fold increase in transcript level (Figure 3-2). In the shoot, ABA, SA and ACC induced *Atα-DOX1* expression (Figure 3-3 A) with the level of *Atα-DOX1* transcript accumulation in response to ABA and ACC treatment being higher than in the shoot of SA treated plants. Salt stress induced the highest *Atα-DOX1* expression in shoots compared to ABA, ACC and SA treated plants (Figure 3-3). *Atα-DOX1* expression was undetected in the shoots of JA or auxin treated plants (data not shown).

In accordance with our results, microarray data analyses revealed that the *Atα-DOX1* transcript level increased in response to salt in roots and shoots; however, the extent of the up-regulation was modest (1.8 and 1.6-fold in roots and shoots, respectively) (Table 3-1). ABA and SA both increased the *Atα-DOX1* transcript level in seedlings (Table 3-1). The relative order of increase for the ABA and SA treatments was the same in both microarray data analyses and northern analyses. For example, up-regulation by ABA was greater than that caused by SA (ABA increased the *Atα-DOX1* transcript 5-fold, SA by 2.8-fold). Treatment with ACC did not result in increased *Atα-DOX1* transcript accumulation, which agrees with the Northern data.

Atα-DOX2 expression was upregulated in the shoots of salt, ABA, ACC and SA treated plants. Up-regulation of *Atα-DOX2* expression by SA was higher than that by ABA or ACC, which were roughly equivalent to each other based on visual inspection of northen blots (Figure 3-3). The *Atα-DOX2* mRNA level was highest in the shoots of salt treated plants compared to that of ABA, SA and

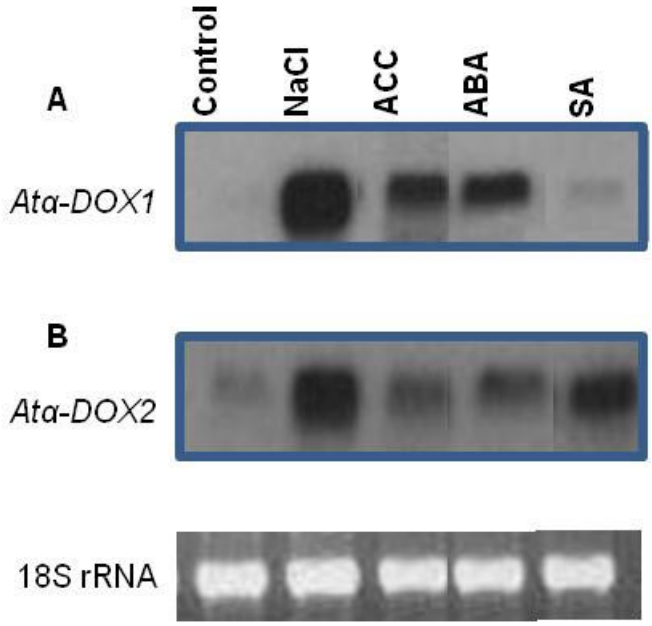


Figure 3-3 ***Atα-DOX*** **expression analysis in shoots of plants treated with salt, ABA, ACC and SA.**

RNA was isolated from the shoots of plants exposed for 24 h to ½ MS media (control), salt (NaCl), ACC (10 µM), ABA (30 µM) or SA (0.5 mM). Each treatment was carried out 2 to 3 times. A representative northern blot and corresponding ethidium bromide stained gel is shown. A, Northern blot analysis with ***Atα-DOX1*** **specific probe. B, Northern blot analysis with** ***Atα-DOX2*** **specific probe.**

3-1 Microarray data for *Atα-DOX* expression in salt- and hormone- treated Arabidopsis plants (Genevestigator V3, Stimulus tool)

Experiment	Anatomical Part	Developmental Stage	Gene Probe	Experimental Ratio (Experimental/Control)
Salt stress (150 mM NaCl for 6, 12, 24 h)	Root	Young rosette	α-DOX1	1.78
			α-DOX2	2.16
Salt stress (150 mM NaCl for 6, 12, 24 h	Root	Young rosette	α-DOX1	1.59
			α-DOX2	2.04
ABA (10 μM for 1, 3 h)	Seedlings	Seedlings	α-DOX1	5.01
			α-DOX2	1.76
SA (10 μM for 1, 3 h)	Seedlings	Seedlings	α-DOX1	2.68
			α-DOX2	0.86
ACC (10 μM for 1, 3 h)	Seedlings	Seedlings	α-DOX1	0.96
			α-DOX2	1.13

ACC. JA or auxin did not enhance *Atα-DOX2* expression in the shoot (data not shown). In microarray data, salt increased the *Atα-DOX2* transcript level in root and shoot tissues and ABA up-regulated *Atα-DOX2* expression modestly in seedlings. Microarray data did not show any effect of ACC and SA on *Atα-DOX2* expression in seedlings (Table 3-1). The discrepancies between the Northern and microarray data with regard to the regulation of *Atα-DOX2* expression by ACC and SA are probably due to differences between the concentrations of applied chemicals, anatomical parts and developmental stages used for the two experiments. Salt imposed the highest *Atα-DOX2* transcript accumulation by microarray analyses, which agrees with the Northern data.

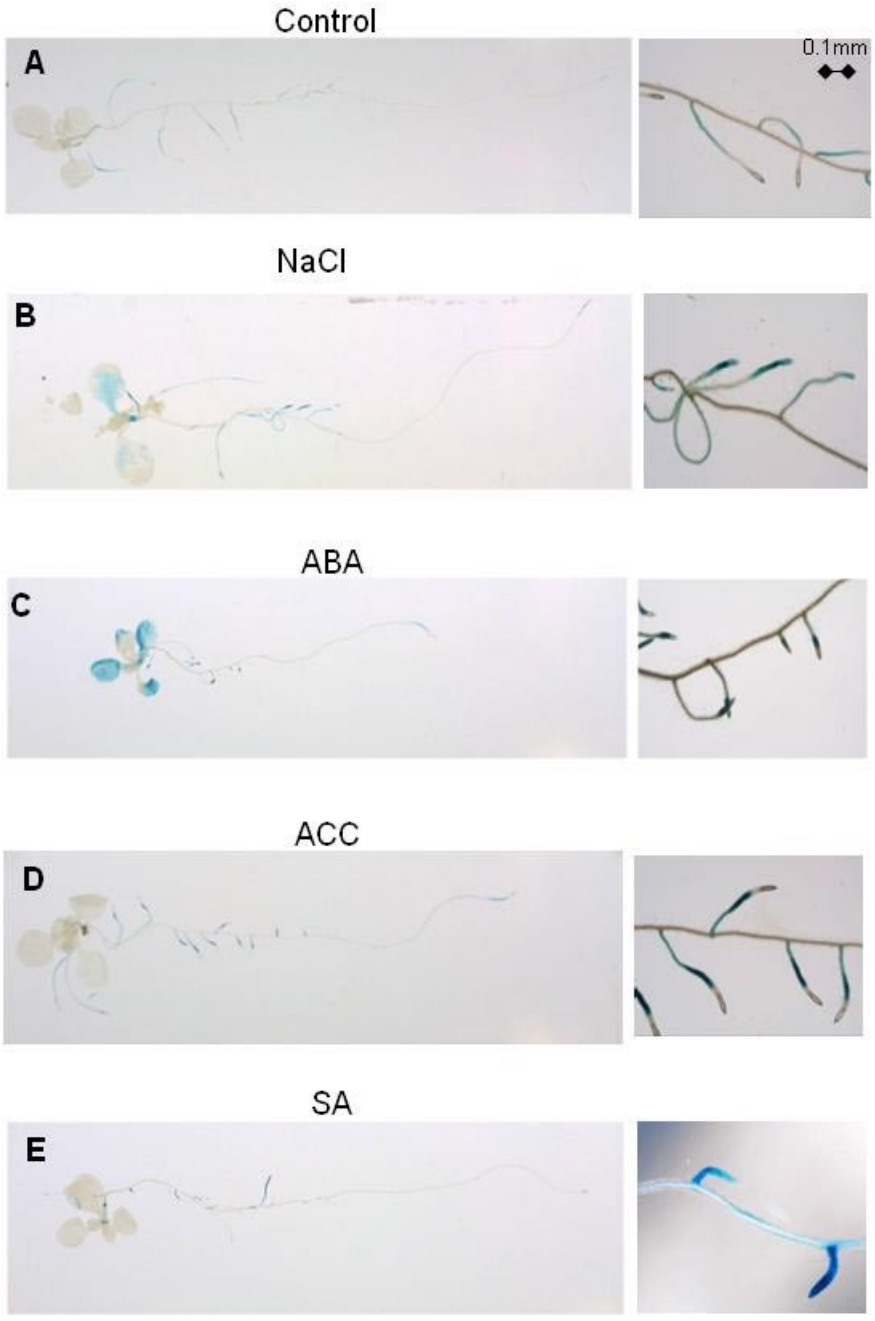
3.3.3 Visualization of *Atα-DOX* expression in Arabidopsis responding to salt, ABA, SA and ACC

To determine the impact of salt stress and hormone treatments on the spatial pattern of *Atα-DOX1* and *-2* expression, the *Atα-DOX1::GUS* and *Atα-DOX2::GUS* seedlings were treated with salt, ABA, SA or ACC. Treatment of *Atα-DOX1::GUS* plants with salt, ABA, ACC or SA intensified the GUS staining in the roots (Figure 3-4 B, C insets). In particular, more intense staining was noted in the zone of cell differentiation where root hairs emerge (Figure 3-4 D and E, insets). In salt treated *Atα-DOX1::GUS* seedlings, *GUS* expression was also observed in the root tips. In SA treated *Atα-DOX1::GUS* seedlings, GUS staining was observed along the length of newly emerged lateral roots; however, in older lateral roots, expression was observed in a region behind the root tip but not at the tip (Figure 3-4 E). A low level of *GUS* expression was observed in a region

Figure 3-4 Histochemical GUS staining of transgenic Arabidopsis plants harboring the *Atα-DOX1* promoter::GUS construct.

A, The *Atα-DOX1* promoter directed GUS expression in a control seedling and in seedlings exposed to B, 150 µM NaCl C, 30 µM ABA D, 5 µM ACC. E, 0.5 mM SA.

***Atα-DOX1* promoter::GUS seeds were germinated in ½ MS (1.5% sucrose) 0.8% agar for 7 days. The seedlings were transferred to ½ MS liquid medium with or without salt or hormones for 24 h. The seedlings were then used for GUS staining. The experiments were carried out at least two times with two replicates for each treatment and four seedlings in each replicate. Representative pictures for the treatments are shown.**


Control
A
0.1mm
NaCl
B
ABA
C
ACC
D
SA
E

0.8 mm behind the root tips in control, salt, ABA and ACC treated *Atα-DOX1::GUS* seedlings. Although more intense GUS staining was observed in salt, ABA and ACC treated seedlings, it was difficult to determine on which treatment resulted in higher GUS expression. GUS staining was observed in the leaves of salt and ABA treated *Atα-DOX1::GUS* seedlings (Figure 3-4 B, C, E). SA elicited a low level of GUS staining in leaves; however, ACC did not.

Under non-stressed conditions, *GUS* expression was observed only in the leaves of *Atα-DOX2*::*GUS* plants (Figure 3-5 A). GUS staining was also observed in leaves of salt, ABA, ACC and SA treated seedlings (Figure 3-5 B, C, D, E) but it was difficult to determine whether GUS staining was more intense. The *Atα-DOX2* promoter directed a low level of GUS staining in a region just behind the lateral root tips of salt and ABA treated plants (Figure 3-5 B, C inset) and a low level of GUS staining was observed in the lateral root tips of SA treated *Atα-DOX2*::*GUS* seedlings (Figure 3-5 E, inset). ACC did not induce *Atα-DOX2* directed GUS expression in roots (Figure 3-5, D).

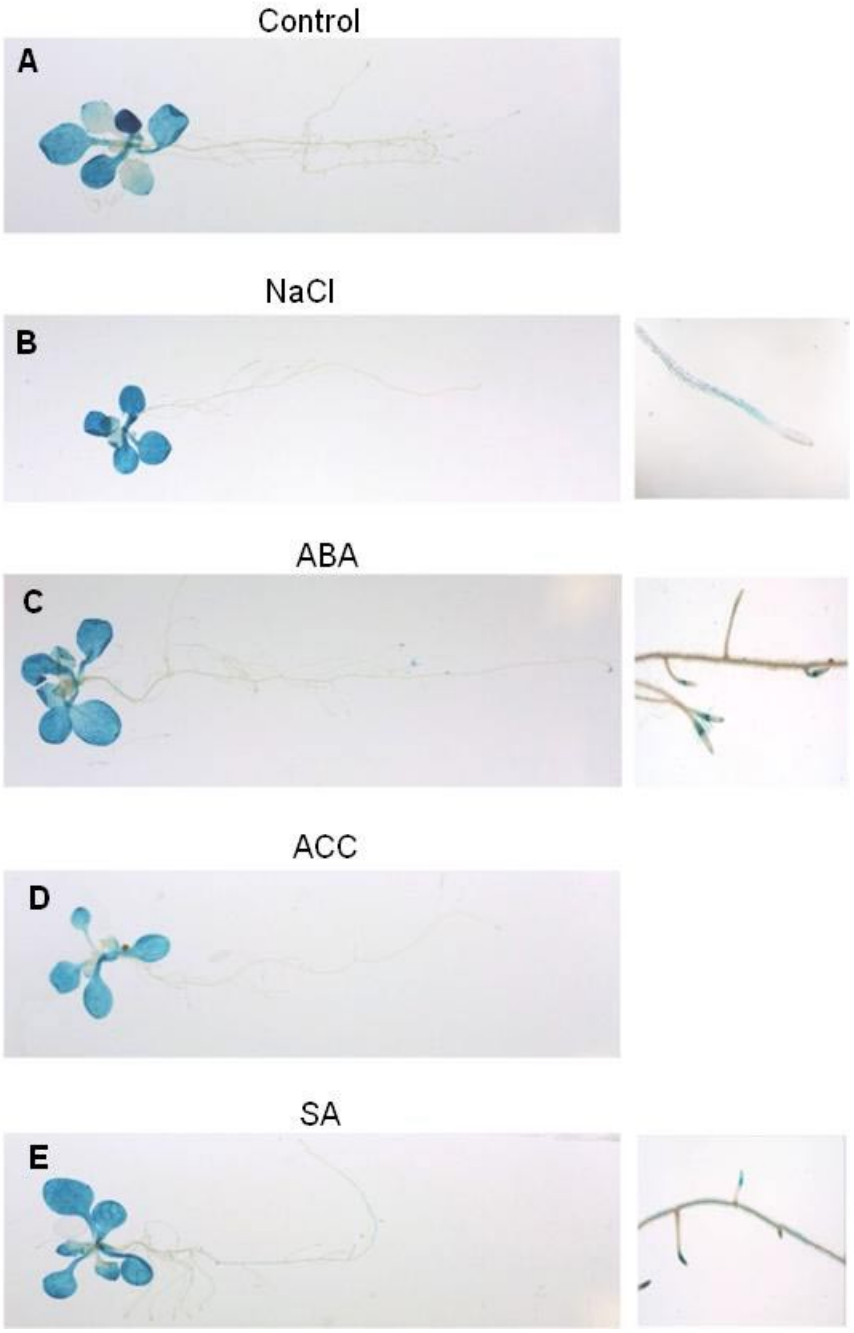
3.3.4 Characterization of *Atα-DOX* expression in hormone mutants

The ABA biosynthetic mutant (*aba1-3*), the ethylene resistant mutants *etr1-1* and *ein2*, the SA induction deficient mutants (*sid2-1* and *eds5-1*), the SA signaling mutant (*npr1-1*) and the ABA resistant mutant (*abi1-1*) were used to further understand the regulation of *Atα-DOX* expression by these hormones during salt stress. In addition, *NahG* transgenic plants expressing the bacterial gene encoding SA hydroxylase were used to explore the importance of SA in regulating *α-DOX* expression. Constitutive expression of *Atα-DOX1* was

Figure 3-5 Histochemical GUS staining of transgenic Arabidopsis plants harboring the *Atα-DOX2* promoter::GUS construct.

A, The *Atα-DOX2* promoter directed GUS expression in a control seedling and in seedlings exposed to B, 150 µM NaCl C, 30 µM ABA D, 5 µM ACC. E, 0.5 mM SA.

***Atα-DOX2* promoter::GUS seeds were germinated in ½ MS (1.5% sucrose) 0.8% agar for 7 days. The seedlings were transferred to ½ MS liquid medium with or without salt or hormones for 24 h. The seedlings were then used for GUS staining. The experiments were carried out at least two times with two replicates for each treatment and four seedlings in each replicate. Representative pictures for the treatments are shown.**


Control
A
NaCl
B
ABA
C
ACC
D
SA
E

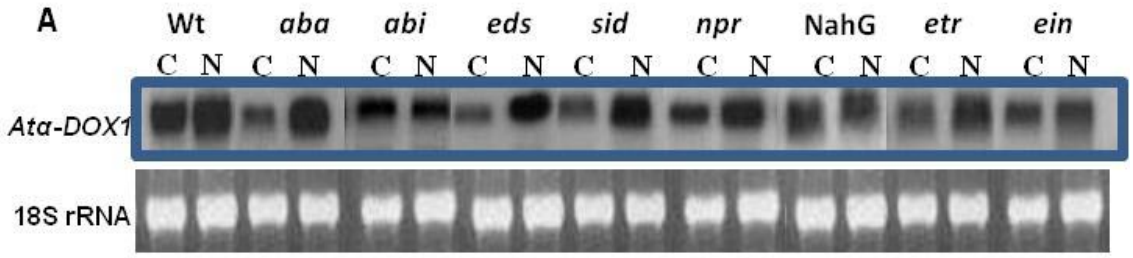
observed in the roots of all hormone mutants; however, transcript accumulation was significantly lower in *abi1-1* and in *etr1-1* and *npr1-1* compared to wild type (Wt) (Figure 3-6 A, B). The *Atα-DOX1* expression level in *aba1-3*, *NahG*, *sid2-1*, *eds5-1* and *ein2* and mutants was similar to that in Wt (Figure 3-6 B). When plants were salt stressed, the *Atα-DOX1* transcript accumulation was significantly lower in the ABA mutants, *aba1-3* and *abi1-1,* and in the SA mutants, *npr1-1* and *nahG,* than in Wt (Figure 3-6 C). The SA mutants, *eds5-1*, *sid2,* and the ET mutants, *etr1-1* and *ein2,* had similar *Atα-DOX1* transcript levels as Wt under salinity stress. In the *abi1-1* and *NahG* mutants the ability of salt to up-regulate *Atα-DOX1* expression was significantly lower than Wt (Figure 3-6 D). In fact, in NahG plants salt did not increase *Atα-DOX1* expression. Salt responsive up-regulation of *Atα-DOX1* in *aba1-3*, *eds5-1*, *sid2-1*, *npr1-1*, *etr1-1* and *ein2* was the same as Wt (Figure 3-6 C).

Microarray data analyses for *Atα-DOX* expression in hormone insensitive or deficient mutants were carried out and presented in Table 3-2. All microarray analyses of *Atα-DOX*1 and *-2* expression reflects that seen in non-stressed hormone mutants. In contrast to the northern results, microarray data revealed that *Atα-DOX1* expression in *abi1-1* and *etr1* was similar to Wt whereas the transcript level in *aba1-1* and *npr1* was higher than in Wt (Table 3-2). However, northern analysis of *Atα-DOX1* expression was carried out in root tissues, whereas microarray analyses were carried out in seeds and seedlings. The JA insensitive mutant, *coil* had a lower *Atα-DOX1* transcript level than Wt (Table 3-2). The mutants that had the lowest and the highest *Atα-DOX1* transcript

Figure 3-6 *Atα-DOX1* expression analysis in roots of hormone mutants.

A, Representative northern blot and corresponding ethidium bromide stained gel. B, Relative *Atα-DOX1* expression in mutant plants under control conditions. C, Relative *Atα-DOX1* expression in mutant plants under salt stress. D, Relative *Atα-DOX1* expression in roots of salt treated Wt and mutant plants was normalized by the expression level of the control for each genotype.

RNA was extracted from the shoot of three-week-old mutant Arabidopsis plants following exposure to ½ MS media (C) or 250 mM salt (N) for 24 h. Genotypes used are *wild type* (*Wt*), *aba1-3* (*aba*), *abi1-1* (*abi*), *eds5-1* (*eds*), *sid2* (*sid)*, *npr1-1* (*npr*), *NahG*, *etr1-1* (*etr*), *ein2* (*ein*). Each experiment was carried out three times. Plot represents mean expression level obtained by dividing the hybridization signal intensity by that obtained for an *UBQ5* probe and normalizing by the expression in the control sample. Bars represent the mean expression level +/- SE. Bars denoted with an asterisk are significantly different.


A
Wt
aba
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NahG
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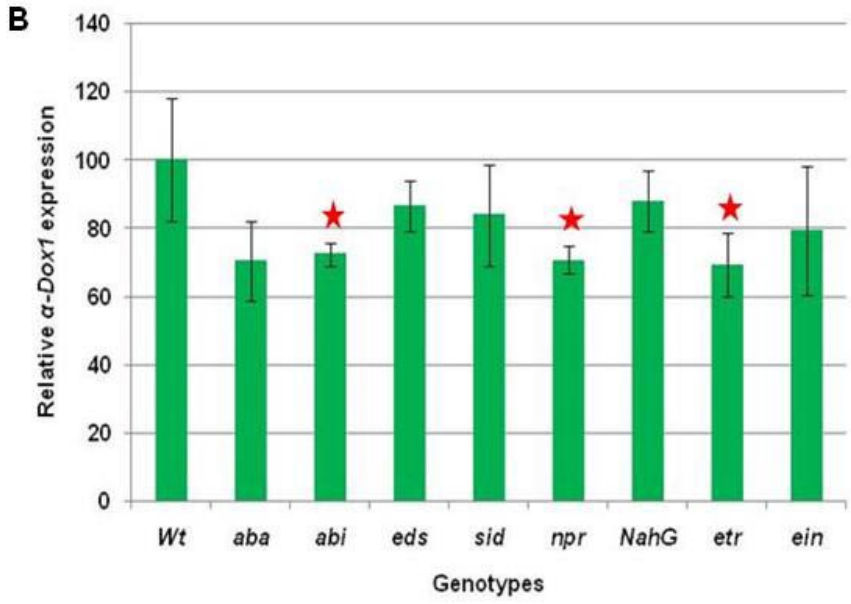
ein
C N C N C N C N C N C N C N C N C N
Ata-DOX1
18S rRNA


B
Relative α-Dox1 expression
140
120
100
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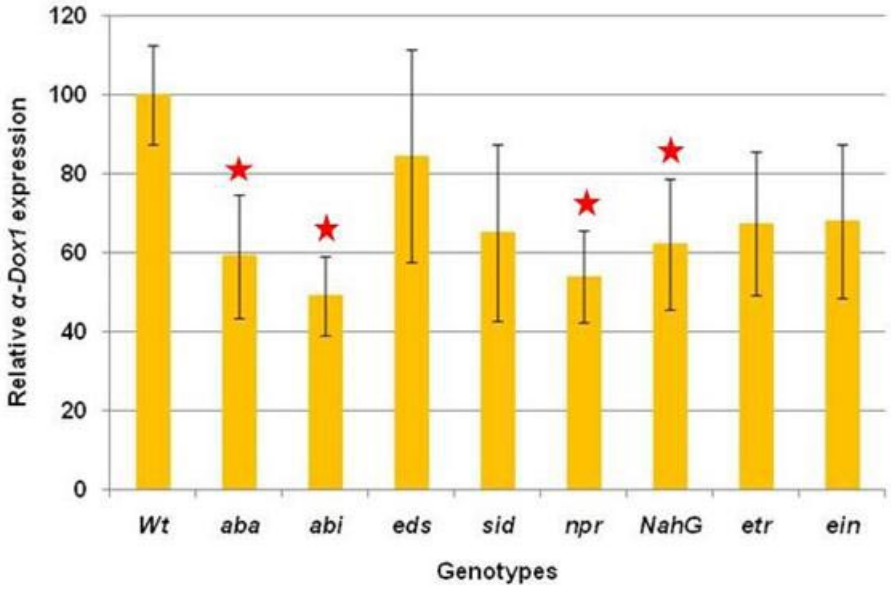
ein
Genotypes

C


120
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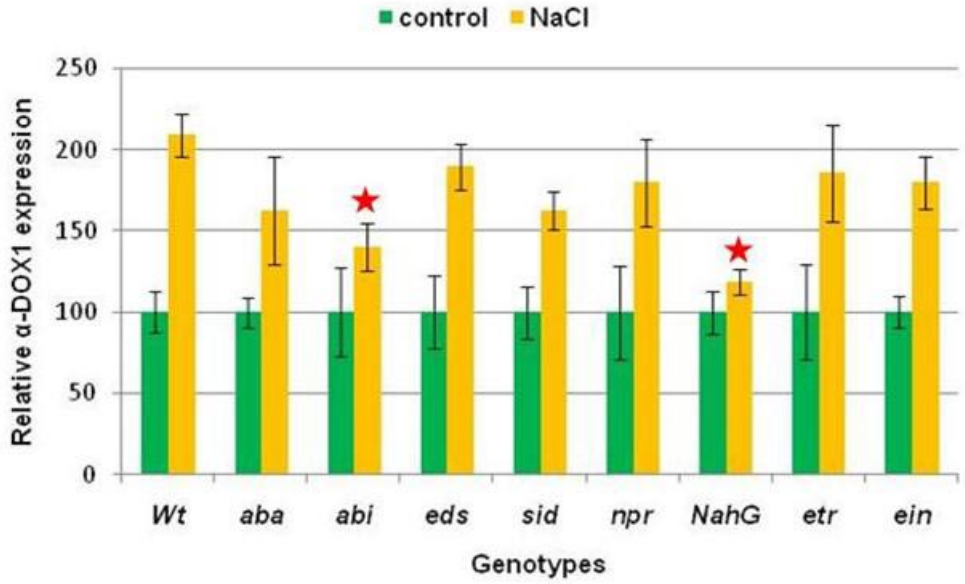
Genotypes

D


control
NaCl
250
200
150
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50
0
Relative α-DOX1 expression
Wt
aba
abi
eds
sid
npr
NahG
etr
ein
Genotypes

3-2 Microarray data on Atα-DOX expression in Arabidopsis mutant plants (Genevestigator V3, mutation tool)

Experimental sample (mutant)	Control sample	Anatomical part	Developmental stage	Gene probe	Expression ratio
rhd2-1	Col-0	Lateral root	Seedling	α-DOX1	0.11
				α-DOX2	0.66
lec1-1	Ws.8	Seed		α-DOX1	0.12
				α-DOX2	0.27
ein2	Col-0	Adult leaf	Developed flower	α-DOX1	0.42
				α-DOX2	1.39
coil	Col-0	Adult leaf	Developed flower	α-DOX1	0.5
				α-DOX2	1.62
abi1-1	Ler-0	Hypocotyl		α-DOX1	0.99
				α-DOX2	1.03
nahG	Col-0	Adult leaf	Developed flower	α-DOX1	1
				α-DOX2	1.06
etr1-1	Col-0	Seedling	Young rosette	α-DOX1	1.06
				α-DOX2	1.32
aba1-1	Ler-0	Seed		α-DOX1	2.3
				α-DOX2	0.67
npr1	Col-0	?	?	α-DOX1	1.58
				α-DOX2	0.88

arf7:arf19	Col-0	?	?	α-DOX1	1.83
				α-DOX2	2.45
gh3.5-1D	Col-0	Adult leaf	Developed rosette	α-DOX1	19.39
				α-DOX2	2.26
cpr5:npr1	Col-0	Adult leaf	Developed rosette	α-DOX1	23.55
				α-DOX2	1.3
cpr5	Col-0	?	?	α-DOX1	51.76
				α-DOX2	1.25

accumulation based on microarray data were included in table 3-2 to provide additional insight into the hormone regulation of *Atα-DOX1*. The lowest level of *Atα-DOX1* expression was observed in *rhd2-1* (*root hair defective*) and *lec1-1* (*leafy cotyledon1*), whereas the highest accumulation was detected in the double mutant *cpr5:scv1* (*cpr5,* constitutive expression of systemic acquired resistance; *scv1,* suppressor of *cpr5*) (Table 3-2). High expression of *Atα-DOX1* was also observed in the auxin response mutant (*gh3.5*), in the double mutant *cpr5:npr,* and in *cpr5* (Table 3-2).

Based on Northern blot analyses, constitutive expression of *Atα-DOX1* was not observed in the shoot tissues of Wt or any hormone mutant except *aba1-3* (Figure 3-7). *Atα-DOX1* expression was induced in all hormone mutants when plants were salt stressed; however, the levels of expression in salt stressed *aba1-3*, *etr1-1*, *eds5-1*and *sid2* plants were lower than that of Wt (Figure 3-7).

Atα-DOX2 was constitutively expressed in the shoots of Wt and in hormone mutant plants. The *Atα-DOX2* transcript accumulation in salt stressed *aba1-3*, *abi1-1*, *npr1-1* and *NahG* was lower than that of wild type. *Eds5-1, sid2-1, etr1-1*, *ein2* mutants and Wt had similar *Atα-DOX2* transcript accumulation under salt stress (Figure 3-8). Based on microarray data analyses, all hormone mutants had a similar level of *Atα-DOX2* expression compared to Wt with the exception of *aba1-1* which had a lower (by almost one half) *Atα-DOX2* transcript accumulation than Wt, however, the *Atα-DOX2* expression data in *aba1-1* was obtained from seeds not from the shoot tissue (Table 3-2). *Atα-DOX2* expression in *coi1* was higher than that of Wt and *Atα-DOX2* expression in this

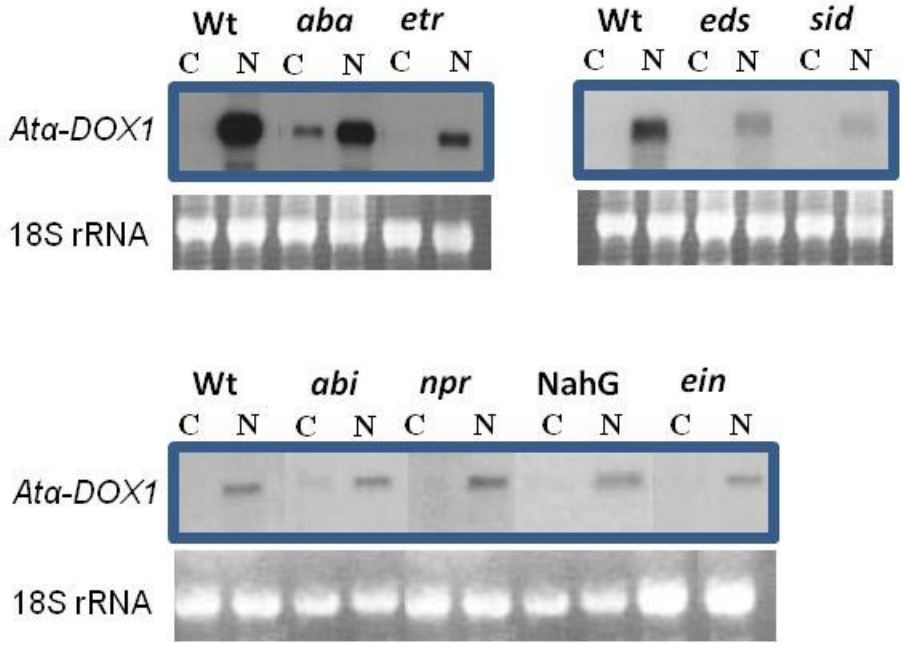


Figure 3-7 *Atα-DOX1* expression analysis in the shoots of hormone mutants.

RNA was extracted from the shoots of three-week-old mutant Arabidopsis plants following exposure to ½ MS media (C) or 250 mM salt (N) for 24 h. Genotypes used are wild type (Wt), *aba1-3* (*aba*), *abi1-1* (*abi*), *eds5-1* (*eds*), *sid2* (*sid*), *npr1-1* (*npr*), *NahG*, *etr1-1* (*etr*), *ein2* (*ein*). Each treatment was carried out 2 to 3 times. A representative northern blot and corresponding ethidium bromide stained gel is shown.

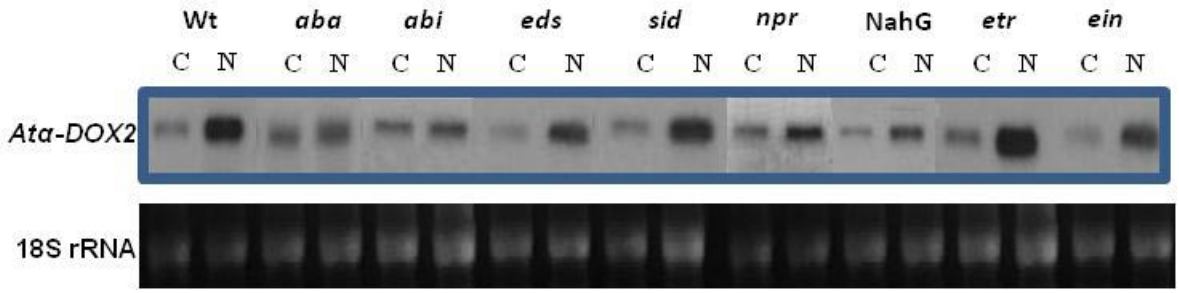


Figure 3-8 *Ata-DOX2* expression analysis in the shoots of hormone mutants.

RNA was extracted from the shoots of three-week-old mutant Arabidopsis plants following exposure to ½ MS media (C) or 250 mM salt (N) for 24 h. Genotypes tested include wild type (Wt), *aba1-3* (*aba*), *abi1-1* (*abi*), *eds5-1* (*eds*), *sid2* (*sid*), *npr1-1* (*npr*), *NahG*, *etr1-1* (*etr*), *ein2* (*ein*). Each treatment was carried out 3 times. A representative northern blot and corresponding ethidium bromide stained gel is shown.

mutant was more than 3 times higher than that of *Atα-DOX1*. The data presented in table 3-2 includes the microarray data for mutants that had the lowest and the highest *Atα-DOX2* expression. The *lec1-1* mutant had the lowest *Atα-DOX2* expression and the double auxin response mutant *arf7:arf19* exhibited the highest *Atα-DOX2* transcript accumulation. Overall, *Atα-DOX2* shows less variability in expression in hormone mutants as compared to *Atα-DOX1*.

3.4 DISCUSSION

3.4.1 Salt stress increases Atα-DOX activity in roots and shoots of Arabidopsis

As shown in chapter 2, *α-DOX* expression was positively regulated by salinity stress in Arabidopsis. In this chapter, I have shown that increased *Atα-DOX* expression resulted in increased α-DOX activity in both root and shoot tissues when Arabidopsis plants were salt stressed. This indicates that salt stress leads to an increase in α-DOX enzyme activity. A two-fold increase in α-DOX activity in the roots and shoots of salt stressed plants suggests that α-DOX products including C17 ald and 2HOT may play a role in mediating a plant's response to salt stress. *Atα-DOX1* was constitutively expressed in the root, which explains the Atα-DOX activity present in the root tissues of non-stressed plants. Furthermore, the α-DOX activity in the roots was 10 times higher than that of the shoot and this is consistent with the higher expression of *α-DOX*, particularly *α-DOX1* in roots compared to the shoots. Since salt stress induced *Atα-DOX2* and up-regulated *Atα-DOX1* expression in roots, α-DOX activity in salt-stressed roots may arise from both α-DOX1 and α-DOX2 action. Roots are the

first organ to experience salinity stress. An exposure to salt reduces root growth and development, which reduces their capacity to explore the soil environment for mineral and water uptake. Possibly, α-DOX products play a role in protecting root cells against damage caused by salt stress.

Atα-DOX2 was constitutively expressed in shoot tissues although *Atα-DOX1* was not. Increased enzyme activity was observed in the shoot tissues of salt stressed plants, implying the possible involvement of α-DOX products, possibly derived from both α-DOX1 and α-DOX2 in salt stress responses in leaves. The levels of α-DOX activity in the shoots was lower than the activity detected in roots and this is consistent with the low level of *Atα-DOX2* expression in shoot tissues. Thus, the main location for α-DOX enzyme activity is in the roots. The preferential occurrence of α-DOX in roots indicates possible roles for α-DOX in 1) root development, and/or 2) root growth adaptation to the soil environment, and/or 3) as a permanent system of protection against infection and damage caused by factors such as soil-borne bacteria. Meisner et al. (2008) detected *α-DOX* expression predominantly in the roots and cotyledons of pea plants during germination, thus suggesting the involvement of α-DOX during development.

Studies with Arabidopsis transgenic lines over expressing *Atα-DOX1* suggest that enhancing the activity of α-DOX1 is part of the defense mechanism induced to protect cells from oxidative stress (Ponce de León et al., 2002). High levels of the α-DOX1 product 2HOT accumulated in tobacco leaves undergoing an HR and this compound may exert a tissue-protective effect (Hamberg et al.,

2003). The ionic and osmotic stresses imposed by high salinity on plants create secondary stresses including oxidative stress (Xiong and Zhu, 2002), which is an important constraint for salt tolerance. Salt tolerance is associated with ROS detoxification. For example, Tsagane et al. (1999) isolated several salt tolerant mutants and found that their enhanced tolerance was associated with increased ROS detoxification as a result of activation of superoxide dismutase (SOD) and ascorbate peroxidase. It is possible that α-DOX plays a role in protecting leaf cells against oxidative stress caused by reduced in photosynthesis due to salt-induced stomatal closure and/or damage to chloroplasts. The products of α-DOX activity might also contribute to salt tolerance by regulating salt stress signalling pathways to protect cells in root and shoot tissues from oxidative stress.

3.4.2 ABA, SA and ET regulate *Atα-DOX* expression

Upon exposure to salt stress, plants undergo a wide range of responses at the molecular, cellular and whole plant levels. Many of these responses are triggered by the primary osmotic/ionic stress signals such as turgor changes whereas others may result from secondary signals such as the phytohormones ABA and ethylene. In this chapter, I first explored the regulation of *Atα-DOX1* and *Atα-DOX2* expression in response to ABA, JA, auxin, ethylene and SA and found that ABA, ethylene and SA had an effect on *Atα-DOX1* and *Atα-DOX2* expression. ABA is the primary hormone associated with abiotic stress (Skriver and Mundy 1990). ABA biosynthesis is increased in the leaves and roots of salt-stressed plants (Xiong and Zhu, 2002; Jin et al., 2000; Iuchi et al., 2000). In tomato, 9-cis-epoxycarotenoid dioxygenase 1 (NCED1*)*, the rate-limiting enzyme

for ABA biosynthesis, is upregulated by salt (Thompson et al., 2007). In salt and drought –stressed plants, ABA is involved in stomatal closure, root growth maintenance and it positively regulates the expression of numerous genes (Seki et al., 2007; Zhu, 2002). Gupta et al. (1998) reported that ABA-inducible genes are predicted to play an important role in the mechanism of salt tolerance in rice. Both northern blot analysis and GUS assays showed that expression of *At*α*-DOX1* and *At*α*-DOX2* was responsive to an exogenous ABA treatment in root and shoot tissues; thus ABA regulated *At*α*-DOX* expression in Arabidopsis.

Mutant plants defective in responding to, or producing ABA, were used to further explore the role of ABA in regulating *At*α*-DOX* expression under salt stress. *Aba1-3* has a mutation in *ZEAXANTHIN EPOXIDASE* that encodes the enzyme for the first step in ABA biosynthesis. As a result, *aba1-3* has impaired ABA biosynthesis. The ABA-insensitive mutant, *abi1-1,* has a mutation in a protein serine/threonine phosphatase 2C involved in ABA signal transduction. In the roots of these mutants, *At*α*-DOX1* expression increased in response to salt; however, the expression level in non-stressed and salt stressed mutant plants was lower than that of wild type. These data imply that ABA influences the basal expression level of *At*α*-DOX1* in roots. In the shoots, the expression of *At*α*-DOX1* in salt stressed ABA mutants was lower than that of the wild type, implying a role for ABA in regulating *At*α*-DOX1* in shoots. Constitutive expression of *At*α*-DOX1* was observed in the *aba1-3* mutant, suggesting that endogenous ABA suppresses the expression of *At*α*-DOX1* in shoots of control plants. However, ABA is known to inhibit ethylene production and activity (Sharp and LeNoble,

2002; Steffens et al., 2006); thus, in *aba1-3* leaves, the low ABA content may trigger ethylene synthesis which in turn regulated the expression of *At*α*-DOX1*. Microarray data for the *aba1-1* and *abi1-1* mutants did not support the Northern data since *At*α*-DOX1* expression in *aba1-1* and *abi1-1* was higher or the same as in wild type, respectively. However, discrepancies may arise due to differences in anatomical parts used in northern and microarray analyses.

The low level of *At*α*-DOX2* expression in leaves of salt stressed *aba1-3* and *abi1-1* indicated that ABA is involved in regulating *At*α*-DOX2* expression in response to salt. Both ABA mutants had a lower level than wild type of *At*α*-DOX2* expression in shoots of salt stressed plants. This suggests that a normal level of ABA and full operation of the ABA signalling pathway are important factors in regulating *At*α*-DOX2* expression in salt-stressed shoots. The lower *At*α*-DOX2* expression in *aba1-1* compared to wild type as revealed by microarray data further supports the importance of ABA in the regulation of *At*α*-DOX2*. These data support the importance of ABA as a major hormone that up-regulates *At*α*-DOX1* and *At*α*-DOX2* expression in both roots and shoots.

Increased ethylene production in salt stressed seedlings has been reported (Achard et al., 2006). Also, in tomato roots, salt stress up-regulated the ethylene response pathway since salt enhanced the expression of *ACS3, ACO1* and *ERF1*, that encode enzymes for ethylene biosynthesis, and action, respectively (Kwok and Plant, unpublished) and recently Wang et al. (2007) reported that *EIN2* is a salt responsive gene. Ethylene markedly increased the accumulation of the *Le*α*-DOX1* transcript in tomato roots (Tirajoh et al, 2005).

However, ACC did not up-regulate *Atα-DOX1* expression in roots as revealed by both northern and microarray analyses. In the shoot, ACC induced and increased *Atα-DOX1* and *Atα-DOX2* expression, respectively, indicating that ethylene may be an important regulator of *Atα-DOX* expression in shoots. However, GUS staining was not observed in leaves of ACC-treated *Atα-DOX1*::GUS transgenic plants. It is possible that the concentration of ACC used in GUS staining experiment was not optimal to induce *Atα-DOX1* expression in leaves.

Atα-DOX1 expression was reduced in the roots of the ET response mutant *etr1-1* under non-stressed conditions and in roots of both *etr1-1* and *ein2-1* under salt stressed conditions. Even though ACC did not increase *Atα-DOX1* expression in roots, the reduced expression in *etr1-1* and *ein2*, which are ET-insensitive, suggests that ethylene plays some role in regulating *Atα-DOX1* expression in roots. The reduced *At-α-DOX1* expression in shoots of salt stressed *etr1-1* further supports a role for ethylene in regulating *Atα-DOX1* expression. Similarly, for *At-α-DOX2,* the decreased expression observed in shoots of *ein2* supports a role for ET. Taken together, these data suggest that ethylene plays a role in regulating *Atα-DOX1* and *Atα-DOX2* in salt stressed plants but it may not be a major signal for inducing/up-regulating *Atα-DOX1* expression in Arabidopsis.

Salicylic acid (SA) plays an important role in the defence response of plants to pathogen attack (Durner et al., 1997). SA mediates the oxidative burst that leads to cell death during the HR, and acts as a signal for the development

of systemic acquired resistance (Verberne et al., 2000). *At-DOX1* expression is induced by SA in leaves (Ponce de León et al., 2002); however, to my knowledge there are no studies on SA responsive *At*α*-DOX* expression in the roots. SA clearly played a role in up-regulating *At-*α*DOX1* expression in the roots and that of *At-*α*DOX1* and *-2* in the shoots of Arabidopsis. SA-induced up-regulation of *At*α*-DOX2* expression in shoots was not supported by microarray data but this can be explained by differences in the developmental stage, tissue type and concentration of SA used. For example, Northern blot analysis was carried out on shoot tissues of 3-week old plants, whereas microarray data was based on seedlings. Only a few studies support a major role for SA in modulating a plant response to abiotic stress (Yalpani et al., 1994; Senaratna et al., 2000). Osmotic stress activates an SA-induced protein kinase (Mikolajczyk et al., 2000) and the salt stress responsive gene, *TOBACCO STRESS-INDUCED GENE 1*, is induced by SA (Park et al., 2001). However, the involvement of SA in salt stressed plants is not well established.

To further understand the importance of SA in regulating *At*α*-DOX* expression under salt stress, *At*α*-DOX* expression in hormone mutants was explored. There are two pathways that lead to SA accumulation in plants. SA can be synthesized from phenylalanine in the cytoplasm and from chorismate by means of isochorismate synthase in plastids (Coquaz, et al., 1998). In this research, the *sid2-1* and *eds 5-1* mutants from the isochorismate pathway were used. In addition to these two mutants, *npr1-1,* which has a mutation in NPR1 needed for the activation of SA induced systemic acquired resistance was

included (Uquillas et al., 2004). Finally, in order to distinguish between SA produced from the chorismate pathway and SA produced in the cytoplasm, *NahG* transgenic plants carrying the bacterial SA hydroxylase gene were used since this mutant lacks SA. The finding that SA plays a role in up-regulating *Atα-DOX1* expression in salt stressed roots was substantiated by the absence of salt-induced up-regulation of *Atα-DOX1* in the *NahG* plants. In addition, the reduced *Atα-DOX1* expression in roots of non-stressed *npr1-1* and salt stressed *npr1-1* and *nahG* plants suggests that SA contributes to *Atα-DOX1* expression in roots. Microarray data revealed that *Atα-DOX1* expression in *npr1* was higher than the wild type. However, as stated before, this difference between northern and microarray data could be due to differences in the developmental stage of the plants or the organs used for analyses. In the shoots, the accumulation of the *Atα-DOX1* transcript in *eds5-1* and *sid2-1* in response to salt stress was lower than in the Wt. In addition, the accumulation of *Atα-DOX2* in *npr1-1* and *NahG* was lower than in the wild type. Thus, SA produced in the cytoplasm plays a role in regulating *Atα-DOX* expression in roots and shoots of salt stressed plants.

3.4.2.1 Microarray data mining reveal regulation of *Atα-DOX* by SA and ROS

Analysis of microarray data for mutations that drastically affect *Atα-DOX1* and *-2* expression revealed additional and novel information regarding the factors that regulate their expression. Very low *Atα-DOX* expression was observed in the *rhd2-1* (*root hair defective*) and *lec1-1* (*leafy cotyledon1*) mutants. ROS production by the NADPH oxidase (NOX) encoded by *AtrbohC/RHD2* is required for root hair growth in *Arabidopsis thaliana* (Jones et al., 2007) and ROS

formation is much lower in root hairs and lateral roots of the *rhd2* mutant (Foreman et al., 2003). Ponce de León et al. (2002) proposed that signals, including intracellular superoxide (O_2^-) or singlet oxygen, acted additively with SA in inducing *At*α*-DOX1* in pathogen inoculated Arabidopsis leaves. However, H_2O_2 which accumulates during oxidative stress, did not induce *At*α*-DOX1* expression in leaves (Ponce de León et al., 2002) or up-regulate its expression in roots of Arabidopsis (data not shown). These data suggest that O_2^- and/or other ROS are required for *At*α*-DOX1* and *-2* expression and the low level of ROS in lateral roots of *rhd2* contribute to the low level of the *At*α*-DOX1* transcript. The low expression of *At*α*-DOX1* and *-2* in the *lec1-1* seeds suggests a possible role for *At*α*-DOX* during early plant development. This is consistent with a proposed role for *Le*α*-DOX3* in development as revealed by the phenotype of the *feebly* tomato mutant. Lec1 is a transcription factor that mediates the switch between embryo and vegetative development (Vicient et al., 2000). Further possible involvement of *At*α*-DOX2* in development is provided by the increased transcript level in the double mutant *arf7*:*arf19*. ARF7 and ARF19 encode transcriptional activators of early auxin response genes and the *arf7:arf19* double mutant is severely impaired in lateral root formation (Okushima et al., 2007).

A possible role for JA in up-regulating *At*α*-DOX1* is suggested by the reduced expression in the JA resistant mutant, *coi1*. However, exogenous application of JA did not increase *At*α*-DOX1* and *-2* expression in the roots and shoots of Arabidopsis (data not shown). High levels of *At*α*-DOX1* and *-2* expression were detected in mutants and mutations with altered responses to

auxin and SA. One of these mutants with a high level of *Atα-DOX* expression is *gh3.5-1D* in which GH3.5 is over-expressed. GH3.5 is a bifunctional modulator in both SA and auxin signalling during pathogen infection (Zang et al., 2007). Similarly *Atα-DOX1* expression was high in the *cpr5* mutant, which constitutively expresses SAR. For both of these mutants, SA is possibly responsible for the increased *Atα-DOX* transcript accumulation since both *gh3.5-1D* and *cpr5* possess elevated levels of SA (Zang et al., 2007; Bowling et al., 1997). An elevated level of *Atα-DOX1* expression and a marginal increase in *Atα-DOX2* expression was also observed in the *cpr5*:*npr1* double mutant; however, the expression level of *Atα-DOX1* was half of that in the *cpr5* mutant. This suggests that NPR1-dependent (SA) and -independent (JA, ethylene) pathways additively up-regulate *Atα-DOX1* expression since blocking the NPR1-dependent pathway in *cpr5:npr1* reduced the transcript level to half that observed in *cpr5*.

In summary, the *Atα-DOX1* transcirpt level in roots was enhanced by ABA and SA and the salt induced up-regulation of *Atα-DOX1* expression in roots was dependent, at least in part, on ABA and SA pathways. In the shoot, both *Atα-DOX* genes were up-regulated by ABA, SA and ethylene. ABA may be involved in supressing *Atα-DOX1* expression in shoots under non-stressed conditions. Based on microarray data only, JA may play a role in establishing the basal expression level of *Atα-DOX1* in leaves under non-stressed conditions.

4: FUNCTIONAL ANALYSIS OF ALPHA-DIOXYGENASE IN SALT-STRESSED ARABIDOPSIS

4.1 Introduction

The enzymatic activity of α-DOX proteins leads to the production of novel oxylipins. The activity of α-DOX is increased by salt stress in the leaves and roots of Arabidopsis and the oxylipins produced presumably influence the response of roots to salt stress. In general terms, oxylipins are lipid derivatives generated by the oxygenation and further transformation of fatty acids. The first step in oxylipin biosynthesis is the oxygenation of a fatty acid, which is carried out by lipoxygenases (LOX) or α-DOX ezymes (Vellosillo, et al., 2007). The α-DOX enzymes are involved in fatty acid α-oxidation whereby stereospecific oxygenation at the α-carbon of the fatty acid chain provides an unstable 2-hydroperoxy fatty acid which is further converted into 2-hydroxy fatty acids and $C_{(n-1)}$ aldehydes. The α-oxidation pathway in mammals is of critical importance for the degradation of phytanic acid (3, 7, 11, 15-tetramethyl hexadecanoic acid) and other β-methyl branched fatty acids (Verhoeven et al., 1998); however, the function of the corresponding pathway in plants is not understood.

Sanz et al. (1998) reported that sequence analysis of plant α-DOX showed significant similarities with prostagladin endoperoxide synthase (PGHS) also referred to as cyclooxygenase (COX), a key enzyme in vertebrates that mediates the conversion of polyunsaturated substrates to prostaglandins (DeWitt and

Smith, 1995). Prostaglandins serve as intracellular and /or extracellular lipid-derived signals and mediate many cellular responses such as the immune response and inflammation (Serhan et al., 1996). The role of α-DOX in plants was first investigated in Arabidopsis plants responding to bacterial pathogens (Ponce de León, 2002). In Arabidopsis, reduction of α-DOX activity is correlated with a higher level of ion leakage, indicative of cellular damage, whereas enhanced α-DOX activity exerted a protective effect and limited the necrotic leaf area affected by bacterial inoculation (Ponce de León, 2002). There are similarities between plant α-DOX and animal COX at the level of biological function. For example, as found for the *Atα-DOX1* gene, expression *of COX-2* is induced in response to the generation of intracellular superoxide (O_2^-) and the production of an active COX-2 enzyme plays a role in protecting cells against ROS (Ponce de León et al., 2002; Kawamoto et al., 2000). Likewise, as with prostaglandins, the lipid-derived compounds synthesized through the activity of α-DOX1, or other derivatives synthesized from them, could act as signalling molecules to reduce damage caused by pathogen attack in plants (Ponce de León et al., 2002).

Jasmonic acid (JA) is a well characterized oxylipin that is involved in plant developmental processes such as flower development and tendril coiling (Li et al., 2004; Stintzi and Browse, 2000; McConn and Browse, 1996; Falkenstein et al., 1991). JA is also a potent inhibitor of root growth (Wasternack, 2007). The involvement of JA and other oxylipins in root growth and development was recently described by Vellosillo et al. (2007) who observed root waving

accompanied by inhibition of the growth of lateral roots, or root growth arrest with a loss of apical dominance when various oxylipins were present in the growing media. This implies a role for oxylipins in regulating root growth and development.

In chapters 1 and 2, I have shown that *Atα-DOX1* is predominantly expressed in roots and that both *Atα-DOX1* and *Atα-DOX2* are salt responsive in roots and shoots. I have also shown that there is an increased level of α-DOX products in salt versus unstressed plants. This chapter explores the functional significance of α-DOX in plants responding to salt stress.

Hypothesis 1. Atα-DOX products increase tolerance to salt stress.

Hypothesis 2. Atα-DOX products increase tolerance to oxidative stress.

Hypothesis 3. Atα-DOX products direct changes in root system architecture.

To test these hypotheses, I undertook a reverse genetics approach and Arabidopsis lines with increased or decreased α-DOX activity were generated and used to assess:

1. The effects of α-DOX on salt stress tolerance
2. The effects of α-DOX on the production of ROS
3. The effects of α-DOX on root system architecture

4.2 Material and methods

4.2.1 Growth Conditions

Seeds of wild type *Arabidopsis thaliana* ecotype Col-4, *Atα-DOX1* knock-out (D1K), *Atα-DOX2* knock-out (D2K), *Atα-DOX1*/*Atα-DOX2* double knock-out (DK), *35S::Atα-DOXI* (*Atα-DOX1* over-expresser, OEI) and *35S::Atα-DOX2* (*Atα-DOX2* over-expresser, OEII) were gas sterilized with NaClO and HCl for 2 h. The sterilized seeds were grown on 1% (w/v) agar plates containing ½ MS (Murashige and Skoog) medium supplemented with 1.5% (w/v) sucrose (for control) or with 1.5% sucrose (w/v) plus 75 mM NaCl (for salt stress) in petri dishes (150 x 25 mm). The plates were sealed with 3M Micropore Surgical tape (3M Health care, D-41433 Neuss, Germany) and incubated at 4°C for two days for stratification. Plates were placed vertically in a growth room under a 16/8 h light/dark cycle (80 µmol/s/m^2) at 22 °C. For H_2O_2 visualization experiments, 7-day-old (visualization in roots) or 14 day-old (visualization in leaves) seedlings were individually transferred to the treatment medium consisting of ½ MS (control) or ½ MS with 150 mM NaCl for salt treatment.

4.2.2 Verifying T-DNA insertion mutants of *Atα-DOX1* and *Atα-DOX2*

T-DNA tagged *Atα-DOX* insertion lines were identified in the SIGnal database (http://signal.salk.edu/cgi-bin/tdnaexpress) and seeds for four lines were obtained from ABRC. The SALK-042813 line has a T-DNA insertion in the last exon of *Atα-DOX1*. SALK-068824 has a T-DNA insertion in the 3'UTR of *Atα-DOX1*. SALK-029547 has a T-DNA insertion in exon 6, and SALK-089649 has a T-DNA insertion in exon 8 of *Atα-DOX2.* Ten seeds from each line were

germinated and grown for 2 weeks after which DNA was extracted from the leaves as described in section 2.2.5. Primer sequences specific for each line together with the T-DNA-specific primer (LBA1) were obtained from the SIGnal database to amplify the junction of the Atα-DOX1::T-DNA or Atα-DOX2::T-DNA insertion and to select homozygous lines. PCR-based screening was performed following the conditions described in 2.2.3.1 and the following primers:
5'-TACATTTTCCTCATCATGGCC -3' (SALK-068824-RP),
5'-TCTCAAGGCAAATAGGCAATG-3'(SALK-068824-LP),
5'-AGTATTCACCCATTCAAGCCC-3' (SALK-042813-RP),
5'-AGTTCGATCTTGGGAGGTGTC-3' (SALK-042813-LP),
5'-AGAAAAGGCTTCGAAACTTGG-3' (SALK-029547-RP),
5'-TCAAGGTGGCAAATGAAAATG-3' (SALK-029547-LP),
5'-GTTTCGAAGCCTTTTCTCCTG-3' (SALK-089649-RP),
5'- GGATCCAGTTCATGATCCATG-3' (SALK-089649-LP),
5'-TGGTTCACGTAGTGGGCCATCG-3' (LBA1). T3 seeds from plants homozygous for the T-DNA insertion were collected for further analysis.

4.2.2.1 Northern Blot Hybridization Analyses

Atα-DOX1 knock-out (D1K), *Atα-DOX2* knock-out (D2K) and wild type seeds were germinated and grown for three weeks in magenta boxes containing 50 mL ½ MS supplemented with 1.5% (w/v) sucrose. Growth conditions and salt stress treatments were described in section 3.2.2. Northern hybridization analyses were performed as described in section 2.3.6 to confirm the absence of

Atα-DOX1 transcript in D1K plants and the absence of *Atα-DOX2* transcript in D2K plants.

4.2.3 Generation of *Atα-DOX* Double Knock-out

The SALK-042813 knock-out line for *Atα-DOX1* (D1K) and the SALK-29547 knock-out line for *Atα-DOX2* (D2K) were used to generate a double *Atα-DOX1*/ *Atα-DOX2* knock-out by crosses. Pistils of D1K plants were pollinated with anthers from D2K plants and vice versa. The F1 seeds were collected from each cross, grown and subjected to PCR analyses for the presence of T-DNA in both *Atα-DOX1* and *Atα-DOX2*. The resulting F1 heterozygous double knock-outs were grown to generate F2 homozygous double knock-outs. Plants with a homozygous T-DNA insertion in both *Atα-DOX1* and *Atα-DOX2* were selected from the progeny as follows. Using DNA isolated from a single leaf, two separate PCRs were performed. The first PCR was performed with SALK-042813 RP, LP and LBA1 primers to amplify the junction of the *Atα-DOX1::*T-DNA insertion. The second PCR was performed with primers SALK-029547 RP, LP and LBA1 to amplify the junction of the *Atα-DOX2*::T-DNA insertion. T3 seeds from plants with homozygous TDNA insertions in both *Atα-DOX1* and *Atα-DOX2* (DK) were collected for further analysis.

Wild type and T3 D1K, D2K, DK (confirmed at DNA level) seeds were grown as described in section 4.2.1. Seven-day old seedlings were transferred to a Petri dish containing ½ MS media (control) or ½ MS media+250 mM NaCl (salt) for 24 h. Total RNA was extracted from control and salt stressed seedlings as described in section 3.2.3. Northern blot analysis was carried out as

described in sections 3.2.4 and 3.2.5. Northern blots were hybridized with an *Atα-DOX1* specific probe or an *Atα-DOX2* specific probe as described in section 2.3.6. One line that showed complete absence of both the *Atα-DOX1* and the *Atα-DOX2* transcript was selected.

4.2.4 Generation of *Atα-DOX1* and *Atα-DOX2* Over-expressers

The Gateway entry vector pENTR/D-TOPO carrying the *Atα-DOX1* cDNA (U15100) and the *Atα-DOX2* cDNA (U16142) were ordered from ABRC. The nucleotide sequence for both clones was confirmed using the M13 forward primer. *E.coli* TOPO10 (Invitrogen) cells were transformed with U15100 and U16142 plasmids as described in section 3.2.7. The *Atα-DOX1* and *Atα-DOX2* cDNA inserts from the pENTR clones were recombined into the Gateway pK2GW7 destination vector downstream of the 35S promoter using the LR clonase reaction to generate the *35S::Atα-DOX1* and *35S::Atα-DOX2* constructs. The ligation was carried out as described in section 3.2.7. One shot® chemically competent *E.coli* cells were transformed with *35S::Atα-DOX1* or *35S::Atα-DOX2* plasmids as described in section 3.2.8. Positive clones were confirmed using the PCR conditions described in section 2.2.3.1 with *Atα-DOX1* and *Atα-DOX2* specific primers, Atα-DOX1 forward (5'-GAATGTCCCTCCTCGATGCC-3') and reverse (5'-GGAGCTTAAGGGACACTTG-3') and *Atα-DOX2* forward (5'-ATGTCGGTCCGATCACGGTC-3') and reverse (5'-CAAGGGGTTCTAGTGTTGAC-3'). The orientation of the α-DOX cDNA inserts within the pK2GW7 destination vectors was determined by PCR using a forward primer that annealed to the *nptII* (Kanamycin resistant gene) (5'-

CTGCGTGCAATCCATCTTGT-3') gene in the destination vector and the reverse primers used to amplify the gene specific probe used for Northern hybridization (see section 3.2.4). The PCR conditions were similar to the conditions described in section 2.2.3.1 with the exception of an extention time of 90 sec at 72°C.

Agrobacterium tumefaciens (strain GV3101) cells were transformed with *35S::Atα-DOX1* or *35S::Atα-DOX2* constructs via electroporation as described in section 2.2.6.1. Positive clones were confirmed using PCR with the same primers used to amplify the gene specific probes. *Arabidopisis thaliana* plants were transformed using *Agrobacterium tumefaciens* carrying the *35S::Atα-DOX1* and *35S::Atα-DOX2* constructs using the floral dip method (Clough and Bent, 1998). Transformation and screening of putative transformants was carried out as described in section 3.2.10 and 3.2.11. Transformed plants were further screened by Northern hybridization to identify lines with high levels of *α-DOX1* and *α-DOX2* expression.

An alternative line with enhanced *Atα-DOX1* expression was obtained from Carmen Castresana, Centro Nacional de Biotecnologia, CSIC, Campus Universidad Autónoma, Cantoblanco, Madrid, Spain. The results reported in this chapter for the *35S*::*Atα-DOX1* plants are based on the line obtained from Spain. The *35S*::*Atα-DOX1* lines that I generated did not produce elevated levels of α-DOX activity, even though transcript accumulation was enhanced.

4.2.5 Root Growth, Lateral Root Density and Root Primordia Analysis

Seeds of wild type, D1K, D2K , DK, *35S::Atα-DOXI* (OEI) and *35S::Atα-DOX2* (OEII) were grown as described in section 4.2.1 for control and salt treatments. Two to three replicates with 12 seeds each were established per genotype per treatment. Petri plates (150X25 mm) were sealed with 3M™ Micropore™ surgical tape (3M Center, St. Paul, MN), incubated at 4°C for 3 days for stratification and then transferred to a growth room (16 h light/8 h dark) and placed vertically under fluorescent lighting (80 μmol/s/m^2) at 23^{o}C.

The position of the tip of each germinated root was marked two days after the transfer from the cold room to the growth room. The seedlings were grown for another 7 days. Root length of seedlings was measured from the marked points (2 days after the transfer) and the root growth rate was calculated for each seedling by dividing the total root growth by the number of days (7 days) (Figure 4-1). The number of lateral roots (LRs) was counted for each seedling on the ninth day and LR density (LRD) was calculated by dividing LR number by root length. Each experiment was repeated at least five times.

To count root primordia, 7 day-old seedlings were mounted onto microscope slides and primordia were counted at 100X magnification using a Carl Zeiss Axio Observer.Z1 inverted microscope. Photographs of representative root primordia were taken using a Hamamatsu 1394 OCRA-ERA camera in conjunction with Volocity 4.3.2 Build 23 software (Improvision Ltd, 2007). The root primordia of five seedlings per treatment per trial were counted. Three trials were conducted.

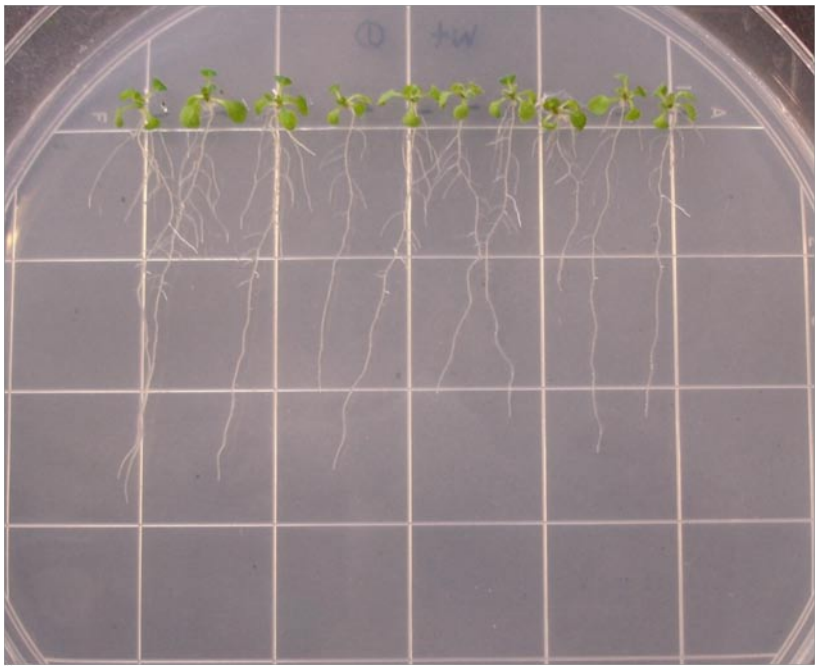


Figure 4-1 The root growth set-up used in this study

Seeds were individually sown on ½ MS + 1.5% sucrose + 0.8% agar with and without 75 mM salt. Seeds were allowed to germinate for 2 days in a growth room and root tips of the germinated seedlings were marked. Root growth was measured 7 days after germination.

4.2.5.1 Oxylipin treatment

Wild type seeds were germinated in ½ MS with 1.5% sucrose and 0.8% agar with and without 75 mM NaCl. The seedlings were exposed to pure oxylipins provided by Dr. Mats Hamberg and linolenic acid ($C_{18}H_{30}O_2$) was ordered from Sigma-Aldrich (Oakville, Ontario). The oxylipins were 2(R)-Hydroxylinolenic acid (2-HOT, $C_{18}H_{30}O_3$), Heptadecatrienal (C17-aldehyde, $C_{17}H_{28}O$) and Hydroperoxypalmitic acid ($C_{16}H_{32}O_4$). Oxylipins were applied at the tip of each primary root two days after germination and the applications were repeated three times every 2 days because of the volatile nature of the oxylipins. Root growth rate and lateral root density were calculated. This experiment was repeated three times with 2 replicates for each trial. The resulting data were inconsistent, possibly due to variations in amount of oxylipin taken up by the roots and the volatility of the compounds. Data are presented in the Appendix.

4.2.5.2 Data Analyses

All data were compiled and initial calculations were performed using Microsoft Office Excel. Data were pooled; mean root growth rate and standard error for each genotype and treatment were calculated. Seedlings with no or minimal growth were excluded from the analyses. LRD and LR primordia density was calculated for each seedling as the number of lateral roots or number of primordia per millimetre root length. The mean and standard error were calculated for LRD, as well as for the number of root primordia present, for each genotype and treatment.

Normalized values for root length, LRD, and LR primordia density were

calculated to determine the impact of salt stress. The values (root growth, LRD, LR primordia density) of salt-stressed seedlings were normalized by the mean control value for each genotype. Data were analyzed using the SAS JMP 7.0.2 software (SAS Institute Inc., Cary, NC, USA) to obtain least squares means and standard errors. Means were compared using student's t-test (P=0.05) to determine if there were significant differences between genotypes for root growth, LRD and LR primordia number or density.

4.2.6 Salt tolerance assay

Seeds for wild type, D1K, D2K , DK, OEI and OEII were grown as described in section 4.2.1. Seedlings were transferred to pots containing Sunshine Mix 4. Each pot contained six seedlings with two replicates for each genotype and for each treatment (control and salt). Three-week old plants were watered with varying amounts of salt (75, 100, 150, 200 mM) solution or water (control) every two days for a week.

Seeds were sterilized as described in section 4.2.1. Sterilized seeds were allowed to germinate on ½ MS media with 0.8% agar and 1% sucrose with or without 150 mM NaCl as described in section 4.2.5. Seeds were allowed to germinate in the growth room for 2 days and the number of germinated seeds was recorded. The number of green seedlings was counted 7 days later and the percent survival (# of green seedlings/# of germinated seeds) was calculated. The experiment was repeated 3 times.

Normalized survival rate was calculated to determine the impact of salt stress. The survival rate for wild type seedlings in salt-containing media was set as 100. The survival value of salt-stressed over-expressers and knock-outs seedlings was normalized by the mean for the wild type salt stressed. Data were pooled and analyzed using JMP 7.0.2 software as described in section 4.2.5.2. Student's t-tests were performed ($\alpha = 0.05$) to determine if there were significant differences between genotypes.

4.2.7 Alpha-DOX enzyme assays

Three-week-old control and salt treated plants were used for enzyme assay analysis. The experimental set up and the enzyme assays were performed as described in section 3.2.13.

4.2.8 Detection of H_2O_2 in salt-treated roots

Seeds were sterilized and grown as described in section 4.2.1. After 7 days, seedlings were transferred to 1/2 MS medium with or without 150 mM NaCl under light (120 μmol/s/m^2) for 12 h. Salt treated and untreated seedlings were incubated with 10 µM 2,7-dichlorofluorescein diacetate (H2DCFDA) (Sigma) for 5 min. Seedlings were washed thoroughly with 1/2 MS medium for 1 min and viewed under an epi-fluorescent microscope (Nikon Eclipse-E6000). Images were obtained with a digital camera. This experiment was repeated at least 3 times.

4.3 Results

The major products of α-DOX assays (C17 aldehyde and 2-hydroxylinolenic acid) were measured by GC-MS by Dr. Mats Hamberg (Department of Medical Biochemistry and Biophysic, Karolinska Institute, Sweden) as described in section 3.2.7. These results are presented in Figures 4-8 and 4-9.

4.3.1 Isolation and molecular analysis of *α-DOX1*, *α-DOX2* and double *α-DOX1*/*α-DOX2* knock-out mutants

4.3.1.1 Knock-out mutants

To understand the effect of decreased α-DOX activity, T-DNA insertion mutants for *α-DOX1* and *α-DOX2* were obtained. To identify *Atα-DOX1* and *Atα-DOX2* knock out lines, genomic DNA isolated from SALK lines carrying T-DNA insertions within the *Atα-DOX1* exon 10 (SALK-042813) (Figure 4-2 A) or 3'UTR (SALK-068824) and T-DNA insertions within the *Atα-DOX2* exons 6 and 8 (SALK-029547, Figure 4-3 A and SALK-089649) was subjected to one PCR with three primers. PCR with the right and left primers specific for the SALK-042813, SALK-068824, SALK-029547 or SALK-089649 lines together with a T-DNA left border primer was carried out to characterize the T-DNA tagged *Atα-DOX* allele. PCR products derived from the left and right primers specific to the *α-DOX1* or *α-DOX2* wild-type alleles were approximately 1000 bp (Figure 4-2 B, 4-3 B). Plants that were homozygous for the T-DNA insertion amplified a 700 bp fragment only and were used for further analysis (Figures 4-2, 4-3). The accumulation of the

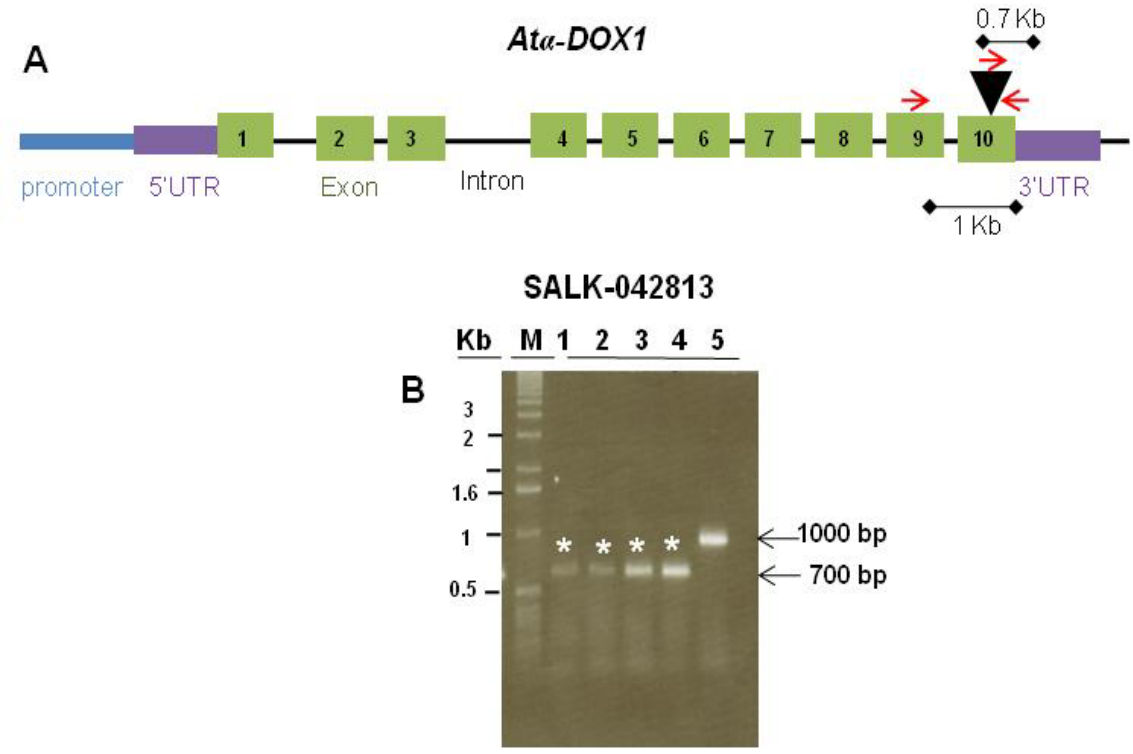


Figure 4-2 Isolation of a homozygous line for the T-DNA insertion in *Atα-DOX1*.

(A) A. Diagram showing the position of the T-DNA in exon 10 of *Atα-DOX1* in SALK-042813. The T-DNA insertion is marked by a triangle. The arrows indicate the position of the primers in the *Atα-DOX1* locus and the T-DNA insertion. (B) PCR screening to isolate a homozygous line. Genomic DNA was isolated form individual plants and subjected to PCR screening. An approximate 0.7 kb fragment was amplified in the homozygous lines of SALK-042813, which is designated with asterisk. M=marker lane.

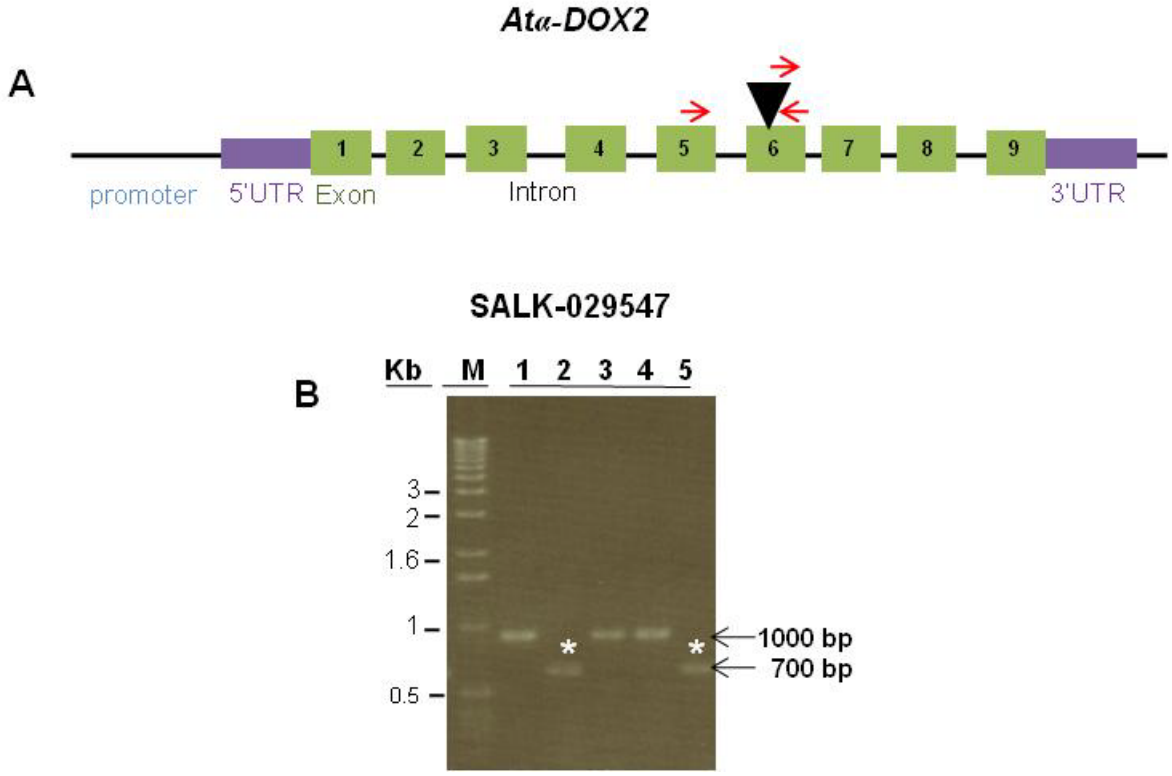


Figure 4-3 Isolation of a homozygous line for the T-DNA insertion in Atα-DOX2.

(A) A. Diagram showing the position of the T-DNA in exon 6 of *Atα-DOX1* in SALK_029547. The T-DNA insertion is marked by a triangle. The arrows indicate the position of the primers in the *Atα-DOX1* locus and the T-DNA insertion. (B) PCR screening to isolate a homozygous line. Genomic DNA was isolated form individual plants and subjected to PCR screening. An approximate 0.7 kb fragment was amplified in the homozygous lines of SALK-029547, which is designated with asterisk. M= marker lane.

Atα-DOX1 and *Atα-DOX2* transcript was assessed in the homozygous T-DNA insertion plants by northern blot analysis. No *Atα-DOX1* transcript was observed in SALK-042813 (Figure 4-4 A); however, the SALK-068824 line accumulated the *Atα-DOX1* transcript (data not shown). Both the SALK-029547 (Figure 4-4 B) and SALK-089649 lines (data not shown) showed complete elimination of the *Atα-DOX2* transcript. The SALK-042813 (D1K) and SALK-029547 (D2K) lines were used for further experiments.

To create a double α-DOX gene knockout mutant, the D1K and D2K plants were crossed to generate a heterozygous double *Atα-DOX1/ Atα-DOX2* knock-out mutant. The resulting F1 plants were selfed to recover F2 homozygous double *Atα-DOX1/ Atα-DOX2* knock-outs from the progeny. Plants that were homozygous for the T-DNA insertion in both *Atα-DOX1* and *Atα-DOX2* were selected (Figure 4-5 A, B) and used for northern analysis to ensure that the *Atα-DOX1* and *Atα-DOX2* transcripts were both absent. Figure 4-6 shows that both Atα-DOX1 and *Atα-DOX2* transcripts were absent in the putative *Atα-DOX1/ Atα-DOX2* knock-out lines. From now on, D1K will represent *Atα-DOX1* knock-out, D2K will represent *Atα-DOX2* knock-out and DK will represent *Atα-DOX1/ Atα-DOX2* double knock-out lines. No phenotypic differences between wild type, D1K, D2K and DK were observed.

4.3.1.2 Isolation and molecular analysis of *Atα-DOX* over-expressors

To elucidate the effects of increased *Atα-DOX* expression in salt stressed Arabidopsis, transgenic plants over expressing *Atα-DOX1* or *Atα-DOX2* were

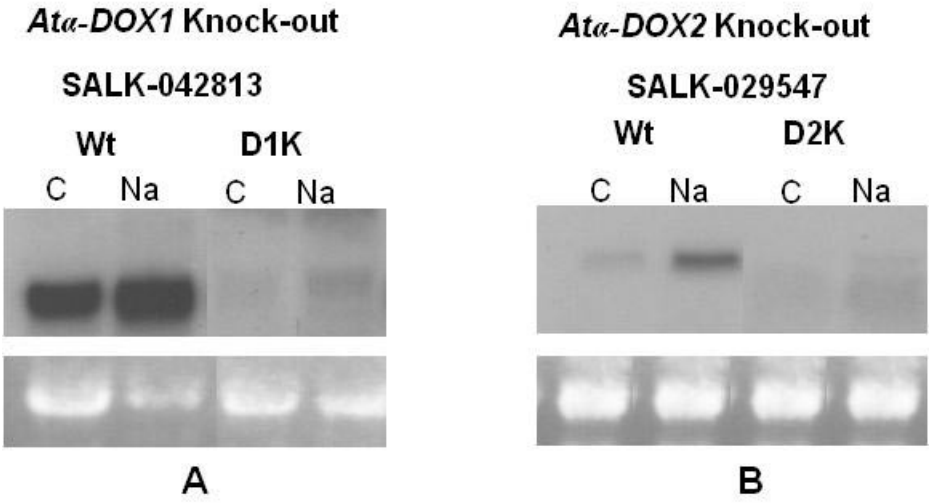


Figure 4-4 Expression analysis of *Atα-DOX1* and *Atα-DOX2* in the SALK-042813 and SALK-029547 lines.

Total RNA from roots of control (C) and NaCl treated (Na) plants was extracted and probed with *Atα-DOX1* specific probe (A) or with the *Atα-DOX2* specific probe (B). Ethidium bromide staining of 18S rRNA is shown below each northern blot.

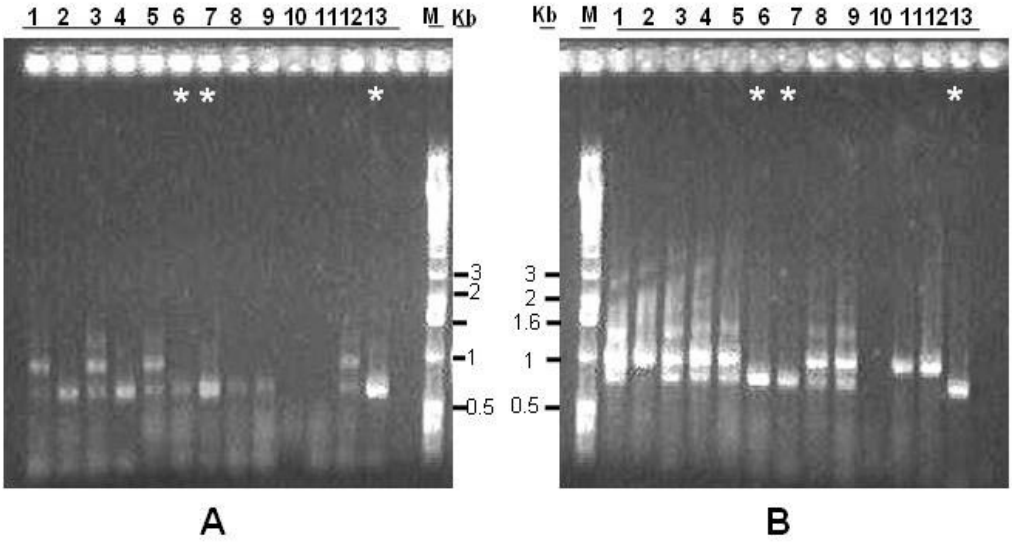


Figure 4-5 Isolation of a homozygous line for the T-DNA insertion in SALK-042813XSALK-029547.

Genomic DNA was isolated form individual F2 plants derived from selfing the F1 SALK-042813 X SALK-029547. (A) DNA was subjected to PCR with the SALK-042813 RP, LP and LBA1 (Wt allele with no TDNA =1 Kb band and T-DNA tagged allelle =0.7 Kb). Homozygous lines which showed the T-DNA tagged allele only are designated with an asterisk. (B) DNA was subjected to the PCR with SALK-029547 RP, LP and LBA1 (Wt allele with no TDNA =1 Kb band and T-DNA tagged allelle =0.7 Kb). Homozygous lines which showed T-DNA tagged allele only are designated with an asterisk.

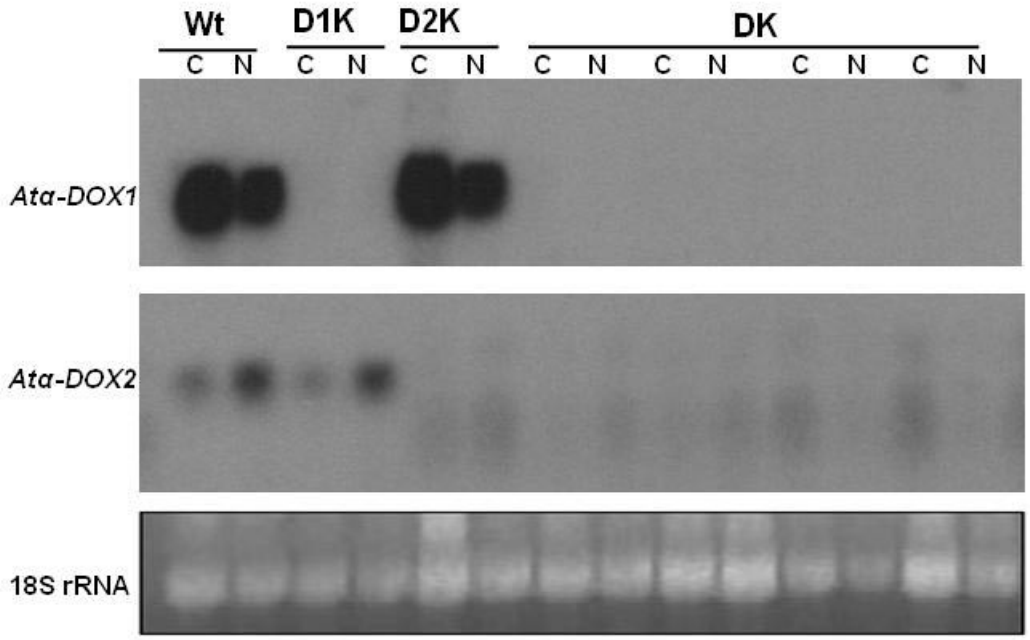


Figure 4-6 Northern blot analysis of putative double homozygous lines for the T-DNA insertion in *Atα-DOX1* and or *Atα-DOX2*.

Total RNA from 7-day-old seedlings of wild type (wt), *Atα-DOX1* knock-out (D1K), *Atα-DOX2* knock-out (D2K) and double knock-out lines (DK) were isolated and probed with the *Atα-DOX1* specific probe (upper panel) and the *Atα-DOX2* specific probe (lower panel).

generated. The constructs contained the cauliflower mosaic virus *35S* promoter sequences to drive constitutive expression of *Atα-DOX1* or *Atα-DOX2* to produce over-expressor transgenic lines: 35S:: *Atα-DOX1* and 35S:: *Atα-DOX2*. The expression of *Atα-DOX1* and *Atα-DOX2* was examined using northern blot analysis in 35S:: *Atα-DOX1* and 35S:: *Atα-DOX1* transgenic plants. All *35S*:: *Atα-DOX1* lines had a higher *Atα- DOX1* transcript level than Wt plants (Figure 4-7 A) whereas for *35S*:: *Atα-DOX2,* lines 1, 2 and 5 had a higher transcript level than wild type. The 35S::*Atα-DOX1* line 1 was selected as an over-expresser of *Atα-DOX1* (OEI) and 35S:: *Atα-DOX2* line1 (OEII) was selected as the over-expresser line for *Atα-DOX2*. I did not observe any phenotypic difference between wild type, OEI and OEII.

4.3.2 α-DOX enzyme assays in D1K, D2K, DK, OEI and OEII

To ascertain the effect of over expressing or knocking out α-DOX1 or α-DOX2 on α-DOX enzyme activity, linolenic acid was incubated with root and shoot homogenates from D1K, D2K, DK, OEI and OEII. The products of the α-DOX assay, 2 HOT and C17 ald were measured in roots and shoots of non-stressed and salt-stressed plants. Enzyme assays performed with root extract from non-stressed plants produced both 2 HOT and C17 ald, and the 2 HOT product level was at least two-fold higher than C17 ald in 3 out of 4 experiments (Figure 4-8 A). Higher α-DOX activity was observed in root homogenates from salt stressed Wt plants. Enzyme assays using extracts from salt stressed roots with linolenic acid produced a two- to three-fold increase in the level of C17ald in

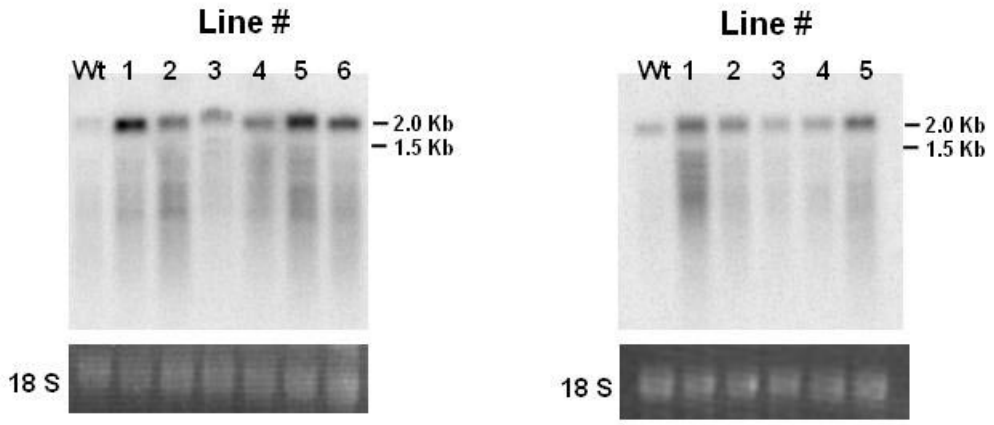


Figure 4-7 Expression of *Atα-DOX1* and *Atα-DOX2* transgenes in Arabidopsis plants.

RNA was isolated from 7-day-old non-stressed seedlings and subjected to Northern blot analysis. A. Blot probed with the *Atα-DOX1* specific probe . Lane Wt contains RNA from Wt. Lanes 1 to 6, contain RNA from individual transgenic lines (*35S*::*Atα-DOX1*). B. Blot probed with *Atα-DOX2* specific probe. Lane Wt contains RNA from Wt. Lanes 1 to 5, contain RNA from individual transgenic lines (*35S*::*Atα-DOX2*). Ethidium bromide staining of 18 S rRNA is shown below each blot.

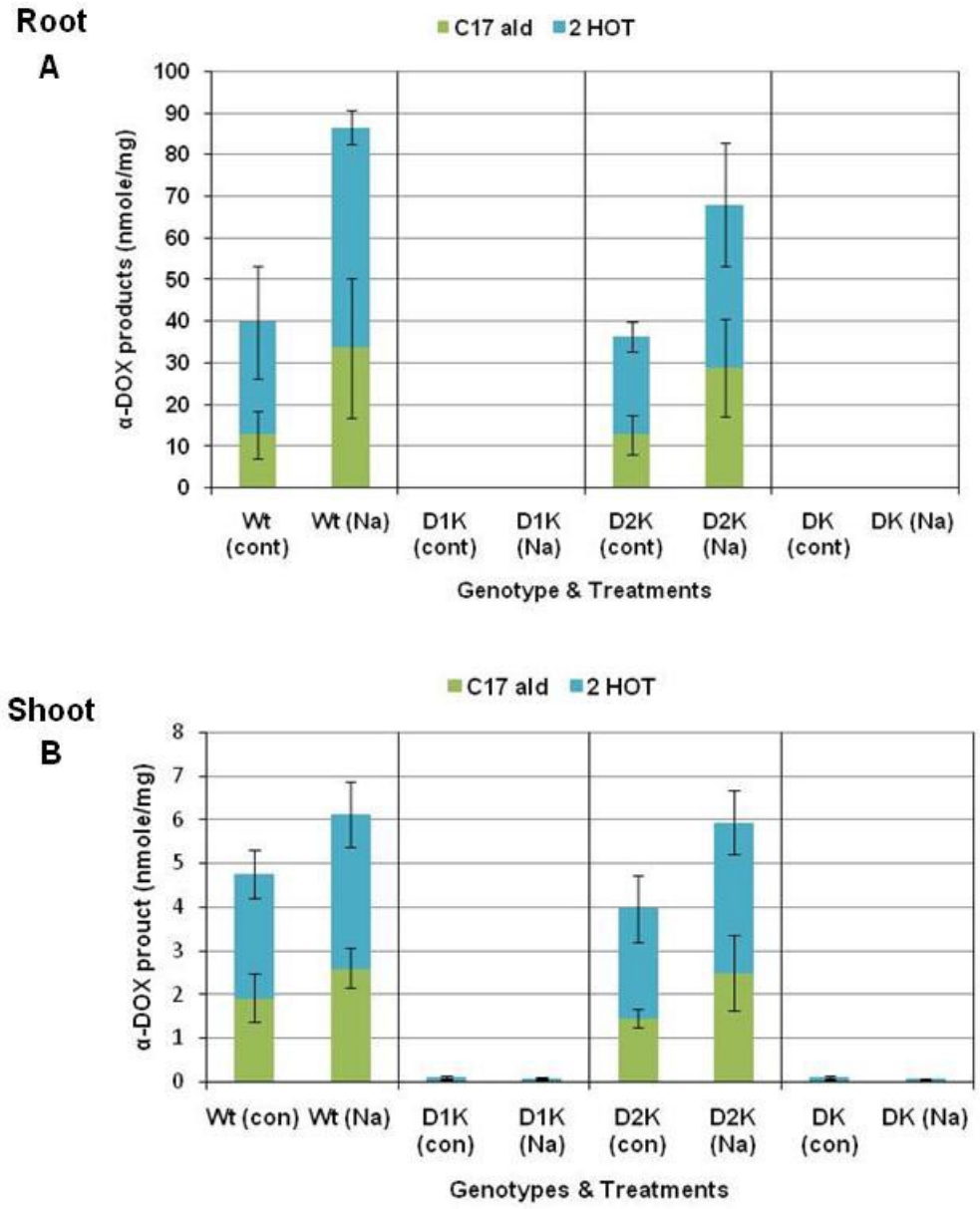


Figure 4-8 α-DOX enzyme activity in control and salt treated wild type, D1K, D2K and DK.

Homogenates from root and shoot tissues of control and salt treated plants were incubated with linolenic acid. The α-DOX products, C17 aldehyde (C17 ald) and 2 hydroxylinolenic acid (2 HOT) were measured using GC-MS. A, roots. B, shoots. The experiment was carried out at least three times. Green bars represent the mean C17 ald nmole/mg ± SE and blue bars represent the mean 2 HOT nmole/mg ± SE.

all experiments; however, the amount of 2 HOT produced varied from experiment to experiment (range = marginal to a 4-fold increase in four experiments). No α-DOX activity was found in roots of D1K and DK. The α-DOX activity in non-stressed D2K roots was similar to that of Wt. Increased α-DOX activity was observed in salt stressed D2K roots but the level of enzyme activity was similar to that of Wt.

In the shoot, α-DOX activity was approximately 10-fold lower than in the root. Enzyme assays performed by incubating linolenic acid with shoot homogenates from non-stressed plants resulted in a higher level of 2 HOT than C17 ald (range = marginal to more than 2 fold higher in all experiments). Salt elicited an increased α-DOX activity in the shoot (Figure 4-8 B). Enzyme assays with extracts from shoots of salt stressed plants produced a marginal increase in the level of C17 ald and a marginal to 1.5-fold increase in the level of 2 HOT relative to non-stressed plants. No Atα-DOX enzyme products activity was detected in shoot extracts from control or salt stressed D1K and DK plants (Figure 4-8 B). Both 2 HOT and C17 ald were detected in enzyme assays performed with extracts from D2K shoots; however, the α-DOX activity in non-stressed and salt-stressed D2K was similar to that of Wt.

Enzyme assays performed using roots homogenates from OEI did not reveal an increase in the level of Atα-DOX products (data not shown). As a result, a 35S::*Atα-DOX1* line was obtained from Carmen Castresana (Universidad Autónoma). This line had increased α-DOX activity in leaves inoculated with *Pseudomonas syringae* (Ponce de León et al., 2002) and was

used for all further analysis. In the OEI line, a higher level of α-DOX activity was detected in the roots as compared to the Wt roots in the absence of salt. However, salt stress did not induce an increased level of α-DOX activity in OEI roots (Figure 4-9 A). In fact, α-DOX activity was lower in salt stressed OEI roots compared to both Wt and non-stressed OEI roots. This was a surprise but the result was identical when the assay was repeated (data not shown). In non-stressed plants, there was a marginal increase of α-DOX activity in roots of OEII compared to Wt. Salt did not induce an increased activity of α-DOX in OEII roots. Enzyme assays performed using shoot homogenates with linolenic acid showed that enzyme activity in the shoot homogenates of OEI was much higher than that in shoot homogenates of Wt and OEII (Figure 4-9 B). Salt stress increased α-DOX enzyme activity in the shoots of OEI plants. There was a slightly higher accumulation of 2 HOT and C17 ald detected in products of enzyme assay with extract from shoots of non-stressed OEII plants compared to Wt (Figure 4-9 A, B). The enzyme activity in the shoots of OEII was similar to that of Wt and , like Wt, salt stress imposed a marginal increase of α-DOX activity in OEII shoots.

Products derived from 9-lipoxygnease (9-LOX) activity were also detected in enzyme assays performed uisng roots and shoots homogenates from control plants with linoleinc acid (data not shown); thus, 9-LOX might be competing for the linolenic acid substrate. In order to eliminate this possibility, I used an

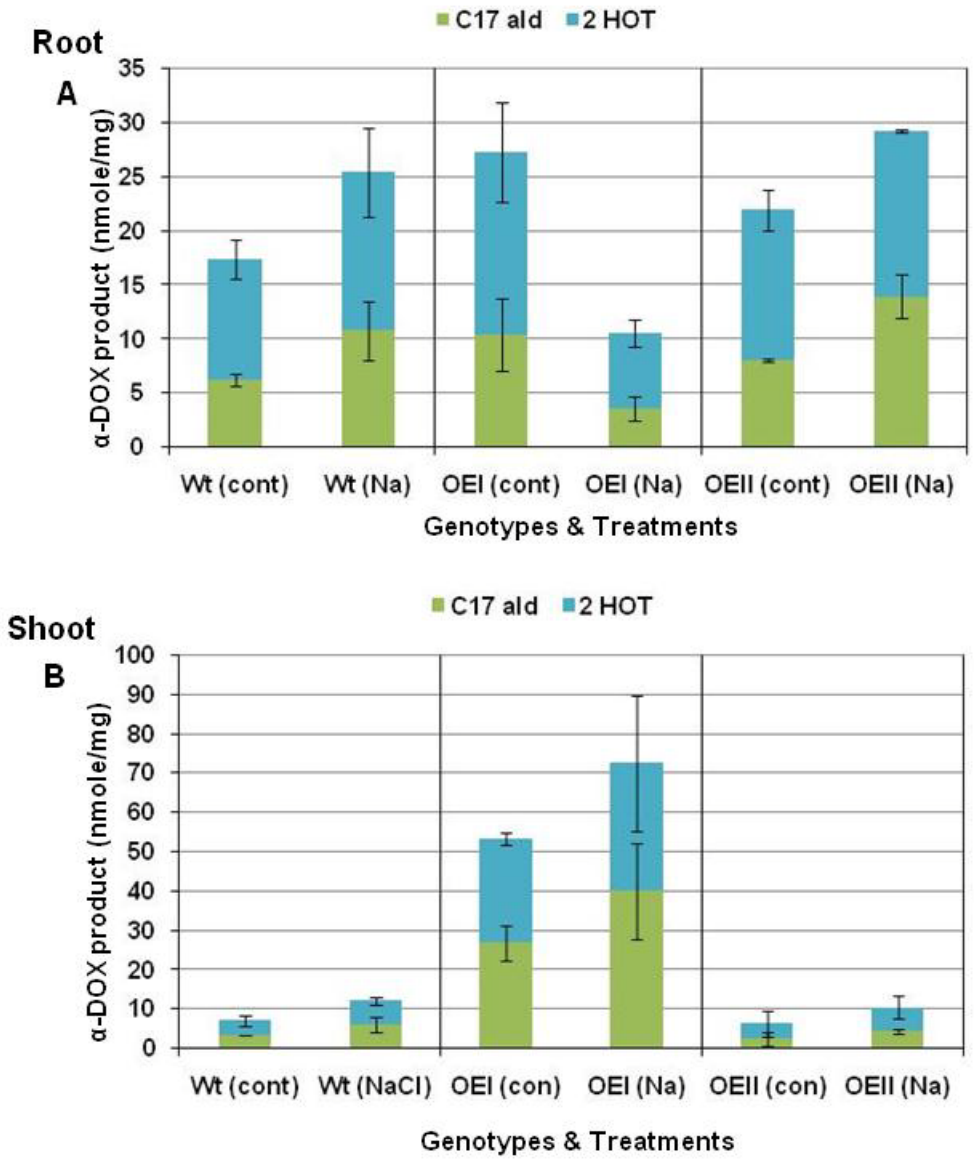


Figure 4-9 α-DOX enzyme activity in control and salt treated wild type, OEI and OEII.

Homogenates from root and shoot tissue of control and salt treated plants were incubated with linolenic acid. The α-DOX products, C17 aldehyde (C17 ald) and 2 hydroxylinolenic acid (2 HOT) were measured using GC-MS. A, roots. B, shoots. The experiment was carried out 2 times. Green bars represent the average C17 ald nmole/mg of protein ± SD and blue bars represent 2 HOT nmole/mg of protein ± SD.

inhibitor of lipoxygenase activity, nordihydroguaiaretic acid (NDGA) in the α-DOX assays. In addition, I performed the α-DOX enzyme assay with palmitic acid, which is not a substrate for lipoxygenase activity. NDGA did not affect the amount of products detected in the α-DOX enzyme assays with linolenic acid (data not shown). Increased accumulation of α-DOX products was detected in α-DOX assay using roots and shoots extracts of salt stressed plants with Palmitic acid. Palmitic acid is not a preferred substrate for the α-DOX enzymes using roots and shoots homogenate since lower levels of α-DOX products were observed in enzyme assays with palmitic acid than with linolenic acid.

4.3.3 Atα-DOX contributes to salt tolerance in Arabidopsis

Plant survival analysis was carried out as a means to determine whether α-DOX1 or α-DOX2 contribute to salt tolerance. To test the role of Atα-DOX1 and -2 in plant salt tolerance, the survival of the DK1, DK2, DK, OEI and OEII genotypes on salt containing media was determined relative to Wt plants. Salt treatment caused seedling death in all genotypes (Figure 4-10). In Wt, salt caused a 20% loss of seedlings. Figure 4-10 shows the percent survival for the DK1, DK2, DK, OEI and OEII lines relative to Wt. The salt sensitivity of D1, D2 and DK was significantly higher than Wt in that fewer seedlings survived the salt treatment. Over-expressing *Atα-DOX1* or *Atα-DOX2* in Arabidopsis did not improve salt survival and the salt sensitivity of OEI and OEII plants was similar to that of Wt (Figure 4-10).

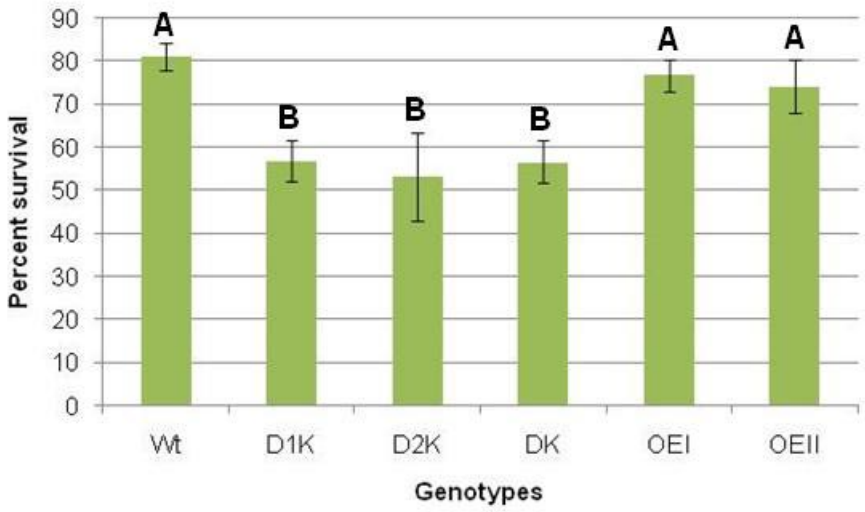


Figure 4-10 The ability of D1K, D2K, DK, OEI and OEII to survive under salt stress.

Seeds were plated on 0.8% agar, ½ MS nutrient medium with or without 150 mM NaCl. Percent survival was determined on day 9 by dividing the number of green seedlings on the media containing salt by the number of seeds that germinated. Bars represent mean percent survival +/- standard error. Bars denoted with the same letter are not significantly different (student's t-test $P<0.05$).

4.3.4 Effect of salt on root growth and lateral root density in D1K, D2K, DK, OEI and OEII lines

To assess whether α-DOX contributes to salt tolerance, plants with increased or reduced α-DOX activity were used in plant growth experiments in the presence of salt. I first observed the phenotype of 3-week-old Wt, D1K, D2K, DK, OEI and OEII watered with varying amounts of salt (75, 100, 150, 200 mM) for one week. No phenotypic differences were observed between the genotypes grown in soil (data not shown). Therefore, root growth assays were carried out to determine the effect of salt on the growth of seedlings on agar media. During the process of measuring root length, I noticed differences in the number of lateral roots (LRs) formed between the genotypes and therefore I broadened my analyses to include LRs. By measuring root length and counting lateral roots, I was assessing the root system architecture (RSA) of the D1K, D2K, DK, OEI and OEII genotypes and the impact of salt on RSA. Root growth rate (RGR) and lateral root density (LRD) of the Wt, D1K, D2K, DK, OEI and OEII genotypes grown in the presence or absence of salt were calculated. In the absence of salt, the RGR for all the genotypes was the same except for the DK and OEI.

Under control conditions, the RGR for the DK seedlings was significantly higher than all other genotypes (Student's t-test $\alpha = 0.05$; Figure 4-11 A). The RGR of OEI was significantly lower than Wt, but that of D1K was not different from the RGR of D2K and OEII. Salt reduced root growth of all genotypes. In the presence of salt, the RGR for all genotypes was the same as the Wt with the exception of OEII, which had a significantly higher RGR than the Wt (Student's t-

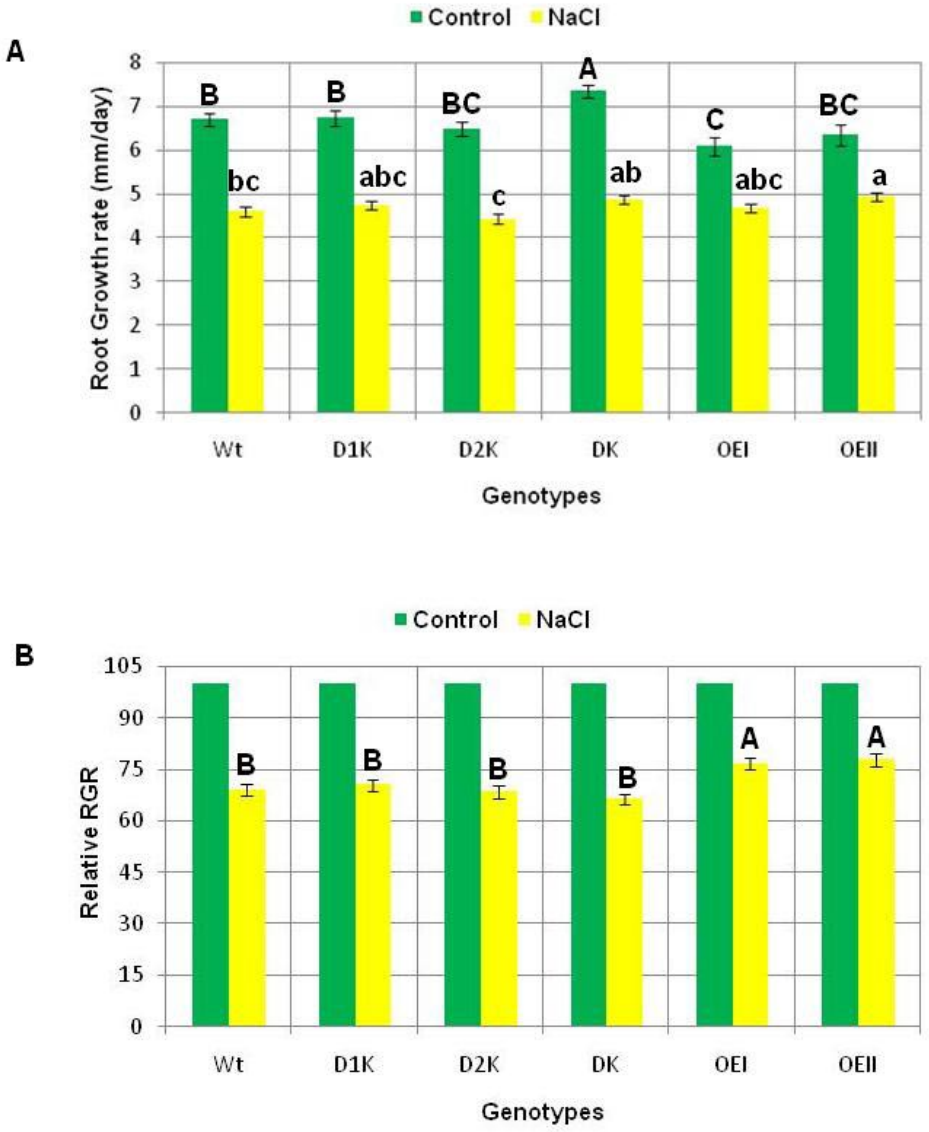


Figure 4-11 Effect of salt on root growth rate of wild type, D1K, D2K, DK, OEI and OEII plants.

Seeds were germinated on ½ MS media with or without 75 mM NaCl. Root lengths were measured 9 days after germination. A, Root growth rate for each genotype. B, Relative root growth rate. Root growth rate (RGR) of each genotype under control conditions was set as 100 and RGR for each genotype in salt was represented relative to their respective control. RGR data were analyzed by ANOVA. Bars represent mean of pooled data +/- SE; green bars, MS media; yellow bars, MS media with salt. Bars denoted with the same letter are not significantly different (Student's t-test $P<0.05$). This experiment was carried out at least 5 times with 2-3 replicates for each genotype for each treatment per experiment.

test α = 0.05; Figure 4-11 A). Salt reduced growth by approximately 30% in Wt. All the α-DOX knock-out genotypes had the same sensitivity to salt as Wt. The OEI and OEII were less sensitive to the ability of salt to reduce root growth (RG) compared to Wt (Student's t-test α = 0.05; Figure 4-11 B). The decreased sensitivity of OEI probably reflects the fact that it grew less than Wt under control conditions.

LRD varied between genotypes. The D1K seedlings had a significantly lower LRD than Wt when seedlings were grown under control conditions (Student's t-test α = 0.05; Figure 4-12 A). The LRDs of D2K, DK, OEI and OEII were not significantly different from the Wt under non-stressed conditions. Salt curtailed LR production in all genotypes. When the seedlings were salt stressed the D1K, D2K and DK seedlings had a higher LRD than Wt, which was statistically significant for the D2K and DK genotypes (Student's t-test α = 0.05; Figure 4-12 A). The LRD for OEI and OEII seedlings grown under non-stressed or salt-stressed conditions were not significantly different from Wt grown under the same conditions. Salt reduced LRD of Wt by approximately 30%. The ability of salt to reduce LRD of all α-DOX knock-out mutants was reduced compared to Wt (Student's t-test α = 0.05; Figure 4-12 B). OEI and OEII were more sensitive to salt-induced LRD reduction than Wt but it was significant only for OEI (Student's t-test α = 0.05; Figure 4-12 B).

To determine whether the increased LRD of the α-DOX knockouts under salt stress correlates with an increased number of lateral root primordia (LR

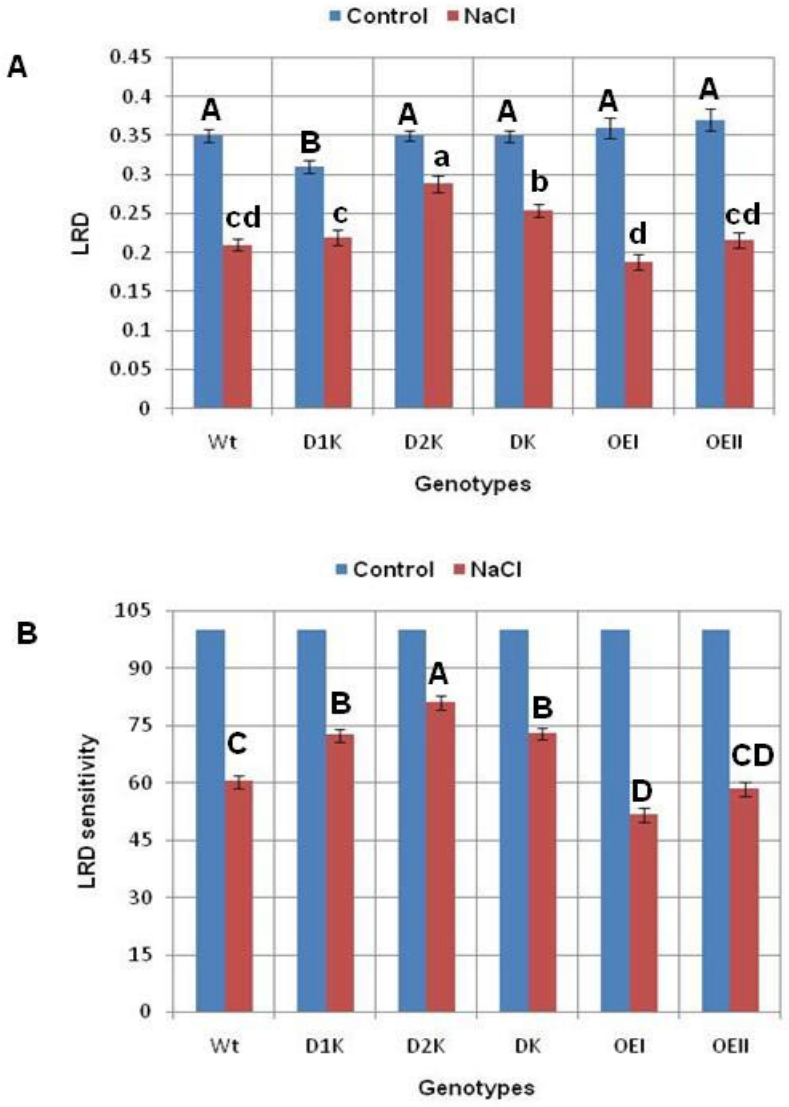


Figure 4-12 Effect of salt on lateral root density of wild type, D1K, D2K, DK, OEI and OEII plants.

Seeds were germinated on plates containing ½ MS media with or without 75 mM NaCl. Root length and the number of visible lateral roots (LRs) were measured. LR densities were calculated by dividing the number of LRs by total root length. A, LR density. B, Sensitivity of LR to salt. LR densitiy in control for each genoptype was set as 100 and LR density in response to salt for each genotype was calculated relative to their respective control. LR data were analyzed by ANOVA. Bars represent mean of pooled data +/- SE; blue bars, MS media; red bars, MS media with

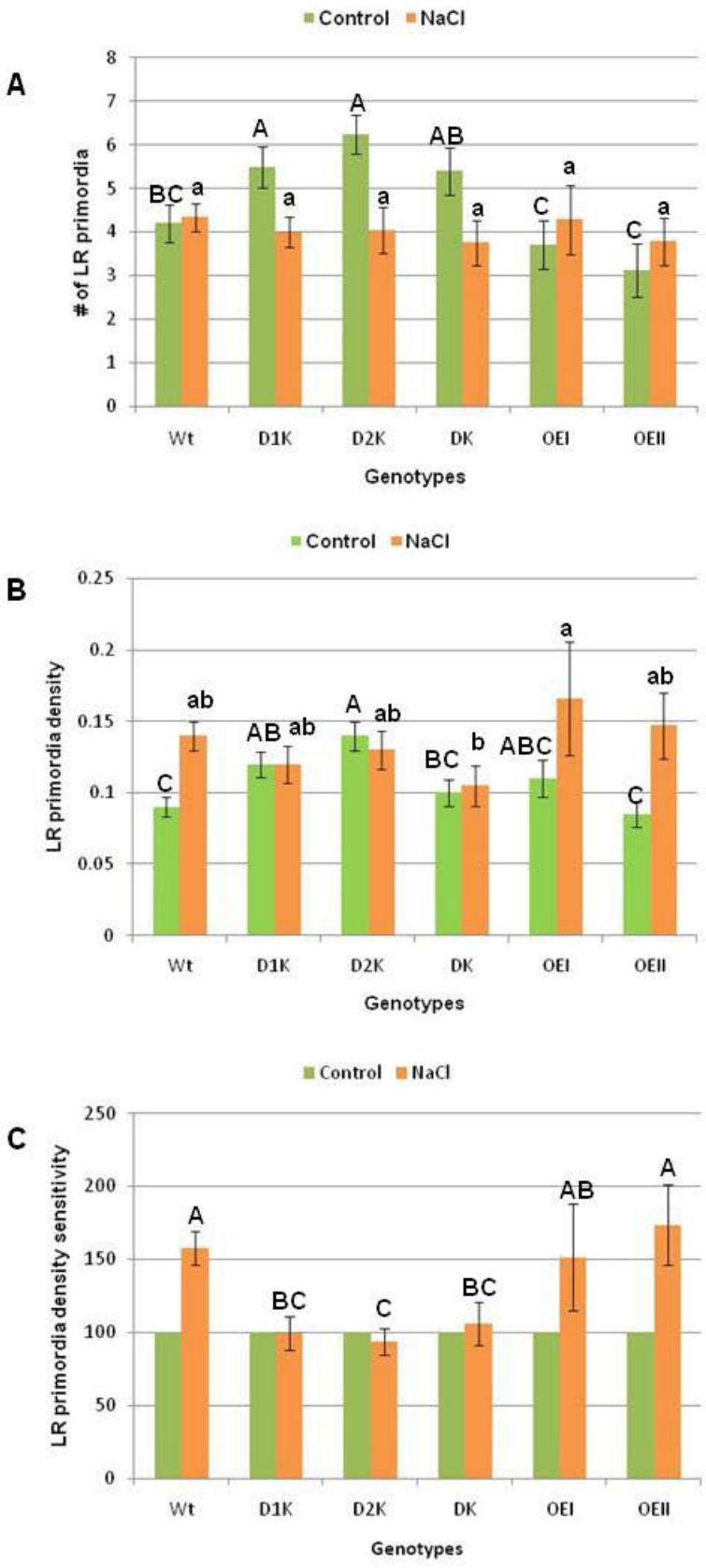
primordia), the number of LR primordia was counted for seedlings of Wt, D1K, D2K, DK, OEI and OEII under non-stressed and salt-stressed conditions and LR primordia density was calculated. Under non-stressed conditions, D1K and D2K genotypes had significantly more LR primordia than Wt (Student's t-test $\alpha = 0.05$; Figure 4-13 A, B). The DK seedlings also had more LR primordia than the Wt; however, the difference was not significant. The LR primordia densities for the OEI and OEII genotypes were not significantly different from Wt under non-stressed or salt-stressed conditions. When seedlings were salt stressed, the number of LR primordia and LR primordia density in all genotypes examined was not different from each other (Student's t-test $\alpha = 0.05$; Figure 4-13 A, B). The LR primordia densities in salt-stressed Wt, OEI and OEII were higher than the non- stressed Wt (Figure 4-13 B, C). Thus, under salt-stressed conditions, fewer LR primordia gave rise to LR, thus increasing the LR primordia density compared to non-stressed plants. Salt-imposed LR arrest from LR primordia was reduced in knock-out mutant plants (Student's t-test $\alpha = 0.05$; Figure 4-13 C).

4.3.5 Detection of H_2O_2 in Wt, D1, D2, DK, OEI and OEII plants

To assess the impact of salt stress on H_2O_2 production in roots of Arabidopsis plants with increased or reduced Atα-DOX activity, 2,7-dichlorofluorescein was used to visualize H_2O_2 production in roots of salt stressed Wt, DK1, DK2, DK, OEI and OEII plants. The conversion of 2,7-dichlorofluorescein diacetate to the fluorescent compound 2,7-dichlorofluorescein occurs in the presence of H_2O_2 (Watanabeet et al., 1986). Control and salt stressed roots were incubated with dichlorofluorescein and the production of the

Figure 4-13 Effect of salt on the number of lateral root primordia of Wt, D1K, D2K, DK, OEI and OEII plants.

Seeds were germinated on ½ MS agar media with or without 75 mM NaCl. The number of LR primordia for 5 seedlings were counted for each genotype for each treatment per trial. This experiment was carried out at least 3 times for each genotype and data from all trials were pooled . The mean and standard error were calculated for the number of primordia, for each genotype and treatment. A, Number of LR primordia. B, LR primordia density C, Relative LR primordia density. The mean LR primordia density in control for each genopype was set as 100 and the LR primordia in salt stressed roots for each genotype was calculated relative to their respective control. LR data were analyzed by ANOVA. Bars represent mean of pooled data +/- std err; green bars, MS media; yellow bars, MS media with salt. Bars denoted with the same letter are not significantly different (Student's t-test P<0.05).


A
Control
NaCl
# of LR primordia
0
1
2
3
4
5
6
7
8
BC
a
A
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AB
a
C
a
C
a
Wt
D1K
D2K
DK
OEI
OEII
Genotypes
B
Control
NaCl
LR primordia density
0
0.05
0.1
0.15
0.2
0.25
C
ab
AB
ab
A
ab
BC
b
ABC
a
C
ab
Wt
D1K
D2K
DK
OEI
OEII
Genotypes
C
Control
NaCl
LR primordia density sensitivity
0
50
100
150
200
250
A
BC
C
BC
AB
A
Wt
D1K
D2K
DK
OEI
OEII
Genotypes

fluorescein indicator of H_2O_2 production was monitored using an epi-fluorescent microscope. In the absence of salt stress, very little fluorescence was present in the root tip (about 0.1–0.2 mm from the root tip) whereas fluorescence was apparent in a region 0.7 mm or further from the root tip, approximately corresponding to the zone of cell differentiation (Figure 4-14). This was observed in all genotypes. Relative to Wt, a higher level of fluorescence was constitutively observed in D1K and D2K roots under non-stressed conditions; however, no differences in fluorescence levels were observed between DK, OEI, OEII and Wt roots. Salt stress enhanced the fluorescence in roots. Enhanced fluorescence was consistently detected in the root tips of the root where the root hairs emerge (0.4 mm behind the tip) and in the distal region (>1.4 mm behind the tip) indicating the accumulation of H_2O_2 in roots of salt stressed seedlings. The fluorescence in the α-DOX knockout seedlings, D1K, D2K and DK compared to the wild type was brighter and this was consistently seen, particularly in roots of the DK salt stressed seedlings. Relative to Wt lower levels of fluorescence were observed in Arabidopsis seedlings over-expressing Atα-DOX1 or Atα-DOX2, especially for the latter genotype.

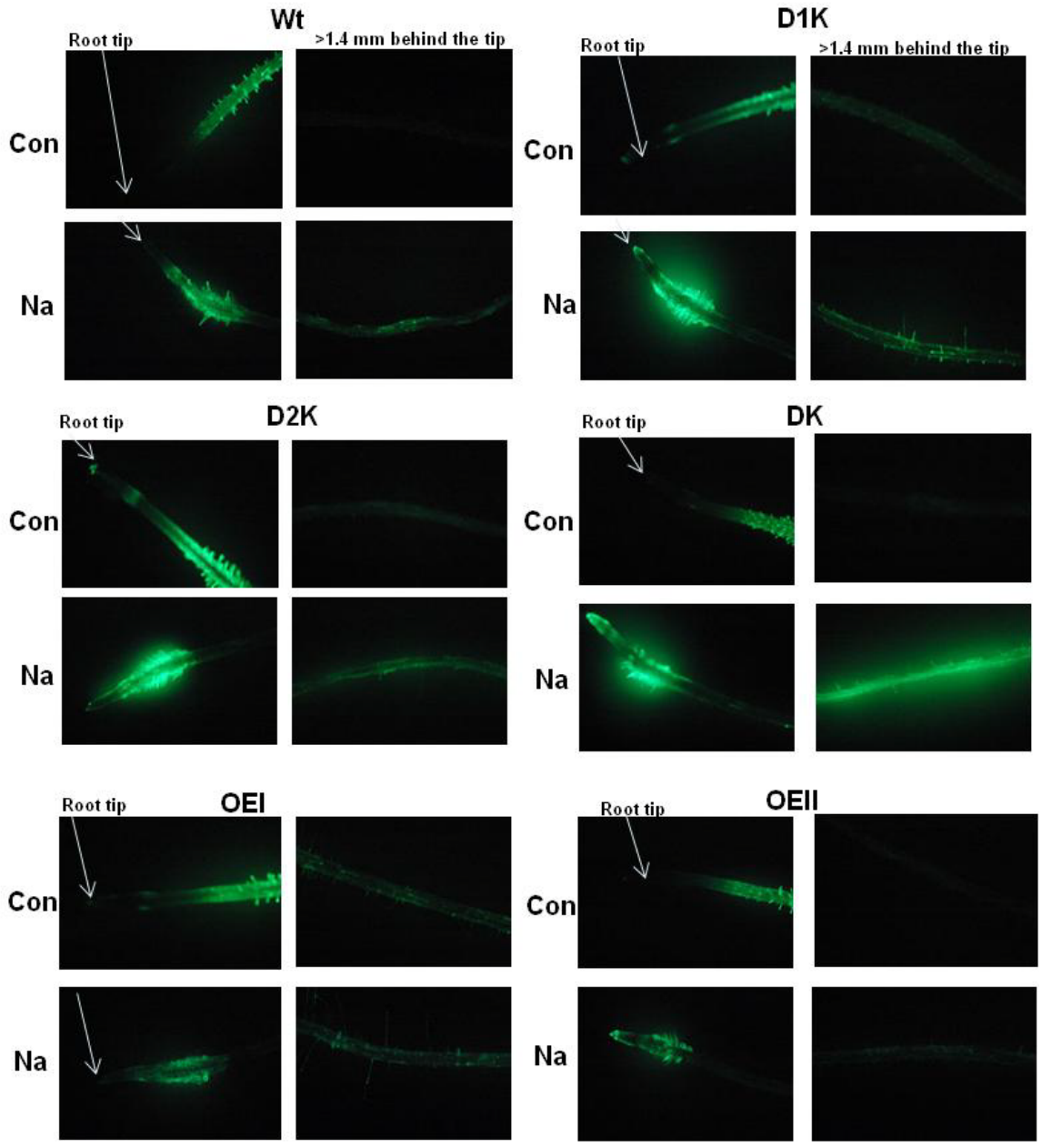

Figure 4-14 H_2O_2 production in roots of salt-stressed Wt, D1K, D2K, DK, OEI and OEII.

Wild-type, D1K, D2K, DK, OEI and OEII were germinated in 1/2 MS (1.5% w/v sucrose) 0.8% agar plates under 16/8 h light/dark cycle (80 µmol/s/m^2) at 22 °C. After 7 day, seedlings were transferred to 1/2 MS liquid medium with or without 150 mM NaCl for 12 h. Seedlings were incubated with 10 µM 2,7-dichlorofluorescein diacetate for 5 min. ROS production was detected with an epi-fluorescent microscope 5 min after addition of the dye. Images were obtained with a digital camera.

4.4 Discussion

In this chapter I examined the accumulation of α-DOX products (C17 aldehyde and 2 Hydroxylinolenic acid) in control and salt stressed Wt, α-DOX knockouts and over-expressor lines. I also examined the possible roles of α-DOX in regulating root system architecture and of α-DOX in alleviating salt stress damage via protection against oxidative stress.

4.4.1 2HOT and C17-ald are the major products resulting from α-DOX activity in Arabidopsis

Salt stress increased the expression of both *Atα-DOX-1* and *Atα-DOX-2* in roots and shoots and increased α-DOX enzyme activity. In this chapter, I examined α-DOX enzyme activity in the root and shoot of α-DOX knockout mutants and in over-expresser lines under non-stressed and salt stressed conditions. The major compounds detected in α-DOX enzyme assays performed with linolenic acid and homogenates from Arabidopsis roots and shoots were C17 ald and 2 HOT, which were the same products reported for tobacco α-DOX and Arabidopsis α-DOX1 by Hamberg et al. (1999). The α-DOX enzyme catalyzes the dioxygenation of linolenic acid into the unstable 2(*R*)-hydroperoxylinolenic acid. The resulting 2(*R*)-hydroperoxylinolenic acids may either decompose into C17 ald and intermediates of the α-oxidation pathway, or undergo enzymatic reduction by peroxidases into 2 HOT (Hamberg et al., 2003). The conversion of 2(*R*)-hydroperoxylinolenic acid into 2 HOT can also occur spontaneously (Hamberg et al., 2003). A large amount of the unstable 2(*R*)-hydroperoxylinolenic acid was converted to 2 HOT in roots and shoots which

might reflect the prevalence of reducing agents in my preparation. In the α-oxidation pathway, the α-DOX enzyme has been suggested to operate together with an aldehyde dehydrogenase (ALD) and NAD^+ to provide a pathway for the stepwise degradation of fatty acids into shorter chain homologs (Hamberg et al., 1999). In this pathway, aldehyde dehydrogenase catalyzes the conversion of C17 ald to C17-fatty acids, which can act as a substrate again for the α-DOX enzyme to repeat the α-oxidation pathway. In support of α-oxidation, the fatty acid α-DOX enzyme complex purified from germinating pea revealed the presence of two subunits, a turgor-responsive NAD^+ aldehyde dehydrogenase (ALD) and the α-DOX enzyme (Saffert et al., 2005). To find out whether the Arabidopsis α-DOX enzymes participate in α-oxidation, α-DOX enzyme assays were performed using a homogenate from an Arabidopsis *ALD* knockout (*ald ko*) mutant. In this mutant, the turgor-responsive *ALD* (*ald7*) was knocked out. A higher level of C17 ald was detected in *ald ko* than in Wt; however, the product profile was not different to Wt (data not shown). This may be because: 1) there is functional redundancy among plant ALDs, 2) the turgor-responsive ALD is not responsible for the catalysis of C17 ald to C17 fatty acid or 3) 2 HOT is the major product for α-DOX in Arabidopsis.

α-DOX activity was much higher in roots than shoots indicating a possible role for α-DOX in root specific functions. The higher α-DOX enzyme activity detected in the root tissues is in agreement with the constitutive expression of *Atα-DOX1* in roots (chapter 3) and the observations of Meisner et al., (2008) that *α-DOX* expression was detected predominantly in the roots and cotyledons of

pea plants during germination. The preferential occurrence of α-DOX activity in roots could indicate a role for α-DOX in establishing a permanent system of protection against multiple negative environmental impacts such as damage caused by soil borne pathogens.

When the *Atα-DOX1* gene was knocked-out, there was a complete absence of α-DOX enzyme activity in roots and shoots. Knocking out the *α-DOX2* gene did not reduce or eliminate α-DOX activity in shoot or root tissues and the activity is similar to that of Wt. This indicates that α-DOX1 was primarily responsible for all α-DOX activity detected using linolenic acid as substrate in assays with root and shoots homogenates. However, these results are inconsistent with its root-specific expression as is the lack of α-DOX activity in the shoot of D1K since *Atα-DOX2* was expressed in the shoot tissues including the shoot of D1K (data not shown). The reason why α-DOX enzyme activity was not detected in the shoot of D1K is not known. It is possible that the enzyme assay was not optimal for α-DOX2 activity or that α-DOX1 generated products, 2 HOT and C17 ald, are needed for α-DOX2 enzyme activity. In addition, the α-DOX enzyme activity detected in shoot tissues of D2K is also puzzling since *Atα-DOX1* was not expressed in D2K shoot tissues under control conditions (data not shown). Possible explanations include the presence of a low level of *Atα-DOX1* expression in shoot tissues or the presence of another enzyme that is capable of producing 2 HOT and C17 ald from linolenic acid. Although *Atα-DOX1* expression was not detected in the shoot of non-stressed plants, microarray analysis revealed a very low level of *Atα-DOX1* expression in rosettes of

Arabidopsis (Table 2-1, chapter 2). Furthermore, in pea a high level of *α-DOX* transcript was detected in roots but the level in the shoot was very low, yet the levels of α-DOX protein in roots and shoots were the same (Meisner et al., 2008). Thus, it is possible that there was a very low level of *Atα-DOX1* expression in shoots that was not detected by northern blot hybridization. Indeed, it has been reported that, for some genes, mRNA levels do not correlate well with protein levels, making it difficult to predict protein expression from mRNA levels (Gygi et al., 1999).Under salt stressed conditions, α-DOX2 activity may have contributed to the α-DOX products detected in shoot tissues since the salt-induced increase in α-DOX activity was absent in D2K.

When *Atα-DOX1* was over-expressed α-DOX activity increased; however, salt had a negative effect on α-DOX activity in roots for reasons that are not clear. The salt-induced enhancement of the endogenous *Atα-DOX1* expression in roots may have pushed total *Atα-DOX1* transcript to a very high level in OEI, which could have resulted in RNA interference and gene silencing. The abundant of *Atα-DOX1* can lead to RNA interference and causes silencing of the gene, which results in decrease α-DOX activity. In shoots, the level of α-DOX products in OEI increased in response to salt and this may have been due to the salt induced increase in the native *Atα-DOX1* or *Atα-DOX2* expression. The level of α-DOX products produced in roots and shoots when *Atα-DOX2* was over-expressed was not substantially different from that in Wt plants. This may be because the enzyme assay was not optimal for detection of α-DOX2 enzyme activity as discussed earlier.

4.4.2 α-DOX products play a role in regulating RSA

Roots are the first organ to encounter salt stress and the root system must modify itself by altering growth and the production of lateral roots. This allows it to avoid high salinity in the growing media by growing towards areas with lower salinity levels. Maintenance of root growth is an indicator of tolerance to salt or osmotic stresses. Towards elucidating whether α-DOX products contribute to salt tolerance by measuring root growth during salt stress I noticed altered lateral root production for the α-DOX knockouts and extended my measurements to include LRD.

Under non-stressed conditions, the longer and shorter roots of DK and of plants over-expressing *Atα-DOX1*, respectively, suggests that α-DOX products may have a slight inhibitory effect on root growth. Salt stress reduces root growth in part by conferring an osmotic stress that reduces cell expansion (Zhu, 2002). Root growth of all α-DOX knockout and over-expresser lines was reduced by salt but this was not substantially different from Wt with the exception of OEII, indicating that the α-DOX2 may aid growth during salt stress. The roots of seedlings with increased *Atα-DOX1* expression were less sensitive to the inhibitory effect of salt on root growth compared to Wt, suggesting a role of α-DOX1 in maintaining root length in Arabidopsis. However overall, the effects of manipulating α-DOX activity during salt stress on root growth were not substantial.

Seedlings with increased or reduced α-DOX activity displayed altered LRD under both non-stressed and salt-stressed conditions. Atα-DOX1 may regulate

lateral root (LR) growth or initiation under non-stressed conditions since the LRD of D1K seedlings was lower than that of Wt. Salt stress reduced LRD in all genotypes. During salt stress, the significantly higher LRD in D2K and DK seedlings relative to Wt suggests the involvement of α-DOX2 in reducing the number of LRs during salt stress. Furthermore, all α-DOX knockouts were less sensitive to the ability of salt to reduce LRD, suggesting that the oxylipin products of α-DOX1 and α-DOX2 mediate the reduction of LRs when plants are under salt stress.

Lateral root formation is initiated from cell divisions in the pericycle of the primary root (Malamy and Benfey, 1997). The root primordium forms a root meristem, which pushes its way through the cell layers of the primary root to generate the lateral root (Malamy and Benfey, 1997). Many environmental and endogenous factors affect this process, among which are the hormones ethylene, auxin and ABA (He et al., 2005). Auxin promotes LR formation and ABA represses the outgrowth of LR (Deak and Malamy, 2005). Deak and Malamy (2005) speculated that a balance between promotive (auxin) and repressive signalling (ABA) pathways determine the fate of LR primordia under all growth conditions. ABA accumulates in roots responding to osmotic stress (Chen and Plant, 1999) and this increased ABA shifts the balance towards repressive signalling, which results in reduced LR formation (Deak and Malamy, 2005). Exogenous ABA inhibits LR primordia development and eliminates auxin promotive activity in the primordia (De Smet et al., 2003). Furthermore, ABA

partly mediates the inhibition of LR elongation in Arabidopsis under drought stress (Xiong et al., 2006).

A role for α-DOX in restricting LR production during salt stress is consistent with a recent report that oxylipins play a role in regulating root system architecture (Vellosillo et al., 2007). Seedlings of insertion mutants lacking lipoxygenase (LOX) activity as well as the *noxy2* mutant, which was isolated based on its insensitivity to 9-HOT, an oxylipin produced via 9-LOX activity, all display an increased emergence of LRs indicating the involvement of 9-HOT in LR arrest (Vellosillo et al., 2007). The oxylipins produced through 9-LOX activity reduced LRs by exerting their effects on the growth of existing LRs but not on their initiation (Vellosillo et al., 2007). Oxylipins may modulate root development through modification of the cell wall since treatment of Arabidopsis seedlings with 9-HOT led to the formation of polysaccharide deposits, composed of callose and pectin, and the production of ROS (Vellosillo et al., 2007). The data presented in this chapter suggest that the oxylipins generated via the α-DOX pathway also contribute to the reduction of LR growth when plants are coping with increased salinity in the growing media. Under salt stressed conditions, this is advantageous because plants need to increase their uptake of fresh water, which is usually available deeper in the soil. The restricted proliferation of LRs in the top soil layers and re-allocation of resources to support the growth of the primary roots offers an advantage to plants by expanding their domains of water supply. ABA up-regulated *Atα-DOX1* and *Atα-DOX2* expression in roots. Thus, it is

possible that the suppression of LR formation by ABA during salt stress is dependent, in part, on its ability to up-regulate *Atα-DOX* expression.

The increased LRD in α-DOX knockouts under salt stress could arise because the oxylipins generated via the α-DOX pathway reduced LR production via effects on LR initiation or emergence. Under non-stressed conditions, the LRD of D2K and DK was the same as Wt and the number of LR primordia in the D1K and D2K was significantly higher than Wt suggesting that α-DOX products repress LR primordium formation under non-stressed conditions. Although the number of LR primordia in all knock-out mutants under salt stressed conditions was not significantly different from Wt, a significant increase in the LRD of D2K and DK relative to Wt was observed. This indicates that LR emergence was not suppressed in the D2K and DK lines during salt stress. This provides evidence for a role for α-DOX2 in checking LR production in salt-stressed plants (knocking out *α-DOX2* but not *α-DOX1* resulted in increased LR production) and suggests that this occurs at the level of LR emergence rather than LR initiation. However, under non-stressed conditions, the average primordia number in α-DOX knock-outs was higher than Wt, suggesting a role for α-DOX in checking LR primordia formation or initiation.

GUS expression was observed in the LRs of salt treated *Atα-DOX1*::GUS and *Atα-DOX2*::GUS plants (Figure 3-4, Chapter 3); however, no GUS activity was observed in their LR primordia (Figure 2-5, Chapter 2). Although Atα-DOX expression was not detected in LR primordia, the salt-induced increase in α-DOX activity was observed in the zone of cell differentiation within which LR primordia

are developing. This is similar to the role of oxylipins produced through the 9-LOX pathway in reducing LR production by exerting negative effects on the growth of existing LRs but not on their initiation (Vellosillo et al., 2007). Therefore, α-DOX appear to have a duel role in regulating LRD, they decrease LR initiation under non-stressed conditions and check LR emergence under salt-stressed conditions. Since ABA represses LR production at the emergence stage and application of the ethylene precursor, ACC, strongly inhibits the initiation of new LR primordia (Fukaki and Tasaka, 2009). It is possible that under non-stressed conditions ethylene reduces LR initiation through α-DOX activity and under salt-stressed conditions, ABA checks LR emergence via α-DOX activity.

4.4.3 Salt tolerance and H_2O_2 production

Oxidative stress is a consequence of many abiotic stresses, including salt stress, and it disrupts the function of organelles with a high oxidizing metabolic activity or with sustained electron flows such as chloroplasts, mitochondria and peroxisomes (Arora et al. 2002; Goel and Sheoran 2003; Apel and Hirt 2004). Several stress-inducible genes that encode proteins involved in osmolyte biosynthesis or ROS scavenging have been over-expressed in transgenic plants to generate stress tolerant phenotypes and in some cases, knocking out the same gene has resulted in a stress sensitive phenotype (Xiong and Zhu, 2002). Over-expressing either *Atα-DOX1*or *Atα-DOX2* did not confer enhanced tolerance to salt stress. However, knocking out either *Atα-DOX1* or *Atα-DOX2* resulted in increased sensitivity to salt stress, which suggests some involvement

of α-DOX1 and α-DOX2 in regulating salt stress tolerance. One possible explanation for the lack of increased tolerance in OE lines is that in Wt plants there may be an adequate amount of Atα-DOX present to confer salt tolerance. Therefore, over expressing *Atα-DOX1* or *Atα-DOX2* did not result in increased tolerance. Atα-DOX1 is involved in protection against oxidative stress in leaves responding to pathogen attack (Ponce de León, 2002). The lipid-derived compounds synthesized through the activity of Atα-DOX1, or derivatives synthesized from them, have been proposed to act as signal molecules to protect tissues from oxidative damage and facilitate the activation of an effective defense reaction (Ponce de León, 2002). The salt sensitivity of α-DOX knock-outs is consistent with the possibility that α-DOX products act as signal molecules involved in protecting cells against salt induced oxidative stress or damage.

Disruption of cellular structures and cell death are frequently correlated with the generation of an oxidative burst, which is known to be part of a general stress defence pathway up-regulated by biotic and abiotic stresses as well as during different plant developmental processes such as senescence (Apel and Hirt, 2004). Lignified xylem cells in Arabidopsis roots undergo cell death during development and analogies have been noted between processes in the lignifying xylem of *Zinnia elegans* and the oxidative burst observed during the HR (Barcelo, 1999). This is consistent with the presence of H_2O_2 in the roots in a region that approximately corresponds to the zone of cell differentiation where xylogenesis is occurring. The *Atα-DOX1* promoter directed GUS expression in roots was also observed in the zone of cell differentiation (Chapter 3) corresponding to the

region of the roots with the highest H_2O_2 accumulation. In salt stressed roots, the size of the zone of cell differentiation was reduced and H_2O_2 accumulation was observed. Salt increased H_2O_2 accumulation in roots, suggesting that, in addition to the production of H_2O_2 during xylogenesis, H_2O_2 must have arisen from salt induced oxidative stress. GUS staining directed by the *Atα-DOX1* or *Atα-DOX2* promoters showed that salt increased *Atα-DOX1* and *Atα-DOX2* expression in the zone of cell differentiation in roots (Chapter 2 and 3). Thus, *Atα-DOX* expression was associated with the region of H_2O_2 production in roots.

The level of H_2O_2 was highest in plants with no α-DOX1 or α-DOX2 activity whereas in plants over-expressing *Atα-DOX1* or *Atα-DOX2*, H_2O_2 accumulation in the roots of salt stressed seedlings was reduced. This suggests that Atα-DOX products might be involved in suppressing ROS accumulation or promoting the removal of ROS in the roots of Arabidopsis plants under salt stress. As such the Atα-DOX products (C17-ald, 2HOT) may act as signalling compounds to activate ROS scavenging enzymes, thus reducing the overall level of oxidative stress in root cells during salt stress. Ponce de León et al., (2002) reported that *Atα-DOX1* expression is confined to the necrotic lesions formed during the HR and that plants with reduced α-DOX1 activity develop a more rapid and severe necrotic response than Wt. Ponce de León et al., (2002) proposed that Atα-DOX1 generates lipid-derived molecules that regulate a process that protects plant tissue from oxidative stress (Ponce de León et al., 2002). This is consistent with my data that reduced α-DOX activity resulted in enhanced or higher H_2O_2 accumulation whereas increased α-DOX activity resulted in lower H_2O_2

accumulation in salt stressed roots. The higher H_2O_2 accumulation in knock-out mutants may hence contributed to the decreased survival of salt-stressed seedlings.

5: SUMMARY AND FUTURE PROSPECTS

5.1 Summary

The research presented in this thesis investigated the role of α-DOX in plants responding to salt stress. Application of DD-PCR was used to isolate novel salt-responsive genes from tomato roots and one of the genes identified was *α-DOX* (Wei et al., 2000). To date, *α-DOX* genes have been identified in several plant species, including *Arabidopsis thaliana*, *Pisum sativum*, *Capsicum annum*, *Nicotiana attenuata* and *Oryza sativa* (Hamberg et al., 2005). Alpha-DOX catalyzes the initial step of the α-oxidation of various C_n fatty acids into C_{n-1} aldehydes together with various amounts of C_n hydroxy fatty acids and C_{n-1} fatty acids (Hamberg et al., 1999). The *α-DOX* genes from *N. attenuata*, *N. tabacum* and *A. thaliana* are induced by wounding and pathogen challenge, respectively, and most of the knowledge of α-DOX is based on responses to biotic stress in the leaves (Hermsmeier et al., 2001; Sanz et al., 1998). Athough *α-DOX* expression has been associated with pathogen and wounding, study by Wei et al. 2000 was the first report on the salt-responsive nature of *α-DOX* expression in tomato. There was no report on the possible functions or roles of α-DOX in salt stressed plants when I started my research. In this thesis, I first explored whether α-DOX (*Leα-DOX1*) expression in tomato was regulated by biotic stress and northern analyses revealed that *Leα-DOX1* was up-regulated in tomato roots in response to the fungal pathogen *Pythium aphanidermatum* and mechanical

wounding. Functional analyses of α-DOX were undertaken in Arabidopsis responding to salt. However, in order to use Arabidopsis for functional analyses I first had to establish that *Atα-DOX* genes are regulated by salt. The expression of both *α-DOX* genes was responsive to salt in the roots and shoots and this salt-responsive expression was accompanied by increased α-DOX activity. 2-Hydroxylinolenic acid (2HOT) and Heptadecatrienal (C17 ald) were the major products detected in α-DOX assays using linolenic acid as a substrate. Both *α-DOX* genes were constitutively expressed, but in distinct locations. *Atα-DOX1* was expressed in roots and *Atα-DOX2* was expressed in the shoot. In addition, *Atα-DOX1* was expressed in stamens and *Atα-DOX2* in sepals, siliques and developing seeds.

Plants employ a variety of signalling pathways to activate defense-related genes, including those mediated by hormones such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The increased transcript level for *α-DOX1* in tomato roots was observed in response to an exogenous application of ABA or ET, suggesting that these hormones may regulate *α-DOX* expression *in vivo* (Tirajoh et al., 2000). Cellular signals such as SA, intracellular superoxide or singlet oxygen and nitric oxide (NO), induce *Atα-DOX1* expression in Arabidopsis leaves (Ponce de León et al., 2005). The nature of the hormone signals that regulate *α-DOX* expression was explored using exogenous applications of hormones and hormone mutants. The results suggested that ABA and SA were major hormone signals that up-regulated *Atα-*

DOX1 and *-2* expression in roots, whereas ABA, SA and ethylene up-regulated *Atα-DOX1* and *-2* expression in shoots.

The role of α-DOX in plants was first investigated in Arabidopsis responding to bacterial pathogens (Ponce de León, 2002). In Arabidopsis, reduction of α-DOX activity is correlated with a higher level of ion leakage indicative of cellular damage, whereas enhanced α-DOX activity exerted a protective effect and limited the necrotic leaf area affected by bacterial inoculation (Ponce de León, 2002). The functional significance of α-DOX in salt-stressed Arabidopsis plants was explored using lines with altered *Atα-DOX1* and/or *Atα-DOX2* expression. The seedlings survival for knockout lines lacking *Atα-DOX1* or *Atα-DOX2* expression was significantly lower than Wt, suggesting that α-DOX products contribute to salt tolerance. In the same lines, increased levels of H_2O_2 were detected in the roots of salt stressed seedlings, indicating that α-DOX suppresses the accumulation of reactive oxygen species or promotes their removal. Since root growth is one of the indicators of salt tolerance in plants, I measured the RGR and LR production in non-stressed and salt-stressed plants. Although there are some differences in RGR between Wt, α-DOX knock-out and over-expresser lines, the differences are not substantial. Analyses of LR density (LRD) and lateral root primordia density have revealed that Atα-DOX1 and Atα-DOX2 play a role in suppressing lateral root (LR) formation under non-stressed conditions and in checking LR emergence under salt stressed conditions. Such a role is consistent with the spatial expression of *Atα-DOX* in roots, which occurred in the zone of cell differentiation within which LR primordia

are known to develop. In Arabidopsis, both *At*α*-DOX* genes were up-regualted by salt and ABA. Therefore, it is possible that the α-DOX products contribute to the known ability of ABA to check LR emergence in osmotically-stressed plants.

5.2 Future prospects

In this study, salt (250 mM) was applied, as a shock treatment to 3 week-old plants or to seedlings grown on solid media containing salt (75 mM). However, plants do not experience this type of stress in their natural environment; instead salinization is a gradual process. Thus, it would be of interest to see if the genes studied here follow the same pattern of expression when plants are subjected to a gradual salt stress. Most crop plants are grown in soil and the soil environment is different from a hydroponic system. Soils contain nutrient ions and diverse groups of living organisms such as fungi and bacteria. The high α-DOX activity in roots suggests that α-DOX products contribute to a permanent system of protection against possible soil borne pathogens. It will be of interest to find out if the same level of constitutive *α-DOX1* expression occurs in roots of soil-grown plants.

Vellosillo et al. (2007) observed root waving and lateral root arrest when Arabidopsis were grown in the presence of oxylipins. Oxylipin treatments were carried out in this research; however, the results obtained did not support the data acquired from the knock-outs and over-expresser lines. For example, RGR for OEI was significantly lower than Wt under control conditions; however, RGR for C17 ald or 2 HOT treated seedlings were higher than non-treated seedlings. This may be due to the differences in up-take or metabolism of the oxylipins *in*

vivo. Of all the oxylipins tested by Vellosillo et al. (2007), the 9-LOX derivative 9-HOT was the most potent inducer of the root waving and LR inhibition phenotype. Increased LR production was observed in *LOX1* and *LOX5* mutants with homozygous T-DNA insertions in 9-LOX encoding genes. In my research, I also found an increased LRD in α-DOX knock-out mutants when plants were salt stressed. Interactions between the LOX and α-DOX pathways was reported by Hamberg et al. (2003); therefore studies to increase our understanding of the role of α-DOX in salt stressed roots will require multiple mutant LOX/DOX plants. Generating multiple *LOX1*/*LOX5*/*α-DOX1*/*α-DOX2* knock-out mutants and comparing the multiple and double knock-out mutant lines under salt stress will be an important approach to further understand the role of oxylipins formed from the α-DOX pathway in regulating root system architecture under salt stress and for determining their importance for salt tolerance. Furthermore, these experiments will also give us insight into the possible roles of LOX in salt stressed plants.

My research indicated that α-DOX1 directed all or the majority of the α-DOX activity detected in roots and shoots of non-stressed and salt-stressed plants. However, there was no *α-DOX1* expression in the shoots of D2K plants, yet the enzyme assays with shoot homogenates from D2K plants possessed α-DOX enzyme activity. Realtime qPCR should be carried out to ascertain whether there is, in fact, a low level of *α-DOX1* expression in non-stressed shoots. Recent work by Meisner et al., 2008 showed that α-DOX protein accumulation was not comparable with transcript accumulation in time course experiments. To

determine whether the α-DOX1 protein is present in the shoot of non-stressed D2K plants, antibodies should be raised against purified α-DOX1 and -2 for western blot analyses. The western blot analyses can also reveal whether the α-DOX2 protein is present in roots and shoots of D1K plants because the enzyme assay we used failed to detect any α-DOX activitiy in this mutant. α-DOX1 and α-DOX2 antibodies can also be used for immunocytochemical studies to determine *in situ* localization of α-DOX1 and α-DOX2 proteins. Furthermore, since little or no significant work has been done with α-DOX2, further investigation is required to ascertain the precise assay conditions needed to reliably detect its activity.

Finally, expanding the studies on the regulation and role of *α-DOX* in salt-stressed plants to other model plants, particularly salt tolerant plants such as *T. halophila* will increase our present understanding of *α-DOX* gene regulation and function in plants exposed to salinity stress. We can compare the expression level or α-DOX activity in *T. halophila* to Arabidopsis and if the activity was different in *T. halophila*, this would suggest a role for α-DOX in salt tolerance.

APPENDIX

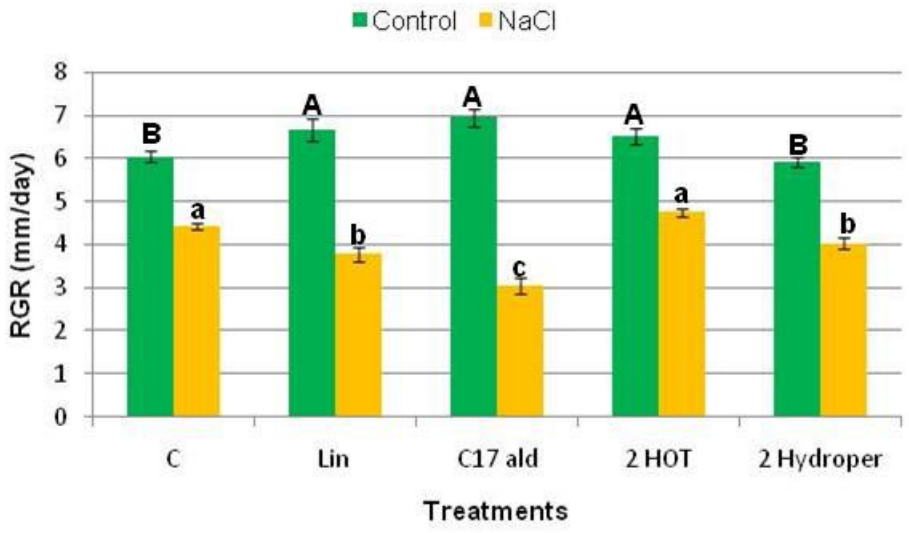

Figure 1 The effect of oxylipins on growth rate of wild type plants.

Seeds were germinated on the plate containing ½ MS media with or without 75 mM NaCl. 15 µM of Linolenic acid (Lin) or C17 aldehyde (C17 al) or 2HOT (2Hot) or 2 Hydroperoxypalmic acid (2Hydroper) were applied to each root tip every 2 days for 6 days. At day 8, root lengths were measured and root growth rate (RGR) was calculated. RGR data were analyzed by ANOVA. Bars represent mean of pooled data +/- SE. Bars denoted with the same letter are not significantly different (Student's t-test P<0.05). This experiment was carried out 3 times with 2-3 replicates for each genotype for each treatment per experiment.

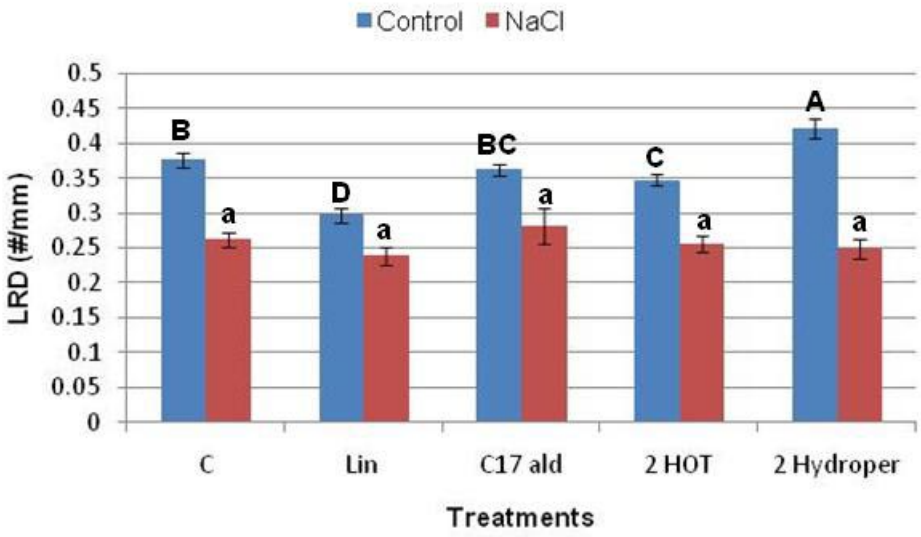

Figure 2 The effect of oxylipins on lateral root density of wild type plants.

Seeds were germinated on the plate containing ½ MS media with or without 75 mM NaCl. 15 µM of Linolenic acid (Lin) or C17 aldehyde (C17 al) or 2HOT (2Hot) or 2 Hydroperoxypalmic acid (2Hydroper) were applied to each root tip every 2 days for 6 days. At day 8, lateral roots were counted and lateral root density (LRD) was calculated. LRD data were analyzed by ANOVA. Bars represent mean of pooled data +/- SE. Bars denoted with the same letter are not significantly different (Student's t-test P<0.05). This experiment was carried out 3 times with 2-3 replicates for each genotype for each treatment per experiment.

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