

**ISOLATION AND CHARACTERIZATION OF NOVEL
SALT-RESPONSIVE GENES FROM TOMATO
(*Lycopersicon esculentum* Mill) ROOTS**

by

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MSc, Simon Fraser University, 1996

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ABSTRACT

In an effort to isolate and characterize novel salt-responsive genes, mRNA differential display-polymerase chain reaction was conducted. Examination of tomato root mRNA profiles revealed that a salt treatment induced, up-regulated or down-regulated the expression of a number of genes. Nine partial cDNAs were isolated and three (JWS19, JWS20 and JWS27) were chosen for further analyses. A full-length cDNA corresponding to JWS27 contained an interrupted open reading frame and two introns, suggesting that JWS27 was potentially derived from either an unprocessed transcript(s) or a pseudogene. Full length cDNAs corresponding to JWS19 and JWS20 encoded polypeptides with similarities to a tomato auxin-regulated protein of unknown function and α -dioxygenase enzymes, respectively. Alpha-dioxygenases catalyse the oxygenation of fatty acids to produce a newly identified group of oxylipins. In tomato, α -DIOXYGENASE is represented by a small gene family, of which only one member (*LE α -DOX1*) was salt-responsive.

The role of ABA in regulating salt-induced changes of gene expression was explored using genetic and chemical approaches to reduce root ABA levels. In salt-treated roots of the ABA deficient mutant *flacca*, the expression of genes corresponding to JWS19, JWS20, and JWS27 was similar to that observed in the wild type. A fluridone (FLU) pre-treatment to reduce ABA content had no effect on the level of salt-induced expression of JWS19 and JWS27. However

following a salt treatment, the expression of *LE α -DOX1* was higher in roots of FLU-pre-treated plants than it was in roots that did not receive FLU. An explanation for this relates to the role of ABA in suppressing ethylene accumulation in osmotically stressed roots. Ethephon and the precursor of ethylene biosynthesis markedly elevated *LE α -DOX1* expression, and this enhanced expression was suppressed by ABA, suggesting that ABA and ethylene can interact to regulate *LE α -DOX1* expression. *LE α -DOX1* expression in salt-stressed roots was not markedly affected by an inhibitor of ethylene biosynthesis, 1-aminoethoxyvinylglycine, AVG, indicating that ABA may be responsible for the enhanced α -DOX expression. However, blocking ethylene signalling with silver ions drastically reduced *LE α -DOX1* transcript levels, suggesting that a functional ethylene signalling pathway may be required for *LE α -DOX1* expression in salt-treated roots.

DEDICATION

UNTUK RANDY & KEIZAR,

PAPA (YANG TAK SEMPAT MENYAKSIKAN KESUKSESAN INI), MAMA DAN AYOT.

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1 LITERATURE REVIEW

Saline soils have long existed and continue to affect land upon which plants are or might be cultivated. Soil salinization is a condition in which there is an increased concentration of salts, mainly sodium chloride or sodium sulphate, within the soil. Salinization can occur naturally, referred to as primary salinization or can be induced by human agricultural practices, referred to as secondary salinization (Szalbocs, 1994).

Naturally occurring saline soils are found in coastal areas and salt marshes all over the world (Chapman, 1974) in which salt from the sea are being carried inland by winds and deposited by rainfall. In addition, naturally occurring soil can also originate from the periodic submergence of land under seawater. Secondary salinization is generally observed within the arid agricultural region. In this region, salt concentration at the soil-root interface increases drastically as soil water is depleted through evaporation and transpiration (Bernstein, 1974). Furthermore, water from lower soil horizons can move upward through the soil profile causing salts to accumulate at the soil surface. In arid regions, irrigation is a major and necessary part of agricultural practices, and most irrigation waters contain more salt than those that will be absorbed by the crop. As a consequence, salts from irrigation water will build up in the soil if continued irrigation has been used with improper drainage.

Saline soils are characterized by high levels of salt and neutral pH levels. Soil salinity is determined by measuring its conductivity; a value of 0-4 (deci siemens) dSm^{-2} is considered non-saline to slightly saline, 4-8 dSm^{-2} is moderately saline and 8-16 dSm^{-2} is severely saline (Shannon et al., 1994). Plant growth is adversely affected when a specific ion concentration exceeds its threshold. High salt concentration can interfere with nutrient uptake, decrease water potential of the growing medium and cause toxicity when ions enter the plant. Although the concentration at which these effects take place varies between plant species, growth stages, environmental interactions and the type of ion, in general salt concentrations higher than 4 dS/m^{-2} can disturb the growth of many plants thereby decreasing their yield.

The exact amount of salt-affected land is unknown. It has been reported that about one fifth of all cultivated land and nearly half of the irrigated area are affected by salt (Rhoades and Loveday, 1990). Salt-affected land is a potential threat to agriculture since most crop plants are not able to grow under high concentrations of salt (Munns, 2002). Thus, ultimately saline soil is a potential threat to our food supply.

It is predicted that the human population will grow by about 50%, from 6.1 billion in 2001 to 9.3 billion by 2050 (Flowers, 2004). As a result, maintaining sufficient food supply is important. According to the United Nations Development Program, about half of the world's land surface is dry lands (see <http://www.undp.org/seed/unso/pub-htm/dryland-population>), which in order to make productive rely heavily on irrigation. Unfortunately, as previously

mentioned, irrigation practices coupled with improper drainage have been linked to soil salinization (Ghassemi et al., 1995). Therefore, applying agricultural practices that are aimed at preventing soil salinization together with efforts to remediate salinized soils are among the approaches that can be used to reduce or minimize soil salinization. However, for optimal results, these approaches need to be complemented with efforts to improve plant salt tolerance either by applying traditional breeding or gene manipulation technologies. In this way, plant productivity can be maintained or may be increased in salt-affected soils that themselves are being remediated.

1.1 Osmotic and Ionic Stresses

Water comprises over 80% of the weight of most plant tissues and is essential as a solvent, a transport medium for nutrients, an evaporative coolant and to provide the turgor pressure to support the growth process. There are two ways in which solutes can move from the outside solution into the root: the extra cellular (apoplastic) and the intracellular (symplastic) pathways. As water passes through the plant, salts are transported through the membranes by active or passive transport mechanisms. All salts can affect plant growth; however the most common cause of ion-specific damage in plants is Na^+ .

What is stress? Literally the word 'stress' is derived from the latin word *stringere* or distress. In most definitions, stress is considered to be a condition in which there is a significant deviation from the optimal condition of life. Stress elicits changes and responses that may be reversible; However they can also become permanent (Larcher, 2003). Under conditions of high salinity, plants

experience two kinds of stresses: osmotic, in which increased amounts of salts reduce water availability by decreasing the osmotic potential of the soil, and ionic, in which high amounts of salts, primarily sodium disrupt cellular ion homeostasis.

Under salinity stress, plants must maintain their water potential below that of the soil in order to maintain turgor and water uptake for growth. To achieve this, when Na^+ levels are high, plants increase their osmoticum either by taking up soil solutes or by synthesizing compatible solutes. This, however, creates a dilemma for plants, since Na^+ and Cl^- are cheap solutes that are readily available in saline soils; however they are toxic if accumulated in the cytosol. On the other hand, compatible solutes are non-toxic but they are energetically expensive to synthesize. Thus to prevent accumulation in the cytoplasm, absorbed Na^+ should be sequestered within the vacuoles and osmotic adjustment within the cytosol is maintained by synthesis of compatible solutes (Flowers et al., 1977; Munns et al., 1995; Tester and Davenport, 2003).

The metabolic toxicity of Na^+ within the plant is mainly due its ability to compete with K^+ for its role as an enzyme activator. The potassium ions can activate more than 50 enzymes, and this function cannot be replaced by Na^+ (Bhandal and Malik, 1988). Therefore high levels of Na^+ or high $\text{Na}^+:\text{K}^+$ ratios are precarious for various enzymatic processes in the cytoplasm. Furthermore, K^+ is required during protein synthesis, particularly for the binding of tRNA to ribosomes (Blaha et al., 2000). As a consequence, the increased level of Na^+ can ultimately disrupt protein synthesis.

1.2 Glycophytes vs Halophytes

Plants can be grouped into glycophytes and halophytes based on their ability to grow on high salt-containing medium (Flowers et al., 1977). Most plants are glycophytes or salt sensitive plants. Although there is a wide range in which glycophytic plants can tolerate salt, the majority of them will be killed if grown in 0.1M NaCl (Smith and McComb, 1981). For their optimal growth, halophytes require much higher Na⁺ and Cl⁻ concentration than are found in non saline soils, for example some halophytes can survive in medium containing 500 mM NaCl (Hasegawa et al., 2000). Halophytic plants can grow and ultimately survive in a saline environment due to osmotic adjustment through intracellular compartmentation that partitions Na⁺ and Cl⁻ away from the cytoplasm through energy-dependent transport into the vacuole (Apse et al., 1999; Binzel et al., 1988; Glenn et al., 1999). Not only are halophytes more effective in partitioning, they also appear to be more effective in coordinating this partitioning with processes controlling growth and ion fluxes across the plasma membrane.

It has been argued however, that halophytes do not necessarily have a special mechanism for dealing with high Na⁺ concentration instead they are more successful in using the mechanisms that exist in glycophytes (Zhu, 2001). Using a comparative genomics approach, this hypothesis was recently tested using *Arabidopsis* and *Arabidopsis*-related halophytic salt cress plants (Taji et al., 2004). *Arabidopsis* is a glycophyte and believed to contain most or nearly all of the salt tolerance genes that exist in halophytes (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2000; Shinozaki et al., 2003). Salt cress (*Thellungiella*

halophila) is a halophyte that is closely related to *Arabidopsis*, and can grow in medium containing 500 mM NaCl. Since salt cress does not have any morphological adaptations such as salt glands that are often present in halophytes, it is hypothesized that its ability to tolerate high salt concentration may be similar to those operating in glycophytes (Bressan et al., 2001; Zhu, 2001). To reveal the mechanism(s) underlying the dissimilarity of salt tolerance regulation between salt cress and *Arabidopsis*, gene expression profiles in both species were analyzed during non-stressed and salt-stressed conditions using an *Arabidopsis* cDNA microarray. The result of this experiment demonstrated that in *Arabidopsis* 40 genes were up-regulated in response to a short term NaCl treatment, whereas, only six genes were up-regulated in salt cress which include genes that encode myoinositol-1-phosphate synthase, putative myoinositol-1-phosphate synthase, galactinol synthase, a putative calcium binding EF-hand protein, late embryogenesis abundant protein LEA-like protein and a protein kinase family (Taji et al., 2004). Thus, one strategy used by salt cress for salt tolerance is its ability to express various abiotic or biotic stress-inducible genes under non-stressed conditions. Therefore, the ability of salt cress to tolerate high salt concentration is not due to its immediate response at the transcriptional level following salt stress; but is likely due to pre-existing mechanisms that are present prior to stress, in which a number of genes that are important for salt stress defense and survival are over-expressed in the absence of the stress which include a putative -1-pyrroline 5-carboxylase synthetase (*AtP5CS*); 9-cis-epoxycarotenoid dioxygenase (*AtNCED*); Fe-superoxide-dismutase (*SOD*);

chitinase, plant defensin1.2 (*PDF1,2*); β -glucosidase; P-protein associated with nitric oxide (NO) production, and the plasma membrane Na^+/H^+ antiporter (SOS1).

1.3 Mechanisms of Salt Entry into Roots

1.3.1 Transmembrane Na^+ Influx

The concentration of Na^+ in the soil is generally much higher than that in the cytosol of a root cell. Consequently, the movement of Na^+ ions into root cells is passive (Cheeseman, 1982). Since no Na^+ -selective channels have been identified to date, current evidence suggests that Na^+ enters root cells mainly through various cation channels (Tester and Davenport, 2003). These channels could be voltage-dependent cation channels or voltage-independent cation channels (VIC).

The non-selective voltage-dependent cation channels (NSCCs) have been suggested to be the most likely pathway for Na^+ entry (Amtmann and Sanders, 1999, Tyerman and Skerrett, 1999; Schachtman and Liu 1999; Demidchik et al., 2002). NSCCs are a large and heterogenous group of channels. In general, they demonstrate a high selectivity for cations over anions, but a low selectivity among monovalent cations under a wider range of ionic conditions (Demidchik et al., 2002). To date there are many candidate genes that encode NSCC's, unfortunately their precise molecular identity remains unclear (Demidchik et al., 2002). The two major 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). In *Arabidopsis*, twenty putative CNGCs have been identified (Maser et al., 2002). Orthologs of some of these putative CNGCs have also been isolated from barley (Schuurink et al., 1998). Studies have indicated that CNGCs are expressed in roots and they affect cation uptake (Sunkar et al., 2000; White et al., 2000). A recent study also showed that the *Arabidopsis* *CNGC1* gene (*AtCNGC1*) is responsible for conducting Na^+ (Hua et al., 2003). The involvement of the glutamate-activated channels (GLRs) in Na^+ influx is derived from a study which showed that upon addition of glutamate there is an increased Na^+ influx (Tester and Davenport, 2003), this phenomenon, however, needs further confirmation.

The voltage-independent pathway that facilitates Na^+ entry is better understood than the voltage-dependent pathway (Xiong and Zhu, 2002a). Voltage independent pathways are considered to be the major route for Na^+ entry into plant cells (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; White, 1999). Due to the similarity between Na^+ and K^+ , potassium influx transporters have long been proposed to mediate sodium influx (Epstein et al., 1963). The K^+ -inward rectifiers are one possible pathway for Na^+ entry into roots cells (Rubio et al., 1995; Blumwald, 2000). In wheat roots, HKT1 was initially isolated from a cDNA library of K^+ -starved roots and belongs to a high affinity K^+ uptake system. Expression analysis of HKT1 in *Saccharomyces cerevisiae* and *Xenopus* oocytes showed that at low external Na^+ , HKT1 functions as an active K^+ transporter whereas at high external Na^+ , it can function as a low affinity Na^+ transporter (Rubio et al., 1995).

The HKT transporter has been successfully identified in *Arabidopsis* (Uozumi et al., 2000), eucalyptus (Fairbairn et al., 2000; Liu et al., 2001), common ice plant and rice (Golldack et al., 2002; Horie et al., 2001). In *Arabidopsis*, HKT is represented by a single member, *AtHKT1*, whereas in rice it is represented by at least nine members: *OsHKT1* – *OsHKT9*, and in eucalyptus it is represented by two members: *EcHKT1* and *EcHKT2* (Uozumi et al., 2000; Horie et al., 2001; Garcideblas et al., 2003). *AtHKT1* has been shown to be responsible for Na⁺ uptake when expressed in *Saccharomyces cerevisiae* or *Xenopus* oocytes (Uozumi et al., 2000 and Rus et al., 2001). Based on analysis of functional properties in *Xenopus* oocytes, plant transporters of the HKT family can be divided into two groups. The first subfamily contains transporters that are similar to wheat HKT1, which is able to mediate both K⁺ and Na⁺ uptake depending on the external Na⁺ concentration. The second sub family appears to mediate only Na⁺ uptake and is not significantly permeable to K⁺ regardless of the external Na⁺ concentration (Rubio et al., 1995; Berthomieu et al., 2003). The second subfamily is hypothesized as a potential Na⁺ specific transporter; *AtHKT1* and *OsHKT1* are members of the second sub family (Berthomieu et al., 2003).

1.3.2 Apoplastic Na⁺ Influx

The apoplastic pathway, also known as the bypass flow, is another possible way in which Na⁺ can diffuse into plants. The physical basis of the apoplastic pathway has not yet been well defined. However, evidence indicates that this bypass flow is likely to be located at points where the root branches. In rice, this pathway appears to be the major pathway of Na⁺ entry (Yeo and Flowers, 1985;

Yeo et al., 1987). The significance of the apoplastic pathway in plants for Na^+ uptake has also been supported by some data from salt-tolerant plants. Halophytic plants appear to have several anatomical adaptations to minimize Na^+ entry via the apoplastic pathway, such as the formation of a second endodermis (Stelzer and Lauchli, 1977). In cotton seedlings, salinization has been shown to induce suberization and the formation of an exodermis (Reinhardt and Rost, 1995).

1.4 Aspects of Plant Salt Tolerance

Salt stress disrupts homeostasis in both water potential and ion distribution which can lead to molecular damage, growth arrest and ultimately death. This disruption can be detected at both the cellular and whole plant levels. Zhu (2001) proposed three interconnected aspects of plant activities that need to be performed in order to tolerate high salt concentration. The first is prevention or alleviation of damage, the second is restoration of homeostatic condition and the third is maintenance of growth albeit at a reduced rate.

1.4.1 Prevention or Alleviation of Salt-Induced Damage

Plants that are growing in habitats that contain high Na^+ cannot escape its effect, and therefore have to develop some strategies to cope with it. Different strategies have been developed by plants for adapting to high levels of Na^+ , for examples, glycophytes are known to use salt exclusion whereas halophytes accumulate salts as a mechanism for maintaining their cellular osmotic potential. Salt exclusion by glycophytes is accomplished by either precluding Na^+ entry into

the plants at the root surface or Na^+ entry into the xylem in the roots. In halophytes, Na^+ is sequestered within the leaf vacuole; furthermore, they also have the ability to accumulate salts in glands and salt bladders that are located on the leaf surface. Other halophytes develop succulence which is a modification in which high water content is maintained per leaf surface area (Longstreth and Nobel, 1979). Succulence adaptation therefore minimizes the effect of excessive salt concentration within the leaf tissue. Additionally, most halophytes distribute Na^+ to older mature leaves rather than to the developing tissues, this phenomenon, however, is not limited to the halophytes since some glycophytes can also compartmentalize salt in older tissues (Flowers and Yeo, 1986, Gorham, 1990).

In response to high Na^+ concentration within the cell, a variety of proteins are synthesized; many of these possess properties similar to chaperones. Chaperones are involved in the maintenance of protein structure during increased salt condition or other conditions in which protein-water interactions are disrupted (Ingram and Bartels, 1996; Campbell and Close, 1997). Additionally, chaperones may function in membrane stabilizing. In general, these proteins are hydrophilic with a random coil structure, and it is believed that they accumulate due to the osmotic component of salt stress.

Genes encoding chaperones belong to the *lea* (late embryogenesis abundant) group (Baker et al., 1988; Dure et al., 1989). These genes were initially identified in cotton during seed maturation and are highly expressed in seeds during the desiccation stage following maturation. A variety of LEA-like

proteins have now been identified in vegetative tissues of plants exposed to stresses that have a water-deficit component. Based on their amino acid sequence homology, LEA proteins have been classified into several groups which are based on their similarity to the prototypical LEA proteins from the cotton plant (D7, D11, D19, D95 and D113, Dure et al., 1993); a new classification system however has recently been proposed by Wise (2003).

Another effect of salt stress in plants is the production of reactive oxygen species (ROS). ROS cause oxidative damage to various cellular components including membrane lipids, proteins, and nucleic acids (Halliwell and Gutteridge, 1986). It has been demonstrated that the activities of enzymes involved in scavenging ROS are higher in salt-tolerant species of tomato than in their salt-sensitive relatives (Shalata and Tal, 1998; Mittova et al., 2004). It is therefore suggested that ROS scavenging activity may be part of the active tolerance mechanisms of the salt-tolerant species rather than part of the secondary response to salt-associated damage (Tester and Davenport, 2003).

Reactive oxygen species are salvaged by osmoprotectants such as proline, mannitol, fructans, trehalose, glycinebetaine, and ectoine (Shen et al., 1997; Xiong et al., 2002b). It is believed that many of the osmolytes and salt-responsive proteins with unknown functions might play a role in detoxification by scavenging ROS or preventing them from damaging cellular structures (Zhu, 2001). Therefore, besides functioning as compatible solutes, these osmolytes can also function as ROS scavengers. This hypothesis is derived from several lines of evidence which indicated that the level of osmolytes in transgenic plants

is generally too low to be significant for osmotic adjustment, yet the transgenic plants are improved in tolerance not only to salt stress but also to other stresses including chilling, freezing, heat and drought all of which generate ROS (Kalir et al., 1981; Alia et al., 1998; Sakamoto et al., 2000; Zhu, 2001). Thus, it is believed that one mechanism that is responsible for the increased level of salt tolerance in transgenic plants engineered with these osmolytes is the increased ability of these transgenic plants for oxidative detoxification (Zhu, 2001).

1.4.2 Ion and Osmotic Homeostasis

1.4.2.1 Ion Homeostasis

Reestablishing ion homeostasis after experiencing salt stress is one response all plants need. In dealing with ionic stress imposed by salt stress, plants can employ several lines of defense mechanisms including restricting salt uptake, increasing Na^+ extrusion and compartmentalization, controlling long distance transport of Na^+ to the aerial parts, and recirculation of Na^+ from shoots to roots (Zhu, 2002; Munns, 2002).

In *Arabidopsis*, several classes of transporters have been shown to be essential in regulating sodium homeostasis during salt stress. AtHKT1 has been proposed to be one of the transporters responsible for Na^+ influx in *Arabidopsis* (Schachtman and Schroeder, 1994; Rus et al., 2001), while a plasma membrane Na^+/H^+ antiporter such as SOS1 is responsible for Na^+ efflux (Shi et al., 2000). The vacuolar membrane Na^+/H^+ antiporter, AtNHX1 (Apse et al., 1999; Gaxiola et al., 2002) is important in regulating cytoplasmic Na^+

homeostasis. The transcript level of some members of the Arabidopsis *AtNHX* gene family (*AtNHX1*, *AtNHX2* and *AtNHX3*) was elevated in response to salt stress (Yokoi et al., 2002). The Na^+/H^+ antiporter activity is also increased following salt stress in barley (Gabarino and DuPont, 1990), tomato (Wilson and Shannon, 1995), and sunflower (Ballesteros et al., 1997). In the salt tolerant species, *Plantago maritima*, the vacuolar Na^+/H^+ antiporter activity is much higher than that observed in the salt-sensitive species *Plantago media* (Staal et al., 1991), and in agreement with this, the vacuolar Na^+/H^+ antiporter activity in salt-sensitive rice was not up regulated following salt stress (Fukuda et al., 1998). The importance of vacuolar sequestration of Na^+ in reducing the damaging effect of Na^+ during salt stress has been tested by over-expressing this gene. Over-expressing vacuolar Na^+/H^+ *NHX1* increased salinity tolerance in Arabidopsis (Apse et al., 1999), tomato (Zhang and Blumwald, 2001) and *Brassica napus* (Zhang et al., 2001).

Unlike animal cells, plant cells do not have Na^+ -ATPases or Na^+/K^+ -ATPases, therefore they rely on V^+ -ATPases and H^+ -pyrophosphatase to create a proton-motive force which then drives the transport of ions and metabolites into the vacuole. In Arabidopsis, the vacuolar H^+ -pyrophosphatase is represented by a single gene, *AVP1* (Sarafian et al., 1992). Transgenic Arabidopsis plants over-expressing the vacuolar H^+ -pyrophosphatase have an increased tolerance to both high NaCl concentration and water deficit stress (Gaxiola et al., 2001), and the increased resistance of these transgenic plants to NaCl is due to their ability to accumulate more Na^+ and K^+ in their leaf tissue.

In Arabidopsis, the Salt Overly Sensitive (SOS) pathway has recently been described as important for dealing with salt stress (Xiong and Zhu, 2002). Excess intracellular or extracellular Na^+ is likely to be the input signal that triggers the SOS pathway (Liu and Zhu, 1998). Increased cytosolic calcium has been suggested to be one of the earliest detectable responses to salt stress (Knight, 2000). It is believed that the salt-elicited calcium signal is sensed by a myriostylated calcium binding protein encoded by *SOS3*, and *SOS3* will interact and activate *SOS2*, a serine/threonine protein kinase (Liu et al., 2000). There are two possible ways in which *SOS3* can mediate *SOS2* activity: (i) kinase activation and (ii) protein targeting (Quintero et al., 2002). Furthermore, the *SOS2/SO3* complex regulates the expression of *SOS1* (Qiu et al, 2002, Shi et al., 2000) and also stimulates *SOS1* transporter activity and Na^+ efflux (Zhu, 2001; Shi et al., 2000; Quintero et al., 2002). The associated transcription factor that is responsible for *SOS1* gene expression however remains to be discovered.

Currently, not much is known about how Na^+ is sensed in the cell. It is believed that salt can be perceived either before or after it enters the cell or both (Zhu, 2003). A membrane receptor may be responsible for sensing extracellular Na^+ , whereas, intracellular Na^+ may be perceived by a membrane protein or any of the Na^+ -sensitive enzymes located in the cytoplasm. In Arabidopsis, the plasma membrane Na^+/H^+ antiporter *SOS1* has been suggested to be a possible Na^+ sensor (Shi et al., 2000), since it possesses 10-12 transmembrane domains and a long 700 amino acid tail that is predicted to be located in the cytoplasm. Membrane transporters with long cytoplasmic tails have been proposed to

function as a sensor of the solute that they transfer. For example, several transporters in yeast and bacteria such as the glucose transporter and the regulator of glucose transporter have long cytoplasmic tails that are sensors (Chen et al., 1997; Ozcan et al., 1998).

Sodium extrusion by root epidermal cells is believed to serve an important role since other cells in the plant are surrounded by neighboring cells and therefore Na^+ extruded by one cell could potentially be a problem to its neighbor. In plants, sodium extrusion is performed by Na^+/H^+ antiporters located on the plasma membrane. In *Arabidopsis*, this task is believed to be performed by *SOS1* (Shi et al., 2000, 2003; Shi and Zhu, 2002). It has been shown that *SOS1* is preferentially expressed both in epidermal cells surrounding the root tip and in parenchyma cells bordering the xylem throughout the plant (Shi and Zhu, 2002). *Sos1* mutation results in a plant that is very sensitive to Na^+ (Wu et al., 1996), whereas over expressing *SOS1* results in a plant with increased tolerance to salt stress (Shi et al., 2003). Further analysis of transgenic plants that over-expressed *SOS1* showed that they had a lower Na^+ content in the shoot following Na^+ treatment, suggesting that the role of *SOS1* as a Na^+ efflux mechanism is important for salt tolerance in plants (Shi et al., 2003; Xiong and Zhu, 2002a).

Recirculation of Na^+ from shoots to roots via phloem sap is thought to be an important mechanism in salt tolerance (Munns, 2002). *AtHKT1* was initially proposed to be involved in Na^+ uptake in roots (Rus et al., 2001); However, this hypothesis was not supported by data obtained by Berthomieu et al (2003) in which they did not observe a reduction in Na^+ uptake in their *Athkt1* mutant

(*sas2*). Moreover, they also found that *AtHKT1* is not expressed in root epidermal cells as demonstrated previously by Rus et al (2001), but rather its expression was restricted to the phloem tissues (Berthomieu et al., 2003). The *sas2* mutant displayed Na^+ over accumulation in shoots, and therefore, they have proposed that *AtHKT1* is involved in recirculation of Na^+ from shoots to roots via the phloem sap. Their electrophysiological analyses showed that *AtHKT1* can fulfill different functions in leaves and in roots: it would mediate Na^+ loading into the phloem sap in leaves and unloading in roots. This recirculation therefore will remove a large amount of Na^+ from the shoot and play an important role in plant tolerance to salt.

1.4.2.2 Osmotic Homeostasis

As Na^+ accumulates in the vacuole, the osmotic potential in the cytoplasm must be balanced with that in the vacuole. Accumulation of compatible solutes is a common strategy used by plants subjected to changes in their external osmotic potential. Several compatible solutes that function as osmolytes include: sugars (sucrose, fructose), sugar alcohols (glycerol, methylated inositols), and complex sugars (trehalose, raffinose and fructans). Additionally, ions (K^+) or charged metabolites (glycine betaine, dimethyl sulfonium propionate, proline and ectoine) are also used. These compatible solutes are relatively small, non toxic compounds which can help stabilize protein and cellular structures as well as increase the osmotic potential (Bohnert et al., 1999; Yeo, AR., 1998). At high concentration, compatible solutes lower the inhibitory effects of Na^+ on enzyme activity (Solomon et al., 1994) as well as preventing dissociation of enzyme

complexes (Galinski, 1995). In the short term, accumulation of compatible solutes would allow the cell to avoid water loss, and maintain an osmotic potential. This is important for sustaining cell turgor, water uptake and cell expansion (Tester and Davenport, 2003).

The metabolic pathways responsible for osmolyte biosynthesis are typically related to basic metabolism pathways that show high output rates (Bohnert et al, 1999; McCue and Hanson, 1992; Nuccio et al., 1999). For example, proline (Delauney et al., 1993; Kavi-Kishor et al., 1995), glycine betaine (McCue and Hanson, 1992; Rathinasabapathi et al., 1997), D-pinitol (Ishitani et al., 1996; Vernon and Bohnert, 1992) and ectoine (Galinski, 1995) are generated from pathways associated with amino acid biosynthesis such as glutamic acid (proline), aspartate (ectoine), choline (glycine betaine) and myo inositol (pinitol). The enzymes that are responsible for the synthesis of these osmolytes are shown to be up regulated following stress. These include enzymes that are responsible for glycine betaine (Hanson et al., 1994), D-pinitol (Ishitani et al., 1996; Nelson et al., 1998; Vernon and Bohnert, 1992 and proline (Nanjo et al., 1999; Yoshida et al., 1995; Zhang et al, 1995) accumulations.

At this moment, the signal transduction pathways responsible for osmolyte production are not well established. Zhu (2002) suggested that there are two possible pathways responsible for osmolyte production: the osmotic stress-activated-protein kinase and osmotic stress-activated phospholipid pathways.

Several osmotic stress-activated plant protein kinases have been successfully identified, for example, the sucrose non-fermenting 1 (SNF1)-related protein

kinase, SnRK2 in Arabidopsis (Umezawa et al., 2004), tobacco (Mikolajczyk et al., 2000) and soybean (Monks et al., 2001). The phospholipid signaling pathways are generally classified based on the phospholipases responsible for the formation of lipid and other lipid messengers (Zhu, 2002). The phospholipase C (PLC) pathway is responsible for the formation of inositol 1,4,5,-triphosphate (IP3) and diacylglycerol (DAG). In Arabidopsis, *AtPLC1* is induced by salt and drought stress (Hirayama et al., 1995). IP3 has been suggested to play a role in releasing Ca^{2+} from internal stores, whereas DAG is responsible for activating protein kinase C. The level of IP3 has been shown to increase in response to hyperosmotic stress (DeWald et al., 2001; Droback and Watkins, 2000; Takahashi et al., 2001). The IP3 precursor, phosphatidylinositol 4,5-bisphosphate (PIP2), is formed by phosphatidylinositol 4-phosphate 5-kinase (PIP5K). In Arabidopsis, the gene responsible for this enzyme is upregulated by both abscisic acid and osmotic stress (Mikami et al., 1998).

Phospholipase D (PLD) generates phosphatidic acid (PA). In suspension cultures of *Chlamydomonas*, tomato and alfalfa, the activity of PLD is increased following osmotic stress (Munnik et al., 1995; Munnik et al., 1998; Munnik and Meijer, 2001). PLD (*AtPLD*) activity in Arabidopsis is rapidly increased in response to dehydration stress (Katagiri et al., 2001). In addition to the PLC and PLD pathways, in algae and yeast, there exists another pathway, phospholipase A2 (PLA2). PLA2 generates lyso-phospholipids and free fatty acids by cleaving phospholipids at the sn-2 position. In algae, PLA2 activity is increased following hyperosmotic stress (Eisenpahr et al., 1988; Meijer et al., 2001). The role of

PLA2 during osmotic stress is not well established yet, however it has been suggested that PLA2-derived products may be involved in osmoregulation by stimulating tonoplast H⁺-ATPase activity (Munnik and Meijer, 2001).

1.4.3 Growth Regulation

One negative effect of salt stress owing to its osmotic stress component is reduced water availability for plants. Since water-generated turgor pressure is a driving force for cell expansion this can result in reduced rates of cell expansion (Munns, 2002). Low water availability is also considered to be one of the major factors that limit photosynthesis, and hence, plant growth and yield. It has been suggested that photosynthesis is predominantly sensitive to salt stress due to stomata closure therefore limiting carbon dioxide uptake. It has, however, been a long-standing debate with regards to whether the negative effect of salt stress on photosynthesis is manifested via stomatal diffusive resistances or metabolic impairments. It was recently demonstrated that salt stress mainly affects CO₂ diffusion in the leaves by decreasing stomatal conductance and not the biochemical capacity to assimilate CO₂ (Flexas et al., 2004).

Slower growth is believed an adaptive feature that is manifested in order to survive stress. By reducing growth, plants can use their energy resources to battle stress (Zhu, 2001). During salt stress, there is a greater inhibition in shoot growth than in root growth (Saab et al., 1990). It is proposed that continued growth in roots albeit at a reduced rate would allow plants to explore more soil volume in order to satisfy their water and mineral requirements.

Salt stress has a direct effect on cell division (Zhu, 2001). In *Arabidopsis*, a cyclin-dependent protein kinase inhibitor 1 (ICK1) was induced by ABA application. ICK1 is believed to inhibit cell division by affecting the activities of cyclin-dependent protein kinases that drives the cell cycle (Wang et al., 1998).

1.5 Abscisic Acid

Abscisic acid (ABA) was initially identified in the early 1960s as a growth inhibitor produced in abscising cotton fruit and leaves of sycamore trees (Addicott, 1983). Since then, ABA has been demonstrated to regulate many aspects of plant growth and development, as well as responses to various environmental stresses including UV radiation, pathogen attack, cold, water deficit and salinity (Leung and Giraudat, 1998; Rock, 2000). Under non-stressed conditions, the level of ABA in plant cells is maintained at low levels. In comparison to the wild type, ABA-deficient mutant plants appear to be less vigorous, and it is therefore believed that a low level of ABA in plant cells is required for normal plant growth (Finkelstein and Rock 2002).

ABA is a sesquiterpenoid ($C_{15}H_{20}O_4$) with one asymmetric, optically active carbon atom at C-1'. The naturally occurring form of ABA is S-(+)-ABA. In addition, R-(-)-ABA has been demonstrated to have biological activities (Zeevaart and Creelman, 1988). In plants ABA exists ubiquitously and can easily enter cells via the plasma membrane. As a weak acid ($pK_a=4.8$), ABA is mostly uncharged when present in the relatively acidic apoplastic compartment. ABA distribution within plant cellular compartments follows the anion trap concept as follows: the dissociated (anion) form of ABA accumulates in alkaline compartments and can

redistribute according to the relative pH of the compartment. Additionally, specific uptake carriers play a role in maintaining a low apoplastic ABA concentration in unstressed plants (Finkelstein and Rock, 2002). Due to the diverse function of ABA in plants, it is believed that there is a complex regulatory mechanism that controls its production, degradation, signal perception and transduction.

Understanding the regulatory mechanism of ABA is therefore critical in order to build or create strategies for breeding plants with an increased tolerance towards adverse environmental conditions.

1.5.1 The ABA Biosynthesis Pathway

Mutants that are defective in ABA biosynthesis have been isolated from a variety of plant species including maize (*Zea Mays*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), and Arabidopsis (*Arabidopsis thaliana*), and these ABA-deficient mutants have been influential in elucidating the ABA biosynthesis pathway. Two possible pathways were initially suggested for ABA biosynthesis, the direct pathway in which ABA is derived directly from the C₁₅ compound farnesyl diphosphate and the indirect pathway in which ABA is synthesized from a C₄₀ carotenoid precursor (Zeevart and Creelman, 1988; Zeevart, 1999). Recent studies have suggested that in higher plants ABA is synthesized via the indirect pathway (Figure 1-1, Taylor et al., 2000; Finkelstein and Rock 2002; Seo and Koshiba 2002; Schwartz et al., 2003).

The carotenoids are synthesized from the C₅ precursor, isopentenyl diphosphate (IPP). In the cytosol, IPP is synthesized from mevalonic acid. However in plastids, IPP is produced via 1-deoxy-D-xylulose-5-phosphate (DXP)

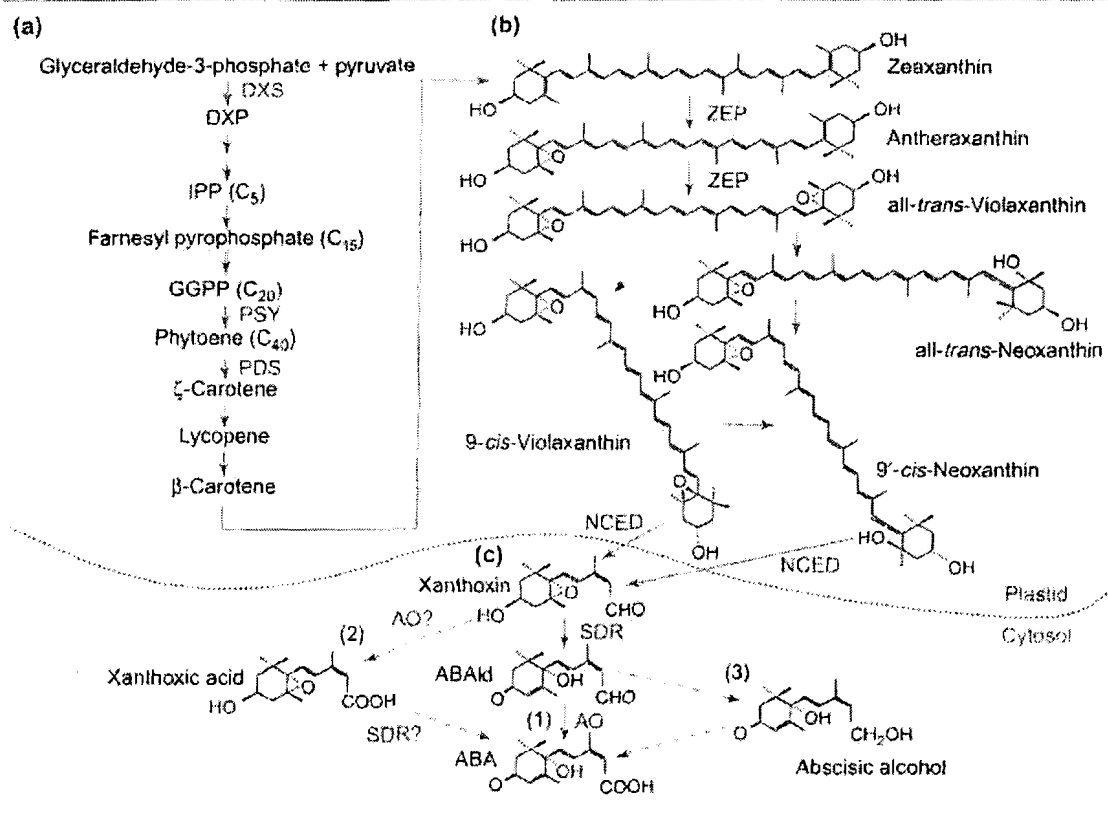


Figure 1-1 The ABA biosynthesis pathway in plants (from Seo and Koshiba, 2002).

- (a)** Carotenoid precursor synthesis in the early steps of ABA biosynthesis.
- (b)** Formation of epoxycarotenoid and its cleavage in plastid
- (c)** Reactions in the cytosol for the formation of ABA

from pyruvate and glyceraldehyde-3-phosphate via the action of DXP synthase (DXS) (Lichtenthaler (1999); Eisenreich et al., 2001). Four IPP are further converted into geranylgeranyl diphosphate (GGPP), a C₂₀ product. The first committed and rate-limiting step of carotenoid biosynthesis is the conversion of GGPP to the C₄₀ carotenoid phytoene, which is catalyzed by phytoene synthase (PSY). Phytoene is subsequently converted to ζ-carotene, lycopene, β-carotene, and then to the xanthophyll zeaxanthin (Cunningham and Gantt, 1998; Hirschberg, 2001). Although it is not a committed step, the epoxidation of zeaxanthin to all-trans-violaxanthin by a two-step epoxidation is believed to be the first step of the ABA-specific synthetic pathway (Seo and Koshiba, 2002; Schwartz et al., 2003). This step is catalyzed by zeaxanthin epoxidase (ZEP) which is the first enzyme identified as an ABA biosynthetic enzyme (Marin et al., 1996). The *Arabidopsis thaliana aba1* and tobacco (*Nicotiana plumbaginifolia*) *aba2* are ABA deficient mutants that are known to be impaired in ZEP (Marin et al., 1996; Rock and Zeevaart, 1991; Duckham et al., 1991). Enzyme(s) that are responsible for catalyzing the conversion of all-trans-violaxanthin to 9-cis-violaxanthin or 9'-cis-neoxanthin have not been identified yet (Seo and Koshiba, 2002).

The first committed step in the ABA biosynthesis pathway is the oxidative cleavage of the xanthophylls, 9-cis-violaxanthin and/or 9'-cis-neoxanthin to yield xanthoxin catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED). The gene encoding NCED was initially identified from the maize *viviparous14 (vp14)* mutant (Tan et al., 1997; Schwartz et al., 1997), and *Notabilis*, an ABA deficient

mutant of tomato is also believed to be defective in NCED (Burbidge et al., 1999). In addition *NCED* cDNAs have also been cloned from bean (*Phaseolus vulgaris*) (Qin and Zeevaart, 1999), cowpea (*Vigna unguiculata*) (Iuchi et al., 2000), avocado (*Persea Americana*) (Chernys and Zeevaart, 2000) and Arabidopsis (Neil et al., 1998; Iuchi et al., 2001). Cellular expression and chloroplast import studies showed that NCED is localized in the chloroplasts (Qin and Zeevaart, 1999; Iuchi et al., 2000., Tan et al., 2001).

The C₁₅ intermediate, xanthoxin is subsequently exported to the cytosol where it is further converted to ABA. Three possible pathways have been proposed to exist between xanthoxin and ABA, each with different intermediates including abscisic aldehyde, xanthoxic acid, and abscisic alcohol (Seo and Koshiba, 2002). In the first pathway, xanthoxin is believed to be converted to abscisic aldehyde and then to ABA. The Arabidopsis *aba2* mutants cannot produce ABA from xanthoxin, however they can oxidize abscisic aldehyde to form ABA (Schwartz, et al., 1997). The *ABA2* gene was recently isolated and demonstrated to encode an enzyme that is related to a short-chain dehydrogenase/reductase (SDR) family. *In vitro* studies using recombinant *ABA2* protein together with xanthoxin resulted in the formation of abscisic aldehyde (A. Endo et al., unpublished *in* Seo and Koshiba, 2002). In Arabidopsis, *ABA2* is constitutively expressed in various organs including roots, leaves, stems and siliques. Furthermore, the *ABA2* transcript level is not affected by ABA, NaCl or mannitol treatment (Gonzales-Guzman et al., 2002; Sindhu and Walton, 1987; Schwartz et al., 1997) but was induced by glucose (Cheng et al., 2002).

Mutants that are affected in the final step of ABA biosynthesis -the conversion of ABA aldehyde to ABA have been identified from a variety of plants. These mutants lack aldehyde oxidase, which may result from a mutation in the aldehyde oxidase (AO) gene or a lesion in the synthesis of a molybdenum cofactor (MoCo) that the enzyme requires for its activity. Arabidopsis *aba3* and *aao3*, tobacco *aba1* and tomato *flacca* are mutants that are unable to convert abscisic aldehyde to ABA. The Arabidopsis *aba3* and tomato *flacca* mutants lack AO activity due to a defect in the formation of a desulfo moiety of the MoCo (Sagi et al., 2002). The expression of Arabidopsis *ABA3* is elevated in response to osmotic stress or ABA (Bittner et al., 2001; Xiong et al., 2001a) suggesting a potential role during osmotic stress. In Arabidopsis, four AO isoforms (*AAO1* to *AAO4*) have been identified (Seo et al., 2000a). *Aao3* is a wilty ABA deficient mutant, and this mutant showed no increased endogenous ABA level following water deficit stress, suggesting that *AAO3* maybe required for ABA production (Seo et al., 2000b). However, *aao3* unlike *aba3*, mutants does not have precocious germination; therefore, it is hypothesized that another AO may be responsible for ABA synthesis in different organs.

Xanthoxic acid has also been suggested to be an intermediate between ABA aldehyde and ABA. It is believed that xanthoxic acid derives from the oxidation of xanthoxin immediately after cleavage and prior to the ring modifications (Cowan, 2000). However, in cell-free extracts, the conversion of xanthoxic acid to ABA is very low (Sindhu and Walton, 1987).

The tomato ABA deficient mutant *flacca* (*flc*) is deficient in ABA accumulation due to the inability to oxidize abscisic aldehyde to ABA. However, *flacca* mutants can synthesize ABA with abscisic alcohol as an intermediate (Linthorpe et al., 1987; Rock et al., 1991b). One of the unique characteristics of *flacca* mutants was their tendency to wilt owing to their lack of control of stomatal closure (Tal, 1966), which is believed to be due to the lower ABA content in their leaves (Imbar and Tal, 1970). The *flc* mutation results in a complete loss of the molybdenum containing aldehyde oxidase enzyme activity in the shoot while minor activity is present in roots (Sagi et al., 1999, Sagi et al., 2002). Consequently, *flc* can accumulate some ABA in roots.

1.5.1.1 ABA Biosynthesis during Seed Maturation and Dormancy

ABA plays a critical role during seed maturation and in the establishment and maintenance of seed dormancy. It is believed that ABA in the developing seeds originates from the maternal tissues or by being synthesized *de novo* within the embryo. Based on studies in *Arabidopsis*, the accumulation of ABA during seed development can be distinguished into two peaks (Koornneef et al., 2002; Finkelstein et al., 2002). The first peak occurs approximately 10 days after pollination and immediately precedes the maturation phase. This ABA has been suggested to be originated from maternal tissues (Karssen et al., 1983), however, there is still debate as to whether this ABA is directly derived from maternal tissues or rather that the maternal ABA serves as a signal for *de novo* synthesis of ABA in developing embryos (Xiong and Zhu, 2003). The second peak of ABA is believed to derive from *de novo* biosynthesis and is responsible

for initiating seed dormancy (Koornneef et al., 2002; Finkelstein et al., 2002). In comparison to the first peak, significantly less ABA is accumulated (about one-third of the first peak) during the second peak. At the later stage of seed maturation, ABA levels fall rapidly and are very low in dry seeds.

1.5.1.2 ABA Biosynthesis and Abiotic Stresses

Salt and drought stresses are the environmental conditions that most significantly activate ABA biosynthesis. Increased ABA levels during these stresses are mainly derived from *de novo* biosynthesis of ABA, and it is believed that the ability of drought and salt stresses to induce ABA biosynthesis is manifested largely via transcriptional regulation of ABA biosynthetic genes since blocking transcription impairs stress-induced ABA biosynthesis (Xiong and Zhu, 2003). Evidence thus far indicates that the regulation of ABA biosynthetic genes varies in different plant organs and developmental stages as well as between plant species.

1.5.1.3 ABA Biosynthesis and Sugar Response

Sugar signals alter many important processes in plants including germination, seedling growth, leaf and root development, and senescence (Sheen et al., 1999; Smeekens 2000). In *Arabidopsis* seedlings, increased ABA levels have been observed in response to sugar treatment. In addition, an ABA treatment resulted in increased sugar sensitivity. These data therefore suggest that regulation of ABA synthesis plays an essential role in the plant sugar response (Arenas-Huertero et al., 2000).

Several identified ABA-deficient mutants were isolated during screens for sugar response mutants (Zhou et al., 1998; Laby et al., 2000; Arenas-Huertero et al., 2000). For example the *Arabidopsis* glucose insensitive1 (*gin1*) (Zhou et al., 1998), and sucrose insensitive4 (*sis4*, Laby et al., 2000) are allelic to *aba2* (Cheng et al., 2002). The expression of *GIN1/ABA2* is up regulated by glucose only in wild type and not in *gin1/aba2*. However, *GIN1/ABA2* expression in wild type plants is not ABA responsive. Since *GIN1/ABA2* is not induced by ABA and ABA deficiency prevented its glucose-activated expression, it is suggested that both glucose and ABA are synergistically required for *GIN1/ABA2* expression.

1.5.1.4 ABA Biosynthesis and Diurnal Variations

In leaves of *Nicotiana plumbaginifolia*, the *ZEP* mRNA level increases dramatically during the light period. A maximum level is reached within three to five hours of the beginning of the light period, and a minimum level is reached during the dark phase. In addition, a small increase of *NpZEP* mRNA was observed thirty minutes prior to the next light period (Audran et al., 1998). However, in spite of the diurnal variations of *NpZEP*, no oscillations were detected at the protein level. It has also been showed that the expression of both *LeZEP1* and *LeNCED1* in tomato leaves is affected by diurnal fluctuations; each however displayed distinctively different patterns (Thompson et al., 2000a). The expression level of *LeZEP1* and *LeNCED1* in tomato leaves was analyzed at 6h intervals throughout 3 days consisting of 12h/12h light/dark period followed by a 2 day period of complete darkness. The *LeZEP1* transcript level peaked during the first five hours of the light period supporting the observation of Audran et al.,

1998. During the 48h complete dark period, the *LeZEP1* transcript level showed additional two oscillations suggesting that circadian oscillators may play a role in regulating the expression of this gene (Thompson et al., 2000a). The *LeNCED1* transcript level peaked at the end of the light period, however, its transcript level stayed low during a complete 48h dark period. It is therefore suggested that the accumulation of *LeNCED1* transcript may be regulated more by light, either by a photoreceptor signal transduction pathway or by indirect effect of light rather than by the circadian oscillator. NCED is believed to be a key regulatory enzyme during ABA biosynthesis. It is therefore interesting to know whether the *NCED* mRNA diurnal oscillation has an effect on its protein level and finally towards the rate of ABA biosynthesis.

1.5.2 Regulation of ABA Biosynthesis

1.5.2.1 At the Level of Carotenoid Biosynthesis

The regulation of ABA biosynthesis at the level of carotenoid biosynthesis has not been well defined thus far. In developing maize seeds, the level of phytoene desaturase (PDS) transcript is not correlated with the level of endogenous ABA (Hable et al., 1998) and over expression of the *phytoene synthase1 (PSY1)* gene in tomato does not result in increased ABA levels (Fray et al., 1995). Recent studies however suggested that there may be a connection between carotenoid biosynthesis and the later stages of ABA biosynthesis. In *Arabidopsis*, endogenous ABA levels are affected by the level of *DXS* expression. Transgenic *Arabidopsis* plants with an increased level of *DXS* contains 295-397% more ABA than that of the wild type. On the contrary,

transgenic plants with reduced levels of DXS contain 44-53% less ABA than the wild type. These results suggest that the early stage of ABA biosynthesis in particular the formation of DXP might play a role towards the regulation of ABA biosynthesis (Estevez et al., 2001).

1.5.2.2 Zeaxanthin epoxidase Gene Expression

In the ABA biosynthesis pathway, *zeaxanthin epoxidase (ZEP)* was the first gene cloned (Marin et al., 1996). *ZEP* genes are expressed ubiquitously in every plants organ. In some plants, however, a higher expression level was observed in leaves (Audran et al 1998; Xiong et al., 2002a). It has been demonstrated that the expression of *ZEP* in tobacco and tomato leaves is not associated with increased endogenous ABA levels during water stress (Audran et al., 1998; Thompson et al., 2000a), This is based on the observation that the amount of 9-cis-epoxycarotenoid in leaves is several times higher than the amount of ABA produced during stress (Thompson et al., 2000a), and therefore it is postulated that *ZEP* does not limit ABA biosynthesis in leaves. In contrast to the expression pattern observed in the leaves, the amount of 9-cis-epoxycarotenoid is lower in roots (Parry and Horgan, 1992). Thus it is speculated that *ZEP* may limit ABA biosynthesis in roots of tobacco and tomato plants. This hypothesis is supported by the fact that the transcript level of *ZEP* in roots of both tobacco and tomato plants were up regulated several fold in response to drought stress (Audran et al., 1998; Thompson et al., 2000a). In Arabidopsis, a basal *ZEP* transcript level is maintained under non-stressed condition. However, the transcript level of *AtZEP* was noticeably increased in response to drought, salt

and polyethylene glycol in both shoot and root (Xiong et al., 2002a). These results indicate that under stress conditions, the regulation of ABA biosynthesis varies in different plant organs and also between plant species.

1.5.2.3 9-cis-epoxycarotenoid dioxygenase Gene Expression

The second major breakthrough in understanding the ABA biosynthesis pathway was the cloning of the maize *9-cis-epoxycarotenoid dioxygenase* (NCED) gene (Tan et al., 1997). Since the *NCED* gene product catalyzes the rate-limiting step in the ABA biosynthesis pathway, the expression of these genes has received substantial attention. Water-deficit stress stimulates *NCED* expression in maize (Tan et al., 1997), tomato (Burbidge et al., 1999), bean (Qin and Zeevaart, 1999), *Arabidopsis* (Iuchi et al., 2001), cowpea (Iuchi et al., 2000), and avocado (Chernys and Zeevaart, 2000). In some cases, substantial increases in transcript level were detected within 15 to 30 min after leaf detachment dehydration treatment (Qin and Zeevaart, 1999; Thompson et al., 2000a).

1.5.2.4 Feedback Regulation of ABA Biosynthetic Genes by ABA

Many biosynthetic pathways are regulated by their end products. ABA can negatively regulate its accumulation via activation of catabolic enzymes that degrade it (Cutler and Krochko, 1999). One of the primary catabolites of ABA is phaseic acid (PA). The conversion of ABA into PA begins with the hydroxylation at the 8' position by ABA 8'-hydroxylase. The 8' hydroxyl intermediate is unstable and therefore can spontaneously rearrange to form PA (Schwartz et al., 2003).

The 8'-hydroxylase is a cytochrome P450 enzyme (Krochko et al., 1998). Evidence has indicated that the activity of ABA 8'-hydroxylase is stimulated by exogenous ABA (Uknes and Ho, 1984; Windsor and Zeevaart, 1997). This observation was further supported by Qin and Zeevaart, 2002. Their work has shown that transgenic tobacco plants that over expressed *NCED* also contained excessive PA levels. Therefore, under non-stressed conditions, ABA might limit its own accumulation by activating its catabolytic pathway. In some tissues, ABA can also be inactivated via the formation of ABA-Glucose esters. Recently, an ABA glucosyl-transferase gene from adzuki bean (*Vigna angularis*) that is up regulated by ABA has been identified (Xu et al., 2002).

One important question with respect to the self regulation of ABA biosynthetic genes is whether ABA can activate or deactivate its own biosynthesis. In tomato (Thompson et al., 2000a) and cowpea (Iuchi et al., 2000), exogenous ABA application failed to induce *NCED* expression. These data suggest that, although ABA can stimulate its own degradation, it is unable to activate its own production. However, in contrast to these data, the expression of *NCED* in *Arabidopsis* (Landsberg ecotype) was found to be induced by ABA treatment (Xiong et al., 2002a; Cheng et al., 2002). Together, these data indicate that the regulation of *NCED* genes in response to exogenous ABA application varies between plant species.

In *Arabidopsis*, the expression of *ZEP*, *AAO3* and *MCSU* is also up regulated by exogenous ABA (Xiong et al., 2001a, 2002a). Moreover, under stress conditions, these genes appear to be regulated by the level of

endogenous ABA, since under osmotic stress conditions, the transcript levels of *ZEP*, *AAO3* and *MCSU* in the ABA-deficient mutants *los5* (low expression of osmotically responsive genes), *aba1*, *aba2*, or *aba3* were considerably lower than that in wild type plants, whereas their basal transcript levels were unaffected under non-stressed condition (Xiong et al., 2002a). Therefore, it is suggested that the expression of *ZEP*, *AAO3* and *MCSU* in response to osmotic stress may also be regulated by the level of endogenous ABA. In addition, in the ABA deficient mutant *los5* and *los6*, the transcript level of *AtNCED3* was drastically reduced under water deficit and salt stress conditions in comparison to the wild type (Xiong et al., 2002a). These data therefore suggested that under stress conditions, the endogenous ABA level may have a positive feedback role towards its own biosynthesis.

1.5.2.5 Differential Regulation of ABA Biosynthetic Genes

Genes that encode enzymes involved in the ABA biosynthesis pathway exists either as a single copy or gene family. It remains unclear however whether all the gene members play a role in ABA biosynthesis. Data so far indicate that the ABA biosynthetic genes that belong to gene families appear to be regulated differentially by different stresses. Furthermore, they also appear to be expressed in a tissue- and developmental stage-specific manner (Xiong and Zhu, 2003).

In avocado (*Persea Americana*), three *NCED*-related cDNAs (*PaNCED1*, *PaNCED2* and *PaNCED3*) have been identified, and two of them (*PaNCED1* and *PaNCED3*) have been demonstrated to convert 9-cis-violaxanthin and 9'-cis-neoxanthin to xanthoxin. *PaNCED1* is highly expressed in leaves, whereas

PaNCED3 expression is not detected in leaves (Chernys and Zeevaart, 2000). In maize, *ZmNCED1* (*Vp14*) is highly expressed in both embryos and roots, and less in leaves. In the embryos of *vp14* mutants, the ABA levels are significantly lower than that of wild type, but no significant difference in ABA level was observed between non-stressed leaves of wild type and *vp14*. However, *vp14* mutants showed faster water loss than the wild type, and stressed leaves of *vp14* accumulated less ABA than the wild type. Taken together, these data indicate that in non-stressed leaves of maize, *NCED* isoform(s) may exist which may be responsible for ABA synthesis (Tan et al., 1977).

In *Arabidopsis*, it appears that *ZEP*, *MCSU* and *SDR* are single copy genes, whereas *NCED* and *AAO* belong to gene families. There are approximately nine *NCED*-related genes in *Arabidopsis*, of which only four have been demonstrated to exhibit *NCED* activity (Iuchi et al., 2001). At least four *aldehyde oxidase* (*AO*) isoforms (*AtAAO1* to *AtAAO4*) have been identified in *Arabidopsis* (Seo and Koshiba, 2002). The *AtAAO4* gene is expressed primarily in siliques (Seo et al., 2000a), whereas *AtAAO3* is expressed in leaves and not in roots (Seo et al., 2000b).

1.5.3 ABA Signaling and Guard Cell Responses

During water deficit stress, one important role of ABA is to regulate transpiration rate by promoting closure and inhibiting the opening of stomata (Schroeder, 2001). Despite much effort dedicated to identify receptor candidates, to date none have been detected. Upon arrival at the guard cells, ABA can be perceived either intra- or extracellularly, and studies have indicated that these

two putative binding sites may reflect distinguishable effects of ABA on guard cells. Extracellular perception of ABA may prevent stomata opening (Jeannette et al., 1999), while intracellular ABA perception may induce stomata closure (Allan et al., 1994).

One of the earliest electrophysiological changes in guard cells exposed to ABA is a transient membrane depolarization that is represented by an increase of Ca^{2+} . ABA mediated increases in Ca^{2+} occurs via ROS production that promotes opening of plasma membrane $\text{Ca}^{2+}_{\text{in}}$ channels. Ca^{2+} release from internal storage is performed via Ca^{2+} channels regulated by IP3 (produced by phospholipase C), cyclic ADP-ribose (cADPR), and Ca^{2+} itself (Schroeder et al., 2001). In addition, many secondary messengers including kinases and phosphatases are involved in stomatal regulation and it is believed that they act upstream of the Ca^{2+} oscillations (Murata et al., 2001). The relationship between these kinases and phosphatases however remains unclear.

The increased Ca^{2+} inhibits both plasma membrane H^+ pumps and K^+_{in} channels and activates Cl^-_{out} (anion) channels, resulting in further depolarization of the membrane (reviewed in Schroeder et al., 2001). Membrane depolarization then activates K^+_{out} movement and further inhibits K^+_{in} channels. ABA is also known to induce phospholipase D-mediated production of phosphatidic acid (PA), which inactivates K^+_{in} channels (Hallouin et al., 2002; Ritchie et al., 2002). An increase in cytosolic pH is another known effect of ABA. The increased cytosolic pH activates K^+_{out} channels and inhibits H^+ pump activity via substrate depletion.

Potassium and anions that are released across the plasma membrane are first released into the cytosol from the vacuoles of guard cells. The movement of K^+ and anions out of the guard cell results in decreased guard cell turgor and the closing of the stomata. These electrophysiological and volume changes of guard cells require reorganization of the actin cytoskeleton and at least a two fold change in membrane surface area (reviewed in Schroeder et al., 2001).

1.5.4 ABA Signaling and Gene Expression

Up regulation of ABA biosynthesis in response to osmotic stress is well understood; however, the signaling cascade(s) that are responsible for up regulating stress responsive genes remains unclear. It has been suggested that the regulation of stress responsive genes occurs via ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2000). Stress responsive genes contain DRE (**D**ehydration **R**esponsive **E**lement), ABRE (**A**BA-responsive **e**lement), MYCRS (MYC recognition sequence), and MYBRS (MYB recognition sequence) *cis*-elements in their promoters (Zhu, 2002). Analyses of the promoters of stress responsive genes and the isolation of transcription factors showed that distinct regulatory mechanisms are responsible for the ABA-dependent and ABA-independent pathways. The ABRE is believed to be responsible for ABA-mediated gene expression (Guiltinan, et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994; Vasil et al., 1995), whereas the DRE is responsible for mediating ABA-independent gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). However, despite differences in transcriptional activation, genetic analyses have indicated that the

ABA-dependent and ABA-independent pathways have extensive interactions in controlling gene expression during abiotic stresses (Ishitani et al., 1997; Xiong et al., 1999; Shinozaki et al., 2003), for example many drought- and cold responsive genes contain both DRE and ABRE elements in their promoter. These cis acting elements are believed to function independently and/or cooperatively as coupling elements (Xiong et al., 2001b).

1.5.4.1 ABA-Dependent Pathway of Gene Expression

The expression of stress responsive genes including *RD29A* (Responsive to Desiccation29A), *RD22*, and 1-pyrroline-5-carboxylate synthetase (*P5CS*) was extremely reduced or completely blocked in the *los5* mutants (Xiong et al., 2001a). *Los5* is an ABA-deficient mutant, which is allelic to *aba3* that encodes a molybdenum cofactor (MoCo) sulfurase. In *los6* mutant plants, the expression of stress responsive genes was lower than that observed in wild type plants (Xiong et al., 2002a). *Los6* is an ABA deficient mutant that is allelic to *aba1*, which encodes ZEP. In the leaves of the tomato ABA deficient mutant *flacca*, many water-deficit-stress responsive polypeptides were either absent or accumulated to a lesser extent following water deficit stress (Bray, 1988). Taken together these data validate the existence of an ABA-dependent pathway for gene expression.

The conserved ABRE (ACGTGG/TC) is present in their promoter region of many ABA-inducible genes (Bonetta and McCourt, 1998; Leung and Giraudat, 1998). The bZIP transcription factors, ABRE-binding proteins (AREB)/ABRE-binding factor (ABF) can bind to the ABRE and activate ABA-dependent gene

expression (Uno et al., 2000; Choi et al., 2000). The AREB/ABF proteins have reduced activity in ABA-deficient *aba2* mutants and in ABA-insensitive *abi1* mutants and enhanced activity in the ABA-hypersensitive *era1* mutants. It is therefore believed that an ABA-mediated signal(s) is required for the activation of AREB/ABF proteins (Uno et al., 2000).

Recent data revealed that both DRE and ABRE elements are interdependent in regulating the expression of *RD29A* (Narusaka et al., 2003). The promoter region of *RD29A* contains two DREs and one ABRE, which are involved in ABA-independent and ABA-dependent gene expression, respectively. Experiments with leaf protoplasts showed that DRE-binding proteins together with ABRE-binding proteins cumulatively activate gene expression (Narusaka et al., 2003), suggesting that the DRE element may function as a coupling element for the ABRE for ABA-dependent gene expression.

The expression of the *RD22* gene is mediated by ABA (Shinozaki and Yamaguchi-Shinozaki, 2000; Abe et al., 1997). A MYC transcription factor, *RD22BP1* (*AtMYC2*), and a MYB transcription factor, *AtMYB2*, have been shown to bind to *cis*-elements in the *RD22* promoter and cooperatively activate *RD22* (Abe et al., 1997). These MYC and MYB proteins are synthesized after the accumulation of endogenous ABA. Over expression of both *AtMYC2* and *AtMYB2* cause not only the ABA hypersensitive phenotype but also improved osmotic-stress tolerance of transgenic plants (Abe et al., 2003).

1.5.4.2 ABA-Independent Pathway of Gene Expression

Several drought-inducible genes are not ABA responsive, thus suggesting the existence of an ABA independent pathway (Shinozaki et al., 2003; Nakashima et al., 1997). The promoter of some stress responsive genes contains cis acting elements, the DRE (also called C repeat), that is involved in ABA-independent gene expression (Yamaguchi and Shinozaki, 1994). Transcription factors that belong to the APETALA2 (AP2) family that bind to DRE have been isolated and named as C-Repeat-Binding Factor (CBF)/DREB1 and DREB2, and they are responsible for trans-activating the DRE element of stress responsive genes (Stockinger et al., 1997; Liu et al., 1998). In Arabidopsis, overexpression of *CBF/DREB1* results in an increased tolerance of plants towards freezing, drought and salt stresses (Liu et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999), whereas overexpression of *DREB2* does not (Liu et al., 1988).

1.6 ABA and Its Role in Maintaining Shoot and Root growth

During water deficit, shoot growth is more sensitive than root growth. Maintaining root growth during water deficit is viewed as an adaptive characteristic that promotes survival of plants under water-limited conditions (Sharp and Davies, 1989; Spollen et al., 1999). ABA levels increase during salt and water deficit stress (Zhu, 2002; Chen and Plant, 1999), and there has been much speculation with regards to its role in inhibiting shoot growth. This is mainly derived from the growth inhibitory effect of ABA when applied to well-watered plants and the fact that the level of ABA increases in plants experiencing water

deficit or other adverse conditions, which is often associated with growth reduction (Sharp and LeNoble, 2002). Ironically, ABA deficient mutants are often shorter and have smaller leaves than their corresponding wild type plants (Imbar and Tal., 1970; Bradford, 1983; Quarrie, 1987). Furthermore, ABA deficient mutants have a typical wilted phenotype, even under well watered conditions, and their reduced shoot growth has been attributed to a disrupted plant water balance (Neil et al., 1986; Nagel et al., 1994).

Earlier reports have indicated that ABA application results in an inhibition of ethylene production (Wright, 1980; Bradford and Hsiao, 1982). It was also reported that ethylene production is greater in ABA-deficient mutants of tomato (Tal et al., 1979) and *Arabidopsis* (Rakitina et al., 1994). The increased ethylene production in tomato ABA deficient mutants results in a phenotype, which is characteristic of excess ethylene such as leaf epinasty and adventitious rooting (Tal, 1966; Nagel et al., 1994). Recently, in a study using wild type tomato together with ABA-deficient *flacca* and *notabilis* mutants, Sharp et al., (2000) demonstrated that normal levels of ABA are required to maintain shoot growth. Under well watered conditions, *flc* showed severely impaired shoot growth together with increased rates of ethylene evolution, leaf epinasty and adventitious rooting. Inhibition of ethylene action in *flc* restored the leaf and stem growth, indicating that ethylene may be responsible for the impairment of shoot growth in this ABA deficient mutant. This hypothesis was recently tested in *Arabidopsis* using ABA-deficient (*aba2-1*) and ethylene-insensitive (*etr1-1*) mutants (LeNoble et al., 2004), and their results demonstrated that under well

watered conditions, wild type endogenous ABA levels are required to maintain shoot development by suppressing ethylene synthesis and/or signaling (Sharp et al., 2000).

Similarly, increased ABA levels have been suggested to be an important factor in maintaining primary root elongation of maize growing at low water potential (Saab et al., 1990; Sharp et al., 1994). Spollen et al., (2000) demonstrated that at low water potential, maize primary root elongation was further inhibited when ABA biosynthesis was blocked by fluridone. Root elongation was recovered by applying inhibitors of ethylene biosynthesis or ethylene signaling. Furthermore, the ethylene level in the fluridone-treated maize roots was high and ABA application reduced ethylene production back to the level observed in the non-fluridone treated roots (Spollen et al., 2000). Together, these results demonstrate that one role of ABA in maintaining root growth at low water potential is to restrict ethylene production or action, and therefore reduce or minimize its inhibitory effect on root elongation.

1.7 Crosstalk between ABA and Ethylene

Recent studies have indicated that an important role of ABA is to limit either ethylene production and/or action (Sharp et al., 2000; Spollen et al., 2000; Le Noble et al., 2004). Currently, interactions between these hormones appear to occur at the level of synthesis as described in the previous section (Sharp et al., 2000; Spollen et al., 2000), and signaling (Beaudoin et al., 2000; Ghassemian et al., 2000; Chiwocha et al., 2005).

The Arabidopsis ethylene insensitive mutant, *ein2*, has been independently isolated from various genetic screens (Fujita and Suyono, 1996, Cary et al., 1995, Oh et al., 1997, Beaudoin et al., 2000, Ghassemian et al., 2000). *Era3* (Enhanced response to ABA3) was initially identified by screening for seeds that could not germinate in the presence of low ABA levels that generally do not inhibit germination (Cutler et al., 1996). The endogenous ABA level of *era3* is two times higher than that of the wild-type. Furthermore, the level of ZEP is also elevated in this mutant. Thus *era3* over accumulates ABA due to increased ABA biosynthesis indicating that ERA 3 may function as a negative regulator of ABA synthesis (Ghassemian et al., 2000). Although the seeds of *era3* have increased sensitivity to ABA during germination, their root growth is less sensitive to ABA than those of the wild type. In the presence of 10 μ M ABA, the root growth of *era3* is better than that of the wild type. Furthermore, root growth of *era3* is not sensitive to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), as indicated by the absence of the triple response phenotype. A similar phenotype is also observed in *ein2* and *era3* was found to be a new allele of *ein2* (Ghassemian et al., 2000).

Concurrent with the identification of *era3-1*, in a screen for mutants with either an enhanced or suppressed *abi1* mutant phenotypes, a new *ein2* allele was also discovered (*ein2-45*) (Beaudoin et al., 2000). *Ein2-45* was initially identified due to its partial reversion of the *abi1* phenotype with respect to ABA inhibition of seed germination. Seed germination of *ein2-45* is sensitive to 3 μ M of ABA, while the same concentration does not impede germination of *abi1*.

Further investigation of the *ein2-45* seedlings growing in the presence of ACC, showed that these mutant seedlings failed to develop the triple response phenotype (Beaudoin et al., 2000).

Seeds of both *ein2-45* and *era3-1* were supersensitive to ABA during germination relative to the wild type. In addition, their seeds also showed dramatically enhanced seed dormancy. Furthermore, both *ein2-45* and *era3-1* are insensitive to ethylene. Therefore, the enhanced dormancy observed in *ein2-45* and *era3-1* may be caused by the defect within the ethylene signaling pathway. Ethylene is believed to promote seed germination mainly by decreasing ABA sensitivity; therefore a mutation that confers lowered ethylene insensitivity results in seeds with an increased sensitivity to ABA (Ghassemian et al., 2000; Beaudoin et al., 2000).

Applications of low concentrations of ABA or ethylene promote root growth, while higher concentrations of either inhibit it (Davis and Zhang, 1991; Abeles, 1992). Both ethylene insensitive mutants and wild type plants showed a 20% increase in root growth when exposed to low ABA concentrations. In the presence of higher ABA concentrations, the roots of ethylene insensitive mutants grow more than the wild type, suggesting that ABA-induced root inhibition requires functional ethylene signaling. Ethylene levels in both ABA-treated and non-treated wild type plants were similar, indicating that ABA inhibition of root growth although requiring a functional ethylene signalling pathway is not mediated by an increase in ethylene production. It is therefore speculated that in

Arabidopsis seedlings, ABA and ethylene may synergistically interact in inhibiting root growth (Ghassemian et al., 2000; Beaudoin et al., 2000).

1.8 Impact of Salinity on Global Gene Expression

Recently, cDNA microarray technology has become a useful tool for the analysis of genome scale gene expression patterns (Schena et al., 1995). This technique allows for the determination of many or nearly all transcripts in a genome in response to specific experimental conditions. To verify the expression profiles obtained from microarray data, this technique is often accompanied by RNA gel-blot analysis and quantitative reverse transcriptase-PCR. Together, they provide transcriptome data that has a high degree of accuracy. The microarray technique has been used to analyze a variety of abiotic stress responses, including salinity stress (Seki et al., 2001, 2002a, b; Kreps et al., 2002; Ozturk et al., 2002; Wang et al., 2003; Kawasaki et al., 2001; Rabbani et al., 2003; Hong et al., 2003; Taji et al., 2004). Together these studies demonstrated that, in plants, many genes were up-regulated or down-regulated following salinity stress. These salinity stress-responsive genes can be classified either as genes that are stress specific or those that are induced by multiple stresses, suggesting the existence of cross talk among signalling cascades in abiotic-stress responses in plants (Seki et al, 2001, 2002a, b).

Currently, little is known about how various abiotic stresses interact and moreover how that interaction will affect the plants ability to respond to these stresses. In an effort to determine the expression profiles during salinity, drought and low temperature conditions, Seki et al., (2002a) found that in Arabidopsis,

there is greater crosstalk between salinity stress and drought than between salinity stress and cold. The role of ABA signaling in mediating drought, salt and cold responsive changes in gene expression and cross talk between them was also investigated by Seki et al., (2002b). Their results showed that a greater degree of cross talk exists between ABA-, drought- and salinity- stress signaling pathways than between ABA- and cold-stress signaling pathways (Seki et al., 2002b).

Gene expression profiles during the initial phase of salt stress were investigated by Kawasaki et al., (2001) in salt-tolerant (variety Pokkali) and salt-sensitive rice (variety IR29). Their results indicated that the Pokkali variety showed immediate changes in gene expression involving either gene up regulation or down regulation as early as 15 min following the applied salt treatment. Additionally, many transcripts that were down regulated at the earlier time points were able to return to their original expression level or even exceeded it. The immediate response showed by the Pokkali variety was delayed in the IR29 variety, resulting in a down regulation of transcripts and death within 24h. Based on these results, it was hypothesized that the delayed response observed in the IR29 variety may be responsible for its salt sensitive phenotype. In a more recent study by Taji et al., (2004) in which they compared *Arabidopsis* and a related halophyte, salt cress, a different hypothesis was derived. They have suggested that the stress tolerance of salt cress is due to the constitutive expression of many genes that are known to function in stress tolerance and which in *Arabidopsis* is stress inducible. Taken together, the results from

Kawasaki et al (2001) and Taji et al (2004) point out that different strategy may exist in different plant species, which allow them to tolerate salinity stress. It would be interesting to see in the future whether one strategy of dealing with salinity stress can outperform others or whether a combination of different strategies is optimal to tolerate salinity stress.

1.9 Research Objectives

The primary objective of the research conducted for this thesis was to isolate and characterize novel salt-responsive genes in tomato (*Lycopersicon esculentum* Mill) roots. A further objective was to explore the role of abscisic acid (ABA) with respect to the regulation of gene expression in order to determine if endogenous ABA mediates changes in gene expression. These objectives were accomplished by:

1. Analyzing salinity-stress induced changes of gene expression at the mRNA level using differential display reverse transcriptase polymerase chain reaction (DD-PCR). To gain insight into the role of ABA in mediating salt-induced changes in root RNA populations, the RNA populations of ABA and combined ABA/salt treatments were compared to that of salt-treated roots.
2. Cloning and sequencing partial cDNA products corresponding to mRNAs whose levels were altered by the applied salt stress.
3. Obtaining full-length cDNAs clones corresponding to the identified salt-responsive partial cDNAs by screening cDNA libraries or sequencing

Expressed Sequence Tag (EST) clone(s) that correspond to the identified salt responsive partial cDNAs.

4. Determining the role of ABA in regulating the salt responsive expression of gene(s) corresponding to the identified salt responsive partial cDNAs using:

- exogenous ABA application
- an ABA deficient mutant, *flacca*, with reduced ABA levels
- Fluridone, an inhibitor of carotenoid biosynthesis, to reduce ABA levels chemically.

2 SALINITY STRESS-INDUCED CHANGES IN mRNA ACCUMULATION IN TOMATO (*Lycopersicon esculentum* Mill) ROOTS

2.1 Introduction

Saline soil is a major environmental stress that can impair plant growth and development and therefore limit productivity. Traditionally, it is associated with arid or semi arid lands in which irrigation practices are very common. Due to their role in absorbing water and nutrients, roots are the first part of the plant to encounter soil salinity. When exposed to saline soils, roots have to cope with two types of stresses: an osmotic stress that results from the relatively high level of dissolved ions in the soil and an ionic stress that results from an excessive accumulation of ions, mainly sodium (Kafkafi and Bernstein., 1996).

Changes in gene expression underpin many of the responses that occur in plants in response to salinity stress. The products of the stress-inducible genes can be classified into two groups: those that may be directly involved in protecting the plants against the salt stress, and those that may be involved in regulating gene expression and signal transduction during the stress response (Shinozaki and Yamaguchi-Shinozaki, 1997). The first group includes genes, which encode products believed to play a role in a number of processes including the biosynthesis of various osmoprotectants (Nanjo et al., 1999; Hu et al., 1992; McCue and Hanson, 1992; Hong et al., 2000), cellular protection (Cohen et al., 1991; Naot et al., 1995; Moons et al., 1997b), ion homeostasis (Niu et al., 1995;

Tsiantis et al., 1996; Dietz and Abringer., 1996; Sze et al., 2004), general defense (Chen et al., 1994; Umeda et al., 1994; Espartero et al., 1994; Ben-Hayyim et al., 2001; Haussuhl et al., 2001), and those with unknown function (Claes et al., 1990; Gulick et al., 1994; Moons et al., 1997b). The second group mostly consists of transcription factors (Urao et al., 1993; Uno et al., 2000; Kang et al., 2002; Sakamoto et al., 2004; Tran et al., 2004) and protein kinases (Urao et al., 1993; Mizogucchi et al., 1996; Hong et al., 1997; Ulm et al., 2001; Chehab et al., 2004; Umezawa et al., 2004).

In salt-affected roots, changes in gene expression have been demonstrated from a variety of plants, including barley (Ramagopal, 1987; Hurkman and Tanaka, 1987; Hurkman et al., 1994), tomato (Chen and Tabaeizadeh, 1991; Chen and Plant, 1999; Wei et al., 2000), rice (Moons et al., 1995; Hashimoto et al., 2004), *Arabidopsis* (Shi and Zhu, 2002; Umezawa et al., 2004), and halophyte, *Aneurolepidium chinense* (Inada et al., 2005). In roots, it is believed that the expression of these genes is regulated by endogenous signals produced by the plant in response to salinity stress. ABA is an important endogenous signal since changes in gene expression are often accompanied by an increase in both the ABA concentration (Zeevaart and Crelman, 1988) and the amount of ABA that is transported from the root to the shoot (Davies and Zhang, 1991). Moreover, many salt responsive genes are expressed in non-stressed roots in response to exogenous ABA application, indicating that ABA may be one of the signals that these genes respond to (Galvez et al., 1993; Hwang and Goodman, 1995; Strizhov et al., 1997; Moons et al., 1997b). Further

evidence for a role for ABA in mediating changes in gene expression in roots during salinity stress was provided by a study that used salt-tolerant and salt-sensitive varieties of rice in which ABA production in the salt-tolerant varieties was higher than in the sensitive varieties (Moons et al., 1995). However, in contrast to the evidence presented above for a prominent role for ABA in regulating changes of gene expression during salinity stress, studies in our lab have demonstrated that an elevated level of ABA may not play a central role in regulating the accumulation of salt-responsive polypeptides (Chen and Plant, 1999; Jin et al., 2000). Together, evidence presented here and elsewhere point toward both ABA-dependent and ABA-independent changes in gene expression in salt-affected roots.

A number of approaches have been undertaken to isolate genes whose expression is affected by salinity stress. These experimental approaches include the differential screening of cDNA libraries, the analysis of protein profiles by two-dimensional polyacrylamide gel electrophoresis, and the differential display reverse transcriptase-PCR technique. Differential display or DD-PCR is a simple technique that allows the identification of differentially expressed genes by detecting individual mRNA species whose levels are altered under different conditions (Liang and Pardee, 1992). The principle of DD-PCR is based on the use of 3' anchored oligo (dT) and 5' arbitrary primers. Messenger RNAs are first converted to first-strand cDNA using 3' anchored oligo-dT primers that differ from each other at the last 3' non-T base. The resulting cDNAs are further amplified and labeled with isotopes by PCR in the presence of 5' arbitrary and oligodT

primers. As a result, a subset of the cDNA transcriptome is amplified, and these cDNA fragments are separated on a denaturing polyacrylamide gel and visualized by autoradiography. Side by side comparisons of cDNA between and/or among relevant RNA samples reveals differences in gene expression. Differentially expressed cDNA bands can then be retrieved, cloned, and sequenced for further characterization. One of the major advantages of DD-PCR is that there is no prior knowledge required for the mRNA sequence (Liang and Pardee, 1992; Liang, 2002).

The DD-PCR method has been successfully used to identify a variety of cDNAs including senescence-associated cDNAs (Kleber-Janke and Krupinska, 1997), developmental-associated cDNAs (Wilkinson et al., 1995; Johnson et al., 1995; Zegzouti et al., 1997), oxidative stress-associated cDNAs (Vranova et al., 2002; Yamaguchi et al., 2003; Hagihara et al., 2004), dehydration-regulated cDNAs (Torres et al., 2003), ethylene receptors of rice (Watanabe et al., 2004) and salt-responsive cDNAs (Rippmann et al., 1997; Ueda et al., 2001; 2002; Wang et al., 2005).

The objectives of the work in this chapter are:

1. To identify novel salt responsive genes through analysis of the mRNA populations in salt-stressed vs non-stressed tomato roots using mRNA differential display.
2. To gain insight into the role of ABA as a regulator of gene expression during salinity stress, by comparing the mRNA populations of ABA- and ABA/salt-treated roots to that of salt-treated and non-treated roots.

2.2 Materials and Methods

2.2.1 Materials

Seeds of wild type tomato (*Lycopersicon esculentum* Mill cv Ailsa Craig (AC)) and the near-isogenic ABA-deficient mutant *flacca* (*flc*) were first surface sterilized with 70% ethanol for 30 sec under vacuum, followed by a 30 sec rinse in sterile water. The seeds were subsequently treated with 10% Javex bleach solution for 3 min under vacuum, followed by one rinse in sterile water for 1 min under vacuum and two five minutes rinses without vacuum. AC and *flc* seedlings were grown as shown in Figure 2-1. The AC and *flc* plants were grown in an aerated hydroponic system with the nutrient solution provided by a 2/3-strength Murashige and Skoog (MS) solution (Murashige and Skoog, 1962). The MS nutrient solution was changed at least once a week and 24h prior to the start of an experimental treatment. Plants were maintained in a growth chamber (Convion Basic Model 125L Incubator) in the light for 16h at 25°C with 70% relative humidity; in the dark for 8h, at 21°C, with 70% relative humidity.

2.2.2 Experimental Treatments

Six-week-old plants were used for all experiments. A salt treatment was imposed by the addition of NaCl to the 2/3 MS nutrient solution to reach a final concentration of 170 mM. Plants were exposed to 170 mM NaCl for varying periods of time ranging from 0 to 24h. Exogenous ABA and combined NaCl/ABA treatments were imposed by exposing plants to 100 µM ABA (mixed isomers, +/- *cis/trans* ABA; Sigma) or 100 µM ABA together with 170 mM NaCl, respectively. Plants were exposed to these treatments for 24h. Control plants were transferred



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

Tomato seeds were germinated in moistened vermiculite placed in a plastic grid (1.5 X 1.5 cm²), lined with a plastic mesh. The plastic grid was placed in a plastic tray that contains the MS nutrient solution. Upon germination the roots grew through the mesh.

to and maintained in 2/3 MS nutrient solution for the duration of the experimental period. Following each treatment the roots were harvested and frozen in liquid N₂ and stored at -80°C until needed. All treatments for DD-PCR were independently performed three times.

2.2.3 Relative Water Content

Relative water content (RWC) measurement was obtained for excised shoot and root samples. Root or shoot tissues were weighed to obtain the fresh weight (W_f) and allowed to attain turgor by immersing in water for 24h. Fully turgid root or shoot tissues were subsequently weighed (W_t) prior to drying them in an oven at 65°C for 24h in order to obtain their dry weights (W_d). RWC expresses the amount of water that is present in the respective tissue sample as a percentage of the water in the fully hydrated tissue as follows:

$$\text{RWC} = 100 \{(W_f - W_d) / (W_t - W_d)\}$$

2.2.4 RNA Extraction

Total RNA for DD-PCR was extracted using the Plant RNeasy System (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's instructions. Total RNA for Northern blot hybridization analyses was extracted using the Lithium Chloride method as described by Prescott and Martin (1987). Frozen tomato roots were ground to a fine powder with liquid nitrogen. Ten milliliter of extraction buffer (50 mM Tris-HCl pH 9.0, 150 mM LiCl, 5 mM EDTA, and 5% SDS) was added per five gram (FW) of sample, and ground until an ice-cream-like consistency was obtained. An equal volume of phenol/chloroform (50:50)

was added, mixed and subsequently transferred to a RNAase-free 50 ml Nalgene™ plastic tube. The sample was then mixed thoroughly by vortexing, incubated on ice for 5 min and centrifuged at 10,000 rpm using a SS34 SORVALL rotor for 15 min at 4°C. Following centrifugation, the top aqueous layer was removed and transferred to a new 50 ml Nalgene™ plastic tube. An equal volume of phenol/chloroform was added to the sample, mixed thoroughly, then centrifuged as before. Following the second centrifugation, the top layer was removed and transferred to a new RNAse free 50 ml Nalgene™ plastic tube. An equal volume of chloroform/isoamylalcohol (24:1) was added, mixed thoroughly, followed by five min incubation on ice and then centrifuged as before. The top layer was removed and transferred to a 15 ml Corex™ tube. An appropriate amount of 8 M LiCl was added to achieve a final concentration of 2 M. The mixture was incubated overnight at -20°C, or alternatively at -80°C for a minimum of 3h. The following day, the sample was centrifuged as before for 30 min at 4°C. Following centrifugation, the supernatant was decanted and the pellet was left to air dry. The pellet was resuspended in two milliliter DEPC-treated ddH₂O and one-third volume of 8 M LiCl was added followed by incubation at -20°C for approximately five hours. Following incubation, the sample was centrifuged as before for 30 min. The supernatant was decanted; the pellet was left to air dry and resuspended in two milliliter DEPC-ddH₂O. Once the pellet was completely dissolved, one-twentieth volume 3 M NaOAc (pH 5.2) and 2.5 volumes ice cold absolute ethanol were added and completely mixed. RNA was precipitated overnight at -20°C or alternatively at -80°C for a minimum of 3 h. The following

day the mixture was centrifuged at 10,000 rpm (SS34 SORVALL) for 30 min at 4°C. The supernatant was discarded, and 70% ethanol was added and the sample was centrifuged at the same speed for 15 min. The pellet was air-dried and dissolved in 100 µl DEPC-ddH₂O and transferred to a 1.5 ml microfuge tube. For measuring RNA concentration, a one to one hundred dilution of RNA was made and RNA concentration was quantified in a spectrophotometer (BIO-RAD SmartSpec™ 3000, Mississauga, Ontario) at 260 nm and 280 nm in water.

2.2.5 Differential Display Reverse Transcriptase PCR

The differential display project was initially started with Dr. Jun Zhi Wei, and it was carried out according to Liang and Pardee (1992) and Bauer et al., (1993). A DD-PCR primer set was obtained from the Biotechnology Laboratory (UBC, Vancouver, BC, Canada). Each reverse transcription (RT) reaction contained 0.2 µg RNA, 20 U RNase inhibitor (Perkin Elmer, Forster City, CA, USA), 20 U reverse transcriptase (Perkin Elmer, Forster City, CA, USA), 1.5 µM T₁₁MN, 5 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂ and 20 µM dNTPs and was incubated at 37°C for 60 min followed by incubation at 95°C for 5 min. For the following PCR reaction, two microliters of the RT reaction was used together with 18 µl of PCR mix consisting of 2 mM MgCl₂, 0.25 µM arbitrary primer, 1.5 µM anchor primer (T₁₁ MN) (Table 2-1), 20 µM dNTPs, 0.074 mBq [α -³³P] dATP (Amersham, B'aie d'Urfe, Quebec, Canada) and 0.5 U DNA Ultratherm Taq Polymerase supplied with its own buffer (Ultratherm, Bio/Can). Each PCR reaction was overlaid with mineral oil prior to 40 cycles of amplification in a MJ

Table 2-1 Primer combinations used for DD-PCR

	<i>Anchor primer</i>	<i>Random Primer</i>
1.	T ₍₁₁₎ CG	#5 (5'GGAACCAATC 3')
2.	T ₍₁₁₎ CG	#1 (5'TACAACGAGG 3')
3.	T ₍₁₁₎ CG	#2 (5'TGGATTGGTC 3')
4.	T ₍₁₁₎ CC	#1 (5'TACAACGAGG 3')
5.	T ₍₁₁₎ CC	#4 (5'TTTTGGCTCC 3')

Research Minicycler. Each PCR cycle consisted of 94°C for 30 sec, 40°C for 2 min and 72°C for 30 sec, with a final 5 min extension at 72°C following the final cycle. One fifth of the PCR products plus 2µl of formamide loading dye (0.02% (v/v) 0.5 M EDTA (pH 8.0), 1% (w/v) bromophenol blue, 1% (w/v) xylene cyanol in formamide) were incubated at 80°C for 5 min then loaded on a 1X TBE (40 mM Tris-borate, 1 mM EDTA) 6% acrylamide gel (bis acrylamide/acrylamide ratio 1:16) containing 8.3 M urea. The gel was run for 3.5h at 55 W constant power, then subsequently transferred to Whatman 3MM paper, dried under vacuum at 80°C for 2h and exposed to an X-ray film (DuPont NEF-395) for 24 - 48h. For cutting the bands of interest, the resulting autoradiography film and the corresponding dried gel were aligned and sterile razor blades were used to excise a slice of the gel. Gel slices were placed in 100 µl dH₂O overlaid with mineral oil and boiled for 15 min. After centrifugation, the supernatant was directly used for re-amplification using the same primer set and PCR conditions as described above except the dNTP concentration was 200 µM and no isotope was added. The re-amplified PCR products were separated on a 1X TBE, 1.2% agarose gel. Each experiment was repeated at least twice using two different RNA batches that originated from two independent treatments. Each batch of RNA was subjected to at least two RT reactions and each RT reaction was subjected to at least two PCR reactions.

2.2.6 Cloning, Sequencing and Analyses of Partial cDNA Products

Re-amplified cDNA products were cloned into the plasmid vector PCR 2.1 using the TA Cloning System from Invitrogen (San Diego, California) following

the manufacturer's instructions. Plasmid DNA was isolated using the Qiagen Plasmid Kit following manufacturer's instruction (Qiagen, Mississauga, Ontario, Canada). Positive clones were further confirmed by using plasmid DNA as a template in a PCR reaction with the same primers used to generate the original partial cDNA. Each PCR reaction contained 0.2 μ M arbitrary primer, 0.15 μ M anchor primer, 20 μ M dNTP mixture, 1.25 mM MgCl₂, 2U Taq DNA polymerase, supplied with its own buffer (GibcoBRL[®], Life Technologies, Burlington, Ontario, Canada) and 15 ng template DNA. Each PCR reaction was overlaid with mineral oil prior to amplification in a MJ Research MiniCycler (MiniCycler, MJ Research, Watertown, Massachusetts, USA). Each PCR cycle consisted of 95°C for 30 seconds, 43°C for 2 min and 72°C for 30 seconds. The reaction was subjected to 36 cycles with a final 5 minute extension following the last cycle. PCR products were separated on a 1.2% agarose gel in 1X TBE (Sambrook et al., 1989). The nucleotide sequence of the cloned partial cDNAs was determined using a Thermo sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc., Cleveland Ohio, USA) following the manufacturer's instruction. This kit uses four [α -³³P] dideoxy-nucleotide (ddNTP) terminators and a Thermo Sequenase DNA polymerase. Both strands were sequenced using the T7 (5' TAA TAC GAC TCA CTA TAG GG 3) and M13 reverse (5' CAG GAA ACA GCT ATG ACC 3') universal primers. Sequencing reaction mixtures were electrophoresed on a 6% 1X TBE acrylamide gel (bis acrylamide/acrylamide ratio 1:16) containing 50% Urea (w/v).

2.2.7 Nucleotide and Deduced Amino Acid Sequencing Analyses

Similarity searches were conducted by submitting the nucleotide sequence of the cDNA insert to the National Center for Biotechnology Information (NCBI) BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). using BLASTN (Altschul et al., 1997) to search against both the non redundant (nr) and expressed sequence tag (EST) databases and BLASTX to search against the nr database. In addition, BLASTP searches were also used with the deduced amino acid sequence of the cDNA insert against the Swiss Protein database.

2.2.8 Preparation of Radiolabelled Probes

Salt responsive partial cDNAs were used as probes for northern hybridization analyses. To amplify the respected salt responsive partial cDNA, PCR was performed (as described in section 2.2.6) using the same primer combination that was initially used to generate them (Table 2-1). The PCR product was purified by adding an equal volume of phenol/chloroform (50:50), vortexing and centrifuging at 12,000 rpm in a Sorvall® MC 12V centrifuge for 5 min. The top aqueous layer was transferred into a new tube and an equal volume of chloroform/isoamylalcohol (24:1) was added followed by centrifugation as before. The top aqueous layer was transferred to a new tube, and one-twentieth volume 3 M NaOAc (pH 5.2) and 2.5 volume absolute ethanol were added and mixed. The sample was incubated for approximately 5 h at -20°C. To recover the DNA, the sample was centrifuged at 12,000 rpm for 20 min, washed in 70% ethanol and centrifuged again for 10 min. The pellet was air-dried and dissolved in 40 µl sterile ddH₂O. The concentration of the cDNA insert was determined

using a spectrophotometer at 260 nm. To verify the concentration, the sample was separated at 80V for 1.5h in a 1.2% agarose gel in 1X TBE together with a DNA Mass Ladder (Gibco, BRL), followed by ethidium bromide staining to allow visualization of DNA under the UV light.

Approximately 30 ng DNA was denatured by boiling for 3 min, followed by cooling on ice. Ten μ l reagent mix and 10U Klenow enzyme (Oligolabelling Kit, Amersham, Pharmacia Biotech, Inc., Piscataway, NJ, USA) were subsequently added, followed by water to a volume of 45 μ l. Fifty μ Ci α -³²P dCTP (at 3000Ci/mmol) (NEN, PerkinElmer Life Sciences, Markham, Ontario, Canada) was added to the mixture, gently mixed with a pipette tip and incubated at 37°C for 1 h. In order to remove unincorporated nucleotide, probe purification was performed by applying the probe mixture to a microspin column (MicroSpin™ S-300 HR Columns, Amersham Pharmacia Biotech) and centrifuging at 3,000 rpm for 5 min. The flow-through was collected, and a 1 μ l aliquot of the flow-through was removed and mixed with liquid scintillation cocktail (BCS Biodegradable Counting Scintillant, Amersham, Oakville, Ontario, Canada) to measure the total cpm from which the specific activity of the probe could be calculated. Prior to use in hybridization, the probe was denatured at 100°C for 3 min and chilled on ice for 4 min.

2.2.9 Northern Hybridization Analyses

Twenty μ g total RNA was separated by electrophoresis in a formaldehyde-denaturing 1.2% (W/V) agarose gel containing 2.2M formaldehyde and 1 X MOPS buffer (20 mM 3-(N-morpholino) propanesulphonic acid, 5 mM sodium

acetate pH 7.0, 1 mM EDTA). RNA in 1X MOPS buffer, 2.2 M formaldehyde and 50% formamide was heated at 65°C for 15 min, and then chilled on ice. RNA-loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 50% v/v glycerol, 1 mM EDTA pH 8.0 and 0.5 µg/ml ethidium bromide) was added, mixed and loaded. The gel was run at 60 V for 3h in 1X MOPS running buffer. Following electrophoresis, the RNA gel was photographed under UV illumination and the RNA gel was rinsed in DEPC-treated ddH₂O prior to soaking for 5 min in 20X SSC (3.0 M NaCl, 0.3 M sodium citrate pH 7.0). A positively charged nylon membrane (Boehringer Mannheim, Laval, Quebec, Canada) was soaked briefly in DEPC-treated ddH₂O, followed by a 5 min soak in 20X SSC. RNA was capillary blotted overnight in 20X SSC as described by Sambrook et al., (1989). Following blotting, 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™ 2400 (Stratagene) for 30 seconds followed by baking at 80°C for 1 h. The membrane was prehybridized at 65°C for a minimum of 1 h in a rotary hybridization oven (Techne Hybridiser HB-2D, Mandel, Guelph, Ontario, ON, Canada) in ten ml of prehybridization buffer (100 mM NaH₂PO₄, 50 mM Na₂P₂O₇, 1 mM EDTA and 7% SDS) containing denatured salmon sperm DNA (100 µg/ml). The radiolabelled probe was added directly to the prehybridization solution and hybridization was performed overnight at 65°C. Following hybridization, the membrane was washed 3X for 5 min each in 10 ml 2X SSC, 0.1% SDS at room temperature. The membrane was then washed twice in 1X SSC, 0.1% SDS at 65°C and at 68°C, each for a period of 45 min. A final

wash was performed in 0.5 XSSC, 0.1% SDS at 65°C for 45 min. Following washing, the membrane was dried briefly, covered with saran wrap before exposure to autoradiography film (Kodak Scientific Imaging Film X-Omat Blue XB1, NEN™ Life Science, Boston, MA, USA), with an intensifying screen at -80°C. Average exposure time was 2 days for JWS-19, 4 days for JWS-20 and 2 days for JWS-27.

2.3 Results

2.3.1 Relative Water Content of Non-treated and Salt-treated Root and Shoot

To examine the impact of a salt treatment on water status, relative water content was determined for salt-stressed and non-treated Ailsa Craig (AC) roots and shoots. One hundred and seventy mM NaCl was used to elicit salt stress, and this concentration was chosen because previous work in the lab has demonstrated that it elicits the accumulation of a number of distinct polypeptides in tomato roots (Chen and Plant, 1999). Salt-treated roots and shoots had a lower RWC compared to that of the non-treated roots and shoots (Figure 2-2). A salt treatment caused an 18% and 22.6% reduction in the RWC of roots and shoots, respectively.

2.3.2 Effect of a Salt Treatment on Root mRNA Populations

RNA isolated from salt-stressed and non-stressed tomato roots was used for DD-PCR. In order to determine how rapidly changes in the mRNA population occurred following the applied salt treatment, a time course experiment ranging

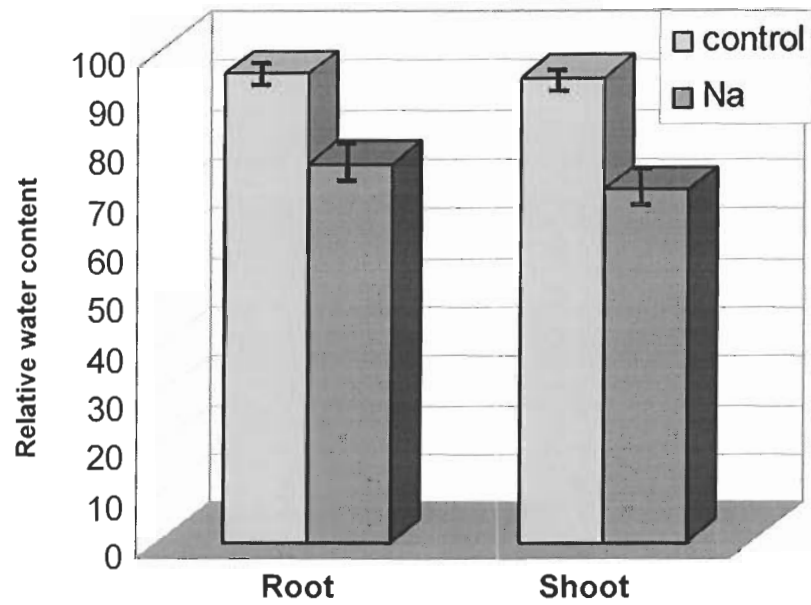


Figure 2-2 Relative water content of non-treated and salt-treated AC roots and shoot tissues.

Data presented are the mean and standard error derived from six shoot and root replications, respectively. This experiment was independently performed three times.

from 0, 0.5, 2, 8 and 24h was conducted. The combination of anchor and arbitrary primers used for DD-PCR are shown in Table 2-1. A total of five primer combinations were used and an average of sixty bands with a size greater than 100 bp was obtained with each primer combination. Figure 2-3 shows representative gels of DD-PCR products generated from RNA isolated from non-treated roots, salt-treated, ABA-treated, and roots that received a combined salt/ABA-treatment. Parallel comparisons of salt-treated and non-treated DD-PCR profiles showed differences suggesting that salt induced changes in the root mRNA population (Figures 2-3 A, B, C, D and E). Based on their expression pattern (salt-induced, salt up-regulated and salt down-regulated) following salt treatment, a total of eight salt responsive partial cDNAs that were consistently and reliably observed on different differential display gels, were chosen and labeled with the name of the anchor and arbitrary primer combinations used to generate them (Table 2-2). The time-dependent accumulation of various members of each group in salt-affected roots is indicated in Table 2-2.

2.3.3 Effect of ABA and a Combined ABA and Salt Treatment on Root mRNA Populations

To investigate the role played by ABA in regulating salt-induced changes of gene expression, roots were exposed to ABA and a combined ABA/NaCl treatment for a period of 24h. An ABA treatment alone resulted in a DD-PCR profile that is different from that caused by a salt treatment (Figures 2-3 A, B, E and Wei et al., 2000). Relative to the 0h control, a 24h ABA treatment resulted in

Figure 2-3 DD-PCR profiles generated using various primer combinations.

RNAs were isolated from non-treated roots (0C) and roots exposed to 170 mM NaCl (NaCl) for 0.5h (0.5NaCl), 2h (2NaCl), 8h (8NaCl) and 24h (24NaCl) or from roots exposed to ABA (A) or combined ABA and NaCl (24A/Na) for 24h and subjected to DD-PCR using the following combination of primers:

(A) T₍₁₁₎CG and primer #5 (5'GGAACCAATC 3')

(B) T₍₁₁₎CG and primer #1 (5'TACAACGAGG 3')

(C) T₍₁₁₎CG and primer #2 (5'TGGATTGGTC 3')

(D) T₍₁₁₎CC and primer #1 (5'TACAACGAGG 3')*

(E) T₍₁₁₎CC and primer #4 (5'TTTTGGCTCC 3')*.

DD-PCR products and gels generated with primers indicated by an asterisk (*) were generated by Dr. Jun-Zhi Wei. Duplicate lanes displaying products generated from two independent PCR amplifications are shown. Arrows indicate partial cDNAs of interest.

Figure 2-3A

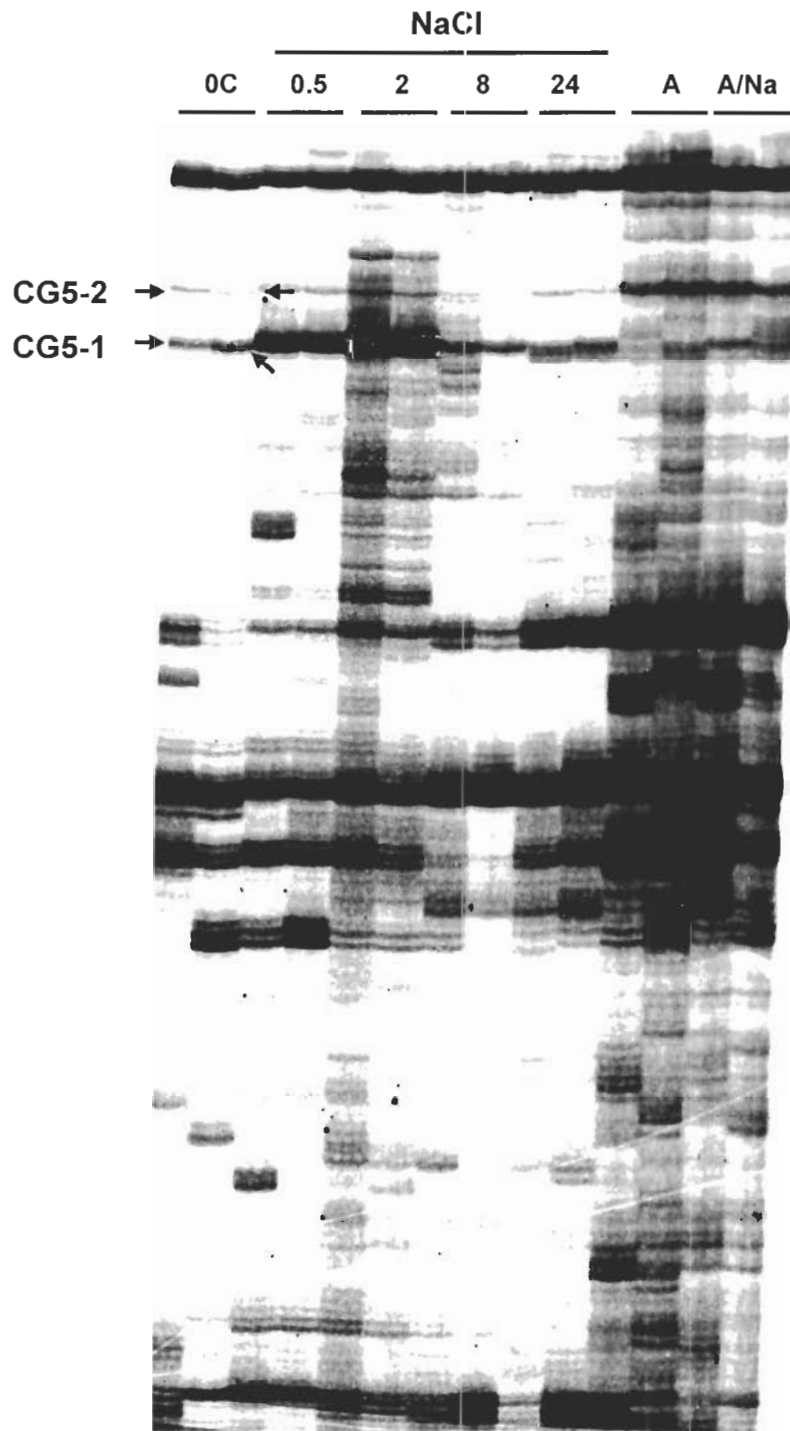


Figure 2-3B

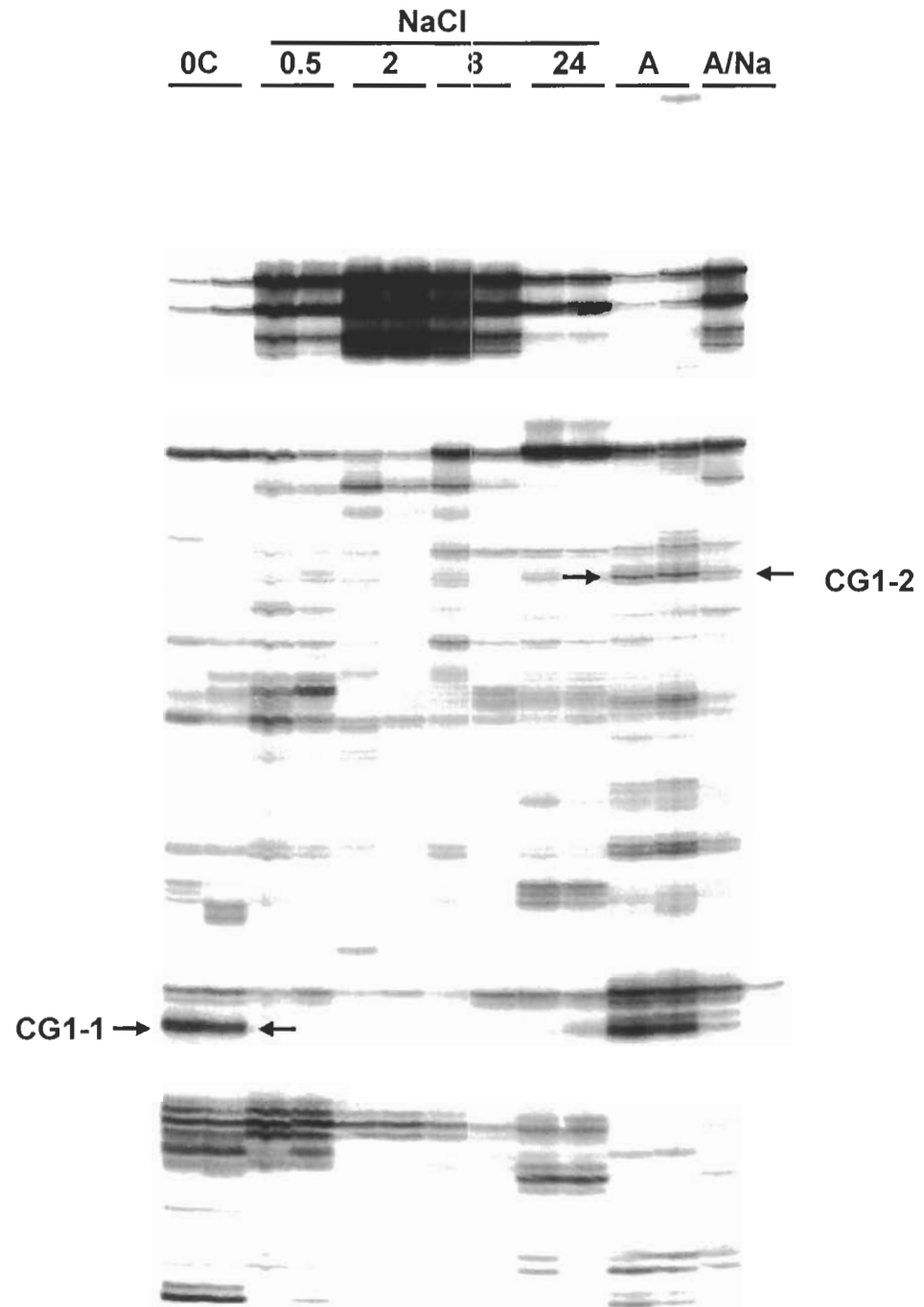


Figure 2-3C

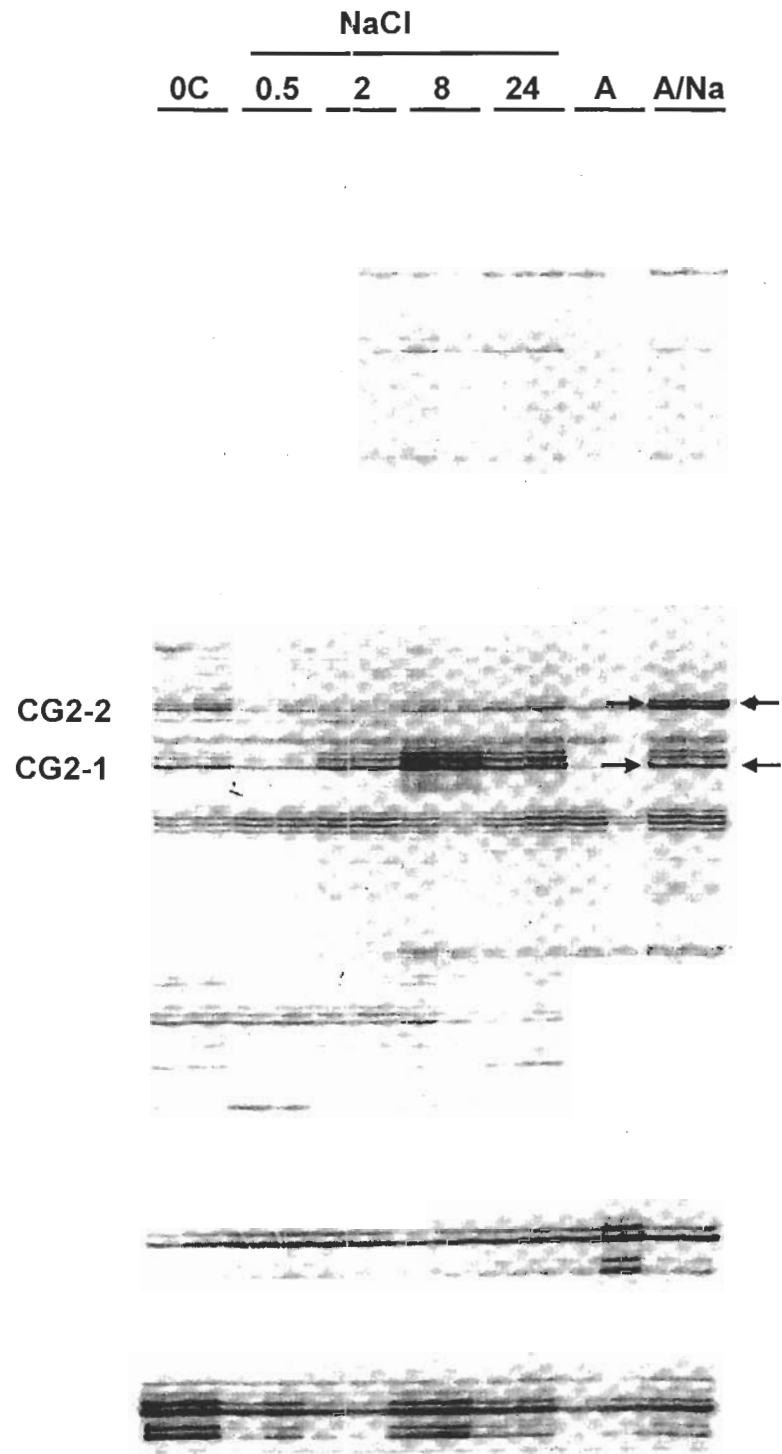


Figure 2-3D

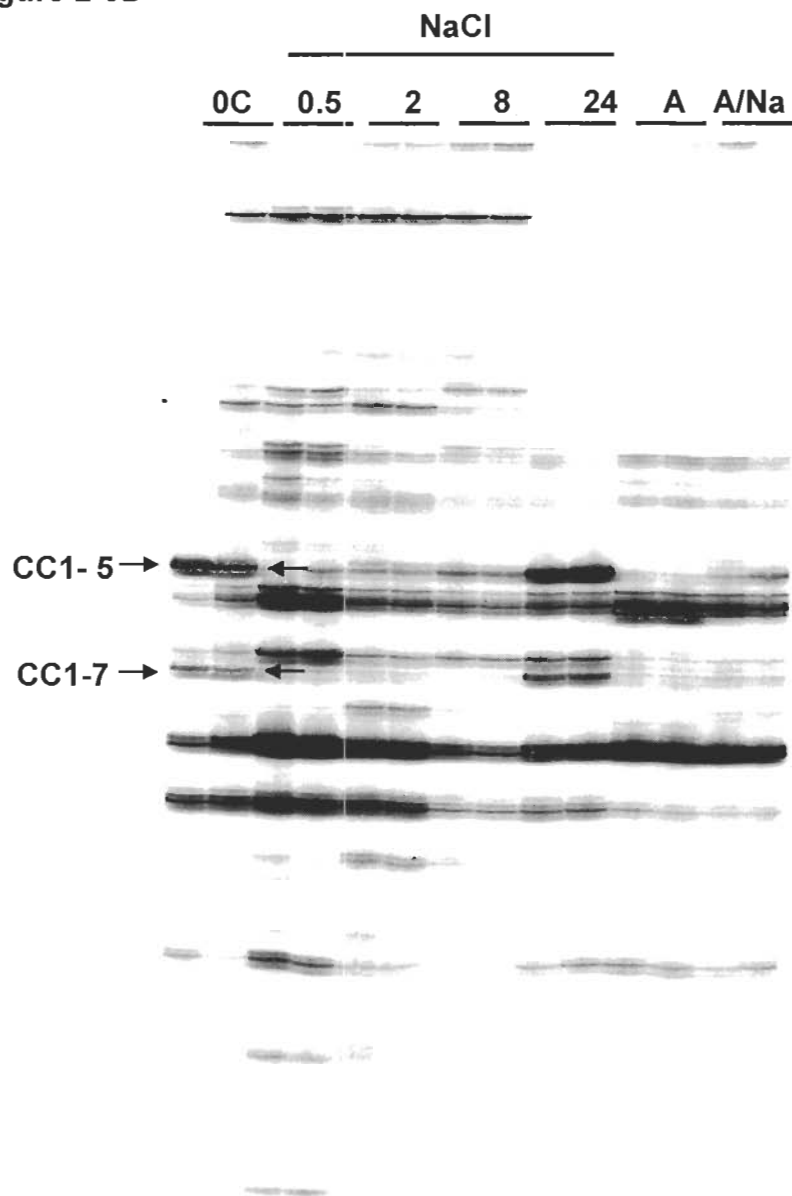


Figure 2-3E

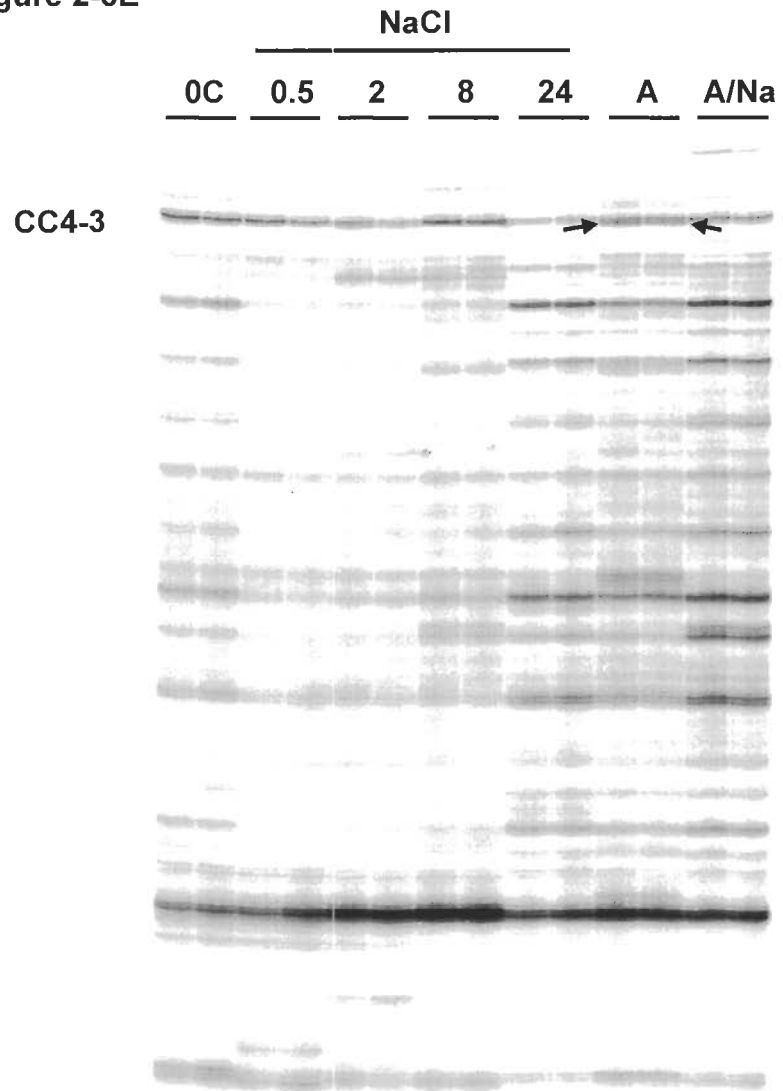


Table 2-2 Accumulation of DD-PCR products corresponding to RNA in salt-, ABA- and ABA/NaCl-treated tomato roots

Group	DD-PCR products	C	0.5Na	2Na	8Na	24Na	ABA	ABA/Na
Salt-induced								
	CC4-3 ^a	-	-	+	++	+	++	+
Salt up regulated								
	CG5-1 ^b	+	+++	++++	+	+	+	+
	CG5-2 ^b	+	+	++	+	+	++++	++++
	CG2-1 ^c	++	+	+++	++++	++++	-	+++
Salt down regulated								
	CC1-5 ^d	+++	+	+	+	+++	+	+
	CC1-7 ^d	+++	+	+	+	+++	+	+
	CG1-1 ^e	++++	-	-	-	++	+++++	++
	CG2-2 ^c	++	+	+	+	++	+	+++
ABA-regulated								
	CG1-2 ^e	+	+	+	+	+	+++	++

^a corresponds to Figure 2-3E, ^b corresponds to Figure 2-3A, ^c corresponds to Figure 2-3C, ^d corresponds to Figure 2-3D, and ^e corresponds to Figure 2-3B. '+', '++', etc denotes the presence and relative intensity of DD-PCR products on differential display gels, and '-' denotes the absence of DD-PCR products.

enhanced expression of three cDNAs, CG5-2 (Figure 2-3A, Table 2-2), (CG1-2 Figure 2-3B, Table 2-2), and CC4-3 (Figure 2-3E, Table 2-2).

Relative to the 0h control, one salt responsive cDNA (CG5-1, Figure 2-3A) was not affected by the applied ABA treatment, and another one, CG2-1 (Figure 2-3C) was not detected in ABA treated roots. In addition, ABA application for 24h down-regulated two partial cDNAs (CC1-5 and CC1-7, Figure 2-3D). A combined ABA/NaCl treatment resulted in changes in root RNA population that were similar to that caused by a salt treatment alone (Figure 2-3C, CG2-1 and CG2-2, Wei et al., 2000).

2.3.4 Cloning and Sequencing of Salt Responsive Partial cDNAs

Differential display clearly illustrated that there were changes in the mRNA population of roots in response to salinity stress. Based on their pattern of expression following salt (salt-induced, salt up-regulated or salt down-regulated) and ABA treatments, a total of six partial cDNAs (CG1-1, CG2-1, CG5-1, CC1-5, CC1-7, and CC4-3) were excised and reamplified using the same primer combinations initially used to generate them (Table 2-2). The sizes of the partial cDNAs ranged from 180 to 520 bp (Figure 2-4). Five of the six clones were successfully cloned (CG1-1, CG2-1, CG5-1, CC1-5 and CC1-7) and three clones (CG5-1, CC1-5 and CC1-7) with inserts of greater than 200 bp were chosen for ongoing work. Among these three partial cDNAs, the accumulation of one partial cDNA, CG5-1, was transiently up regulated by salinity stress (Figure 2-3A), while the other two, CC1-5 and CC1-7 (Figure 2-3D), were down regulated by salt.

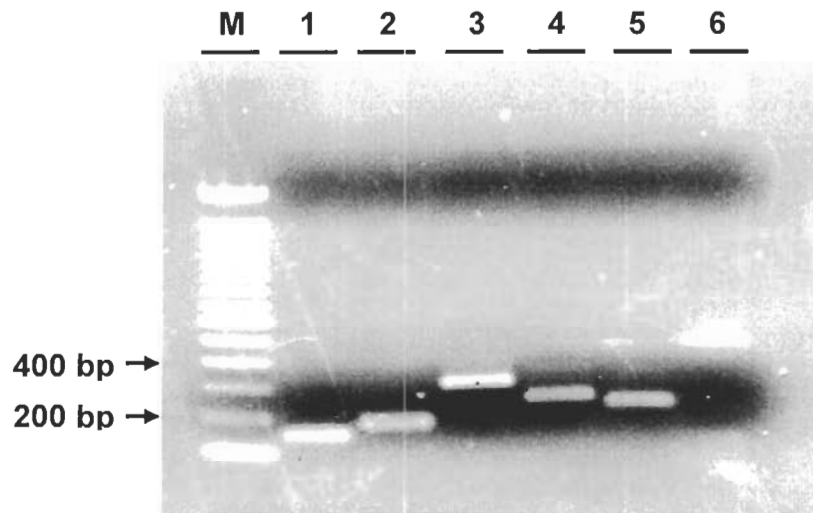


Figure 2-4 Reamplification of six partial cDNA isolated from salt-treated tomato roots. M represents a 100 bp DNA marker, Lane1: CG1-1, lane 2: CG2-1, lane 3: CG5-1, lane 4: CC1-5, lane 5: CC1-7, and lane 6: CC4-3.

The insert of each of these clones was manually sequenced for both strands using the T7 and M13 reverse universal primers. Nucleotide sequence data revealed that CC1-5 and CC1-7 contained related cDNA inserts, with the CC1-5 partial cDNA clone containing a longer 3'UTR. The CG5-1 partial cDNA clone contained a distinct cDNA insert. The nucleotide sequences of CC1-5/CC1-7 and CG5-1 together with the corresponding deduced open reading frame are presented in Figures 2-5 and 2-6, respectively. The CC1-5, CC1-7 and CG5-1 clones contain partial open reading frame (ORF) that includes the stop codon. The CC1-5 clone was chosen for ongoing work due to its larger insert size. Both CG5-1 and CC1-5 were renamed as JWS19 (Gen Bank Acc.: AW062237) and JWS20 (Gen Bank Accession: AW062238), respectively. In addition, another salt responsive partial cDNA clone, JWS27, which was identified and isolated by Dr. Jun-Zhi Wei (Figure 2-7, Gen Bank Accession: AW062242) was also included for ongoing study. The gene(s) corresponding to JWS27 was transiently expressed following the applied salt treatment (Figure 2-7). The nucleotide sequence is presented in Figure 2-8.

2.3.5 Nucleotide and Deduced Amino Acid Sequence Comparison

The nucleotide sequences of JWS19, JWS20 and JWS27 were submitted to the NCBI database for comparison to sequences deposited in various database using the BLASTN program against both the expressed sequenced tag (EST) and non redundant databases and the BLASTX program against the non redundant database (Altschul et al., 1997). The result of BLASTN and BLASTX

CC1-5

```
          10          20          30          40          50
TACAACGAGGAGACATACACAAAGAAAGGATTAGAATGGGTGAATACTAC
  Y N E E T Y T K K G L E W V N T T

          60          70          80          90          100
TGAGAGTTTGAAGATGTGTTAGATCGTCATTATCCAGAAATGACTGATA
  E S L K D V L D R H Y P E M T D

          110         120         130         140         150
AATGGATGAATTCAAACAGTGCCTTCTCTGTTTGGGATTCTTCTCCACAA
  K W M N S N S A F S V W D S S P Q

          160         170         180         190         200
CCTCACAATCCTGTTCCACTCTACTTTTCGTGTTCCCTAAACATTAATACTAA
  P H N P V P L Y F R V P K H *

          210         220         230         240         250
TAATACCTAATTTATTTACTTATCGAAAATGAAATATGAAGAATTGTGTG
                
          260
GAAAAAAAAAA
```

CC1-7

```
          10          20          30          40          50
TACAACGAGGAGACATACACAAAGAAAGGATTAGAATGGGTGAATACTAC
  Y N E E T Y T K K G L E W V N T T

          60          70          80          90          100
TGAGAGTTTGAAGATGTGTTAGATCGTCATTATCCAGAAATGACTGATA
  E S L K D V L D R H Y P E M T D

          110         120         130         140         150
AATGGATGAATTCAAACAGTGCCTTCTCTGTTTGGGATTCTTCTCCACAA
  K W M N S N S A F S V W D S S P Q

          160         170         180         190         200
CCTCACAATCCTGTTCCACTCTACTTTTCGTGTTCCCTAAACATTAATACTAA
  P H N P V P L Y F R V P K H *

          210         220         230
TAATACCTAATTTATTTACTTATCGAAAAAAAAAAAA
```

Figure 2-5 The nucleotide and deduced amino acid sequences of the CC1-5 and CC1-7 partial cDNAs.

The locations of both the random and anchor primers are underlined.

CG5-1

```
      10      20      30      40      50
GAACCAATCGACCCGAGAAGAAGGAGACAGTGACTTGTCTAATGCCCTGC
  N  Q  S  T  R  E  E  G  D  S  D  L  S  N  A  L

      60      70      80      90     100
AGTAGCATTTGCTATTATTTCTAGTATTACTAGCTAGTTATCAAAATGGT
Q  •

     110     120     130     140     150
AGCAAGATGTTATCAAAATGGTAGCAATATGAGTAGATGATAGTGGTTAA

     160     170     180     190     200
GCAGTAGATTAGGGAAAATGGTAGGAAAAAATTGTGGAAACAATTGCTCCTG

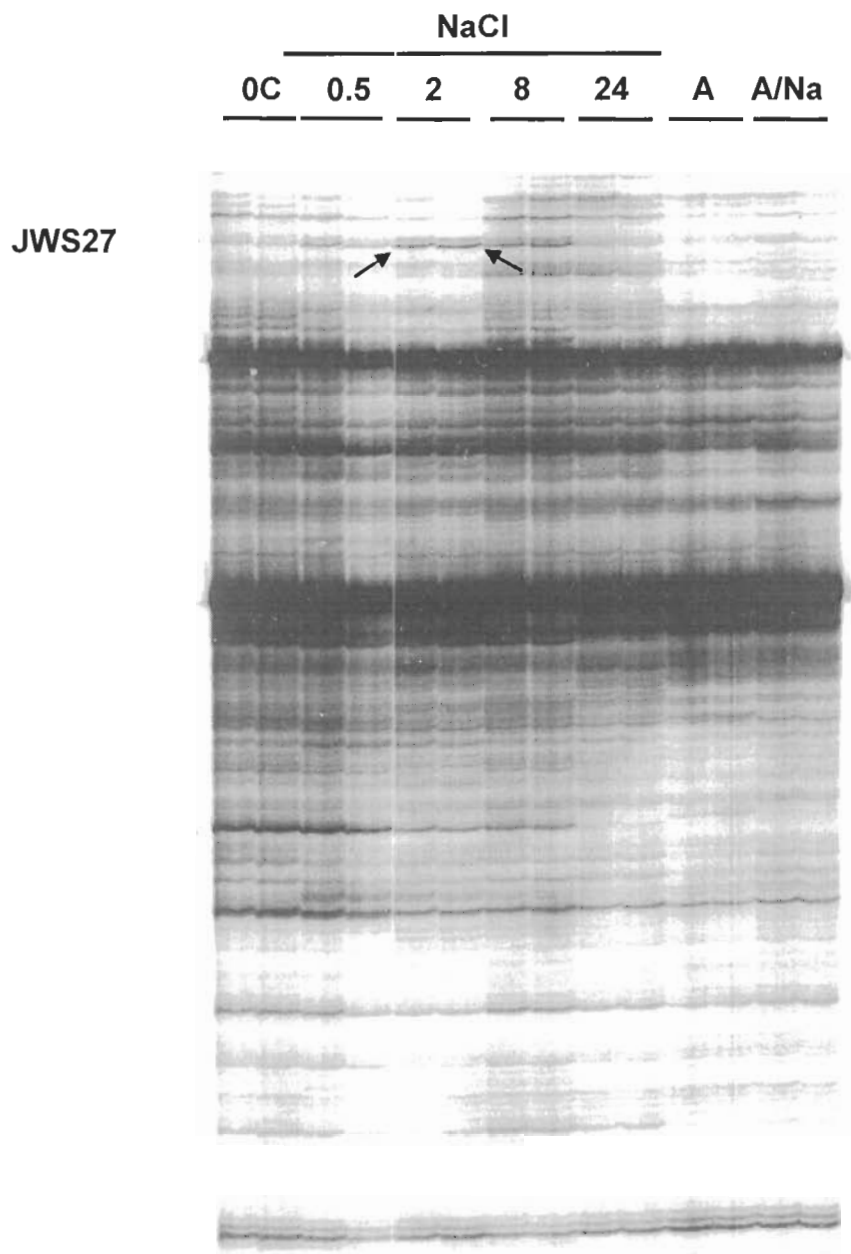
     210     220     230     240     250
ATCATGATCTCAGTTGTAGTTATTGTTTGTGTGGATTTCGACAGTAAAAA

     260     270     280     290     300
TAACTAAATTATCTGTATCAAGTGTTCATCAAAGAAAATGATATGCGAG

     310     320     330
TGCGACATACTATTGAGCCAAAAAAAAAAAA
```

Figure 2-6 The nucleotide and deduced amino acid sequences of CG5-1 partial cDNA. The locations of both the random and anchor primers are underlined.

Figure 2-7 DD-PCR profile generated using T₍₁₁₎GG and primer #4 (5'TTTTGGCTCC 3'). RNA was isolated from non-treated roots (0C) and roots exposed to 170 mM NaCl (NaCl) for 0.5h (0.5NaCl), 2h (2NaCl), 8h (8NaCl) and 24h (24NaCl) or roots exposed to ABA (A) or combined ABA and NaCl (24A/Na) for 24h and subjected to DD-PCR using the following combination of primers: T₍₁₁₎GG and primer #4 (5'TTTTGGCTCC 3'). Both the DD-PCR products and the gels were generated by Dr. Jun-Zhi Wei. Duplicate lanes displaying products generated from two independent PCR amplifications are shown. Arrow indicates partial cDNA of interest: JWS27.



```

          10          20          30          40          51
GCACAAAAAGTATATACTAATCCTCATTTCTAACCAGGTATAATTAATAA
+3:  T K K Y I L I L I S N Q V * L I N
+2:  H K K V Y T N P H F * P G I I N K
+1:  A Q K S I Y * S S F L T R Y N * *

          60          70          80          90          102
ATCCACACAAAAGGCTTACAAAAACGTCAATTTATCCCAGCGCAAATCAAAA
+3:  P H K R L T K T S I Y P S A N Q I
+2:  S T Q K A Y K N V N L S Q R K S N
+1:  I H T K G L Q K R Q F I P A Q I K

          110          120          130          140          153
TCCTACAAACCCTAAATCCAGGTTGACAACCAATGCACAACCCACGAAAAAC
+3:  L Q T L N P G * Q P M H N P R K P
+2:  P T N P K S R L T T N A Q P T K T
+1:  S Y K P * I Q V D N Q C T T H E N

          160          170          180          190          204
CACAAATTTTCCTTACAGATTTACATAGAGTATAGATCCCTGCTAGATTAT
+3:  Q I F L T D L H R V * I P A R L Y
+2:  T N F P Y R F T * S I D P C * I I
+1:  H K F S L Q I Y I E Y R S L L D Y

          210          220          230          240          250
ATCAGGCAACAAGAGCGTAAACACATAAATTCAAGTGCTCTTTGTTTCAAC
+3:  Q A T R A * T H K F K C S L F Q Q
+2:  S G N K S V N T * I Q V L F V S T
+1:  I R Q Q E R K H I N S S A L C F N

          260          270          280          290          300
AAACCTTAATCGATTTCGATCAAGGCTTCATTGCTGGATCTATTCAGCGAAT
+3:  T L I D S I K A S L L D L F S E *
+2:  N L N R F D Q G F I A G S I Q R I
+1:  K P * S I R S R L H C W I Y S A N

          310          320          330          340          350
AGCCAGGTGGTGGGTATGGGAAGCACCGAAGACATCCTCAATACACAGACA
+3:  P G G G Y G K H R R H P Q Y T D K
+2:  A R W W V W E A P K T S S I H R Q
+1:  S Q V V G M G S T E D I L N T Q T

          360          370          380          390          400
AATCCCTTTTCCGATTTTCAGATCTGAGACTTCCCTCCCTTTCTTT
+3:  S L F R F Q I * D F P P F L
+2:  I P F P I S D L R L P S L S L
+1:  N P F S D F R S E T S L P F F

```

Figure 2-8 The nucleotide and deduced amino acid sequences of JWS27.

Neither the arbitrary primer nor a poly A tract was present in the JWS27 insert. A potential location for the T₁₁GG anchor primer is underlined.

searches with the nucleotide sequence of JWS19 is presented in Table 2-3. The nucleotide sequence of the cDNA insert of JWS19 shares high similarities with several ESTs from a tomato ovary cDNA library and with an EST generated from a *Pseudomonas* susceptible tomato cDNA library (Table 2-3). At the amino acid level there was 100% identity between the amino acid sequence derived from JWS19 and the C terminus of *Lycopersicon esculentum* auxin regulated (LE-ARP, Gen Bank Acc. : AF416289) protein. The alignment of the deduced amino acid sequence derived from JWS19 to that of LE-ARP is presented in Figure 2-9.

The result of BLASTN and BLASTX searches with the nucleotide sequence of JWS20 is presented in Table 2-4, 2-5, and 2-6. Against the EST database, the nucleotide sequence of JWS20 shares high similarity with various ESTs that were generated from a variety of plants including potato, poplar and *Nicotiana attenuata* (Table 2-4). Against the non-redundant database, the nucleotide sequence of the JWS20 cDNA shares similarity to the nucleotide sequence of α -dioxygenase enzymes isolated from various plants including *Nicotiana tabacum*, *N. attenuata*, *Capsicum anuum*, *Populus balsamifera* subspecies *trichocarpa*, *Arabidopsis thaliana* and *Cicer arietinum* (Table 2-5). BLASTX searches revealed that there was 93%, 92%, 91%, 79%, 72%, 70%, 61%, 59%, and 82% identity between the amino acid sequence derived from JWS20 and the C terminal portion of α -DOX from *N. attenuata*, *C. anuum*, *N. tabacum*, *A. thaliana*, *Oryza sativa*, *A. thaliana* FEEBLY-like protein, *L. esculentum* FEEBLY, *Zea mays*, and *P. balsamifera* subspecies *trichocarpa*, respectively (Table 2-6, Figure 2-10).

Table 2-3 Results of JWS19 BLASTN searches (May, 2005) against both the EST and the non-redundant (NR) databases.

BLAST results	GenBank Accession Number	Bit Score	E value
BLASTN (EST data base)			
cDNA library			
Tomato ovary	AI771919	636	0
Pseudomonas-susceptible	AI899669	620	e-177
tomato leaves			
Tomato ovary	AI771852	519	e-147
Tomato ovary	AI486194	519	e-147
Tomato ovary	AI487359	511	e-144
BLASTN (NR database)			
Tomato auxin-regulated protein	AF416289	599	e-168

```
JWS19 :      1 NQSTREEGSDLSNALQ 17
          NQSTREEGSDLSNALQ
LE-ARP : 1253 NQSTREEGSDLSNALQ 1303
```

Figure 2-9 Alignment of the deduced amino acid sequence of JWS19 with the C-terminal amino acid sequence of *Lycopersicon esculentum* auxin regulated protein (LE-ARP).

Numbers on the alignment corresponding to the number of the nucleotide sequence of JWS19 and LE-ARP, respectively.

Table 2-4 Results of JWS20 BLASTN search against the EST database (May, 2005).

BLASTN results*	GenBank Accession Number	Bit Score	E value
cDNA library			
Tomato root at preanthesis stage	AW224575	482	e-133
Late blight-treated potato tubers	DN590415	268	6e-69
Mixed potato tissues	BQ515397	268	6e-69
Mixed potato tissues	BQ120527	268	6e-69
<i>M.sexta</i> -induced	CA591872	202	3e-49
<i>Nicotiana attenuata</i>			
<i>M.sexta</i> induced	BU494500	202	3e-49
<i>Nicotiana attenuata</i>			
Mixed potato tissues	BQ121693	178	4e-42
Mixed potato tissues	BQ115240	157	1e-57
Poplar terminal bud	CV243714	133	2e-28
Aspen root	CA927439	125	6e-26

* only ESTs with an E value equal to or less than 6e-26 are presented in this table.

Table 2-5 Results of JWS20 BLASTN search the NR database (May, 2005).

BLASTN results*	GenBank Accession Number	Bit Score	E value
<i>N. tabacum</i> mRNA for oxygenase	AJ007630	210	1e-51
<i>N. attenuata</i> pathogen-induced oxygenase	AF229926	202	3e-49
<i>C. anuum</i> cyclooxygenase-like protein	AY040869	198	4e-48
<i>P. Balsamifera</i> Trichocarpa	AC149545	133	2e-28
<i>P. sativum</i> alpha-DOX1	AJ784963	90	3e-15
<i>A. thaliana</i> alpha-DOX1	AF334402	74	2e-10
<i>A. thaliana</i> feebly-like protein	AY064666	74	2e-10
<i>Cicer arietinum</i> putative alpha-DOX	AJ487467	68	1e-08

* only ESTs with an E value equal to or less than 1e-08 are presented in this table.

Table 2-6 Results of JWS20 tBLASTX search against the NR database (May, 2005).

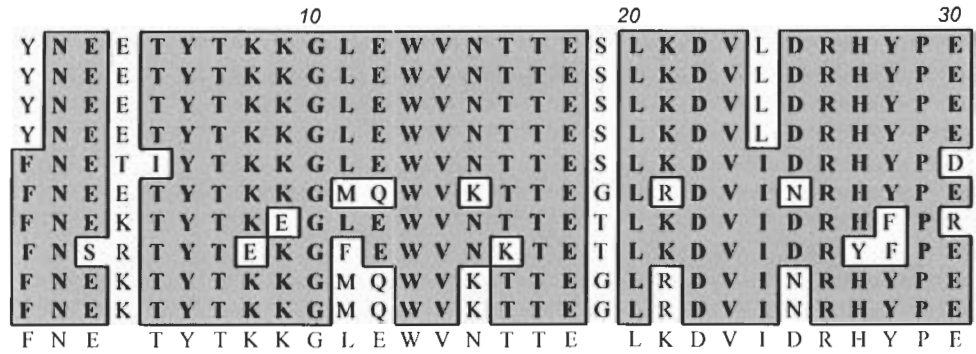
tBLASTX results*	GenBank Accession Number	Bit Score	E value
<i>C. annuum</i> cyclooxygenase-like protein	AY040869	134	1e-30
<i>N. attenuata</i> pathogen-induced oxygenase	AF229926	134	1e-30
<i>N. tabacum</i> mRNA for oxygenase	AJ007630	132	4e-30
<i>P. sativum</i> mRNA for alpha-DOX1	AJ784963	138	4e-31
<i>P. Balsamifera</i> subsp. Trichocarpa	AC149545	115	5e-25
<i>A. thaliana</i> alpha-DOX1	AF334402	114	1e-24
<i>Cicer arietinum</i> putative alpha-DOX	AJ487467	112	7e-24
<i>A. thaliana</i> feebly-like protein	AY128743	100	3e-20
<i>Medicago truncatula</i> clone	AC012679	96	7e-19
<i>Coffea arabica</i> putative oxygenase	AF343970	94	2e-18
<i>L. esculentum</i> feebly-like protein	AJ850958	89	8e-17
<i>Z. mays</i> mRNA	AY108782	85	1e-15
<i>O. sativa</i> fatty acid alpha-oxidase	AF229813	84	2e-15
<i>Z. mays</i>	AY108782	96	2e-18

* only ESTs with an E value equal or less than 2e-18 are presented in this table.

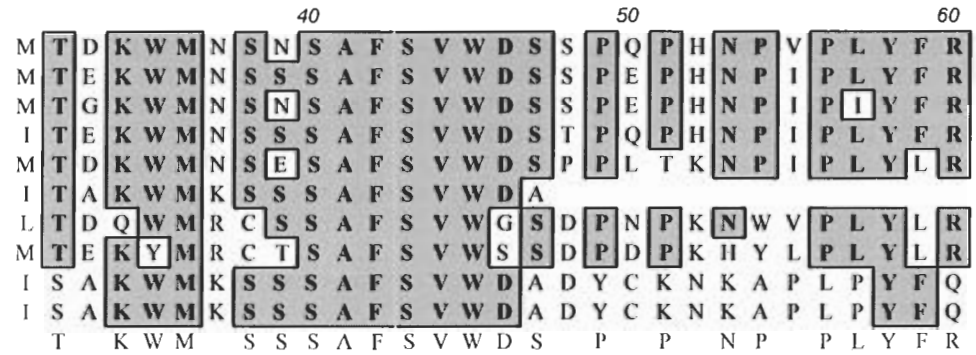
Figure 2-10 Alignment of the deduced amino acid sequence of JWS20 with the amino acid sequences of the C-terminal portion of α -DOX.

Alignment of the deduced amino acid sequence of JWS20 with the amino acid sequences of the *N. attenuata* (*Na* α -DOX), *C. annuum* (*Ca* α -DOX), *N. tabacum* (*Nt* α -DOX), *A. thaliana* (*At* α -DOX1), *O. sativa* (*Os* α -DOX), *A. thaliana* FEEBLY-like protein (*At* α -DOX2), *L. esculentum* FEEBLY-like protein (*Le* Feebly) , *Z. mays* (*Zm*) and *P. balsamifera* subspecies *Trichocarpa* (*PTrichocarpa*). Identical amino acids to the majority are shaded and the consensus amino acid sequence is given at the bottom of the alignment. Alignment was constructed using MacVector 7.1.1 using a 10.0 open gap penalty, 40% delay divergent and Blosum similarity matrix

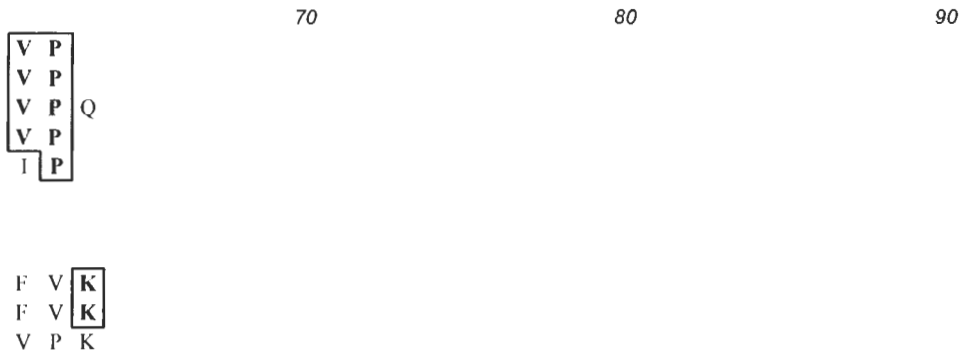
JWS-20
Na alpha-DOX
Ca alpha-DOX
Nt alpha-DOX
At alpha-DOX1
Os alpha-DOX
At alpha-DOX2
Le Feebly
Zmays
Ptrichocarpa



JWS-20
Na alpha-DOX
Ca alpha-DOX
Nt alpha-DOX
At alpha-DOX1
Os alpha-DOX
At alpha-DOX2
Le Feebly
Zmays
Ptrichocarpa



JWS-20
Na alpha-DOX
Ca alpha-DOX
Nt alpha-DOX
At alpha-DOX1
Os alpha-DOX
At alpha-DOX2
Le Feebly
Zmays
Ptrichocarpa



The result of BLASTN and BLASTX searches with the nucleotide sequence of JWS27 is presented in Table 2-7. BLASTN searches against the EST database showed that the nucleotide sequence of JWS27 shares high similarities with a variety of tomato ESTs which were generated from various tissues including fruits, flowers, flower buds, nutrient-deficient roots, germinating seedlings, callus, crown gall tissues, shoots and potato ESTs generated from microtubers, stolons, tuber skins and mixed tissues subjected to salt, drought and cold-stresses (Table 2-7). BLASTN and BLASTX searches against the non redundant database showed that the nucleotide sequence of JWS27 shares 66% identity with a hydrogen peroxide-induced *N. tabacum* cDNA-AFLP fragment (Table 2-7). An amino acid alignment of JWS-27 and the hydrogen peroxide-induced *N. tabacum* cDNA-AFLP fragment is presented in Figure 2-11.

2.3.6 Northern Hybridization Analyses

Differential display is a sensitive technique to isolate differentially expressed genes but is not without its drawbacks. These arise due to the low annealing temperature used during PCR, which can result in a lowered priming specificity which can produce differences in cDNA bands that do not reflect actual differences in gene expression (Baldwin et al., 1999). It is therefore important that the differential expression of genes corresponding to partial cDNAs isolated using DD-PCR is validated by northern hybridization analyses.

RNA northern hybridization analyses were therefore conducted to confirm that the gene(s) corresponding to JWS19, JWS20 and JWS27 are expressed in a

Table 2-7 Results of JWS27 BLASTN search against the EST and Non-redundant databases (May, 2005).

BLAST results	GenBank Accession Number	Bit Score	E value
BLASTN (EST database)*			
cDNA library			
Tomato breaker fruit	BM409848	759	0
Tomato flower (anthesis)	BI935258	759	0
Tomato flower (anthesis)	BI935181	759	0
Tomato flower buds	BE463307	743	0
Tomato root deficiency	AW979397	739	0
Tomato germinating seedling	AW649586	696	0
Tomato callus	AW031912	686	0
Tomato crown gall	BG135811	579	e-162
Tomato shoot	AI483154	579	e-162
Potato microtubers	BI919844	476	e-131
Potato stolon	AW906406	468	e-129
Potato tuber skin	CN514812	440	e-120
Potato: salt, cold, drought-treated leaves and roots	CK274732	428	e-117
BLASTN (Non-redundant)			
<i>N. tabacum</i> hydrogen peroxide-induced cDNA	AJ538962	70	4e-09

* only EST with E value equal to or less than e-117 are presented in this table.

```
JWS27: 281 RCFYPPPGYSLNRSSNE 334
          RC PYPPPGYSL ++N+
Nt      : 23 RCLPYPPPGYSLYTAAND 76
```

Figure 2-11 Alignment of the deduced amino acid sequence of JWS27 and *Nicotiana tabacum* hydrogen peroxide-induced cDNA-AFLP fragment.

Identical amino acids are presented between the alignment, + represents conservative changes, and blank spaces represent non-conservative changes.

salt-dependent manner. RNA was isolated from tomato roots treated with 170 mM NaCl for 0, 0.5, 2, 8 and 24h. In salt treated roots, genes corresponding to JWS19, JWS20 and JWS27 are clearly expressed in response to the applied salt treatment (Figure 2-12 and Figure 2-13). Two transcripts hybridized to the JWS19 partial cDNA probe. The size of the larger (JWS19U) and smaller (JWS19L) transcripts was estimated to be 2.1 kb and 1.8 kb, respectively. The gene(s) corresponding to JWS19 was transiently induced in response to the applied salt treatment. Expression was detected at 0.5 and 2h and had disappeared by 8h following the salt treatment. There was no expression of genes corresponding to JWS19 in non-treated roots (Figure 2-12).

The expression of the gene(s) corresponding to JWS20 was detected in non-treated roots (Figure 2-12). The estimated size of the transcript corresponding to JWS20 is 2.3 kb, which corresponds to the reported size of the *N. tabacum* α -DOX mRNA (Sanz et al., 1998). Following a salt treatment, the gene(s) corresponding to JWS-20 was up regulated at 2h, 8h and 24h.

At least two transcripts hybridized to the JWS27 probe (JWS27U and JWS27L), both of which accumulated transiently following a salt treatment (Figure 2-13). The estimated size of the transcript that hybridized to JWS27 is 4.5 kb (JWS27U) and 2.0 kb (JWS27L). The JWS27U transcript was undetected in control roots, whereas the JWS27L transcript was present (Figure 2-13). Following a salt treatment, the level of JWS27U increased at 0.5, 2 and 8h, whereas the level of JWS-27L only increased at 2h.

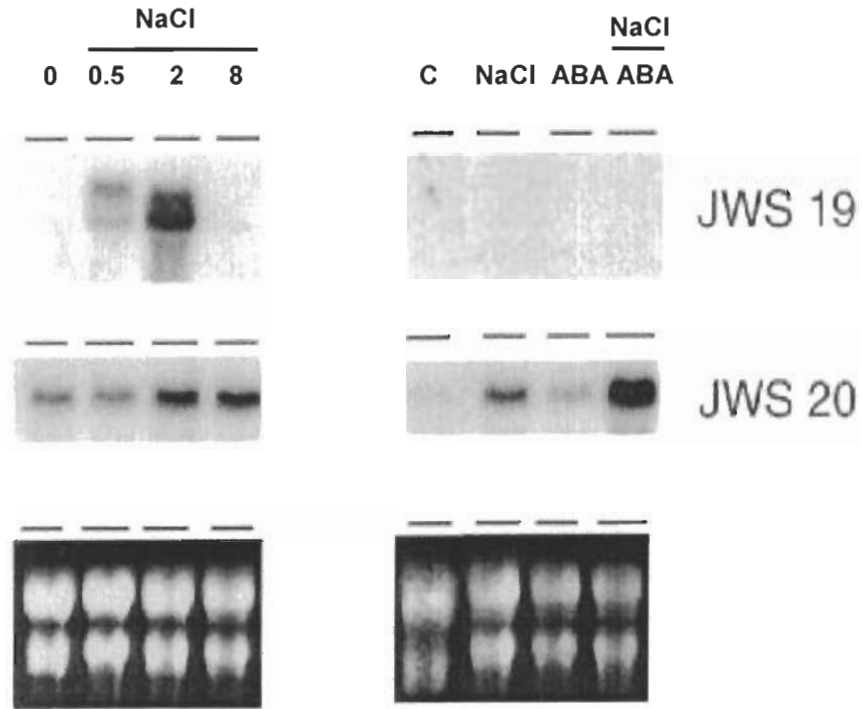


Figure 2-12 Expression of genes corresponding to JWS19 and JWS20 in salt-treated wild type roots.

RNA was isolated from root of plants exposed to MS nutrient solution containing no NaCl for 0h (0) and to MS nutrient solution containing 170 mM NaCl for 0.5h (NaCl, 0.5), 2 h (NaCl 2), 8 h (NaCl, 8), 24 h (NaCl, 24) or to MS nutrient solution containing no salt (C), ABA (ABA) and NaCl plus ABA (ABA/NaCl) for 24 h. A representative ethidium bromide-stained agarose gel is shown to indicate the approximate equal loading of RNA samples. Plants were grown and stressed by both Dr. Jun-Zhi Wei and Ananchanok Tirajoh. RNA extraction and blots were generated by Dr. Jun-Zhi Wei, and hybridization was performed by Ananchanok Tirajoh. This experiment was independently performed for two times.

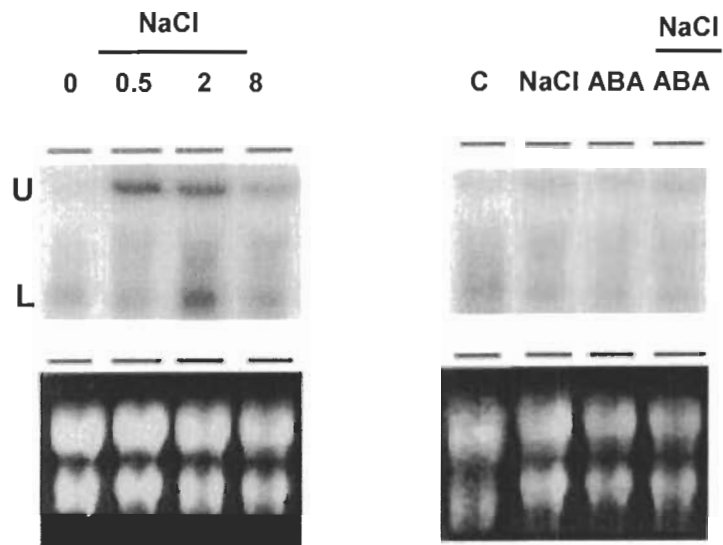


Figure 2-13 Expression of the genes corresponding to JWS27 in salt-treated wild type roots.

RNA was isolated from root of plants exposed to MS nutrient solution containing no NaCl for 0h (0) and to MS nutrient solution containing 170 mM NaCl for 0.5h (NaCl, 0.5), 2 h (NaCl 2), 8 h (NaCl, 8), 24 h (NaCl, 24) or to MS nutrient solution containing no salt (C), ABA (ABA) and NaCl plus ABA (ABA/NaCl) for 24 h. A representative ethidium bromide-stained agarose gel is shown to indicate the approximate equal loading of RNA samples. Plants were grown and stressed by both Dr. Jun-Zhi Wei and Ananchanok Tirajoh. RNA extraction, blot and hybridization were performed by Dr. Jun-Zhi Wei. This experiment was independently performed for two times.

2.3.7 Regulation of Gene Expression by ABA

ABA has been proposed to regulate the changes of gene expression in salt-stressed roots (Galvez et al., 1993; Moons et al., 1995). In order to gain an insight into the role of ABA in regulating the changes of gene expression, the expression of genes corresponding to JWS19, JWS20 and JWS27 was examined in the roots of ABA-treated plants as well as those exposed to a combined ABA/Na treatment. In addition, the expression of genes corresponding to JWS19 and JWS27 was also investigated in salt-treated roots of the ABA-deficient mutant, *flacca* (*flc*). At 24h following the application of exogenous ABA, the expression of genes corresponding to JWS19 and JWS27 was not ABA responsive (Figures 2-12, 2-13). The expression of gene(s) corresponding to JWS20 was up regulated following a 24h exogenous ABA application.

No expression was detected for genes corresponding to JWS19 and JWS27 in response to a combined ABA/Na treatment after 24h. However, for gene(s) corresponding to JWS20, exogenous ABA together with a salt treatment elicited a higher expression level over that present when either treatment was applied alone (Figure 2-12).

In the roots of salt-treated *flc*, the expression level of genes corresponding to JWS19, JWS20 and JWS27 was similar to that observed in salt-treated wild type roots, with one exception (Figure 2-14, 2-15). In non-treated roots of *flc*, the expression of the gene(s) corresponding to JWS20 was higher than that observed in non-treated roots of the wild type; this result was consistently observed in at least two independent treatments.

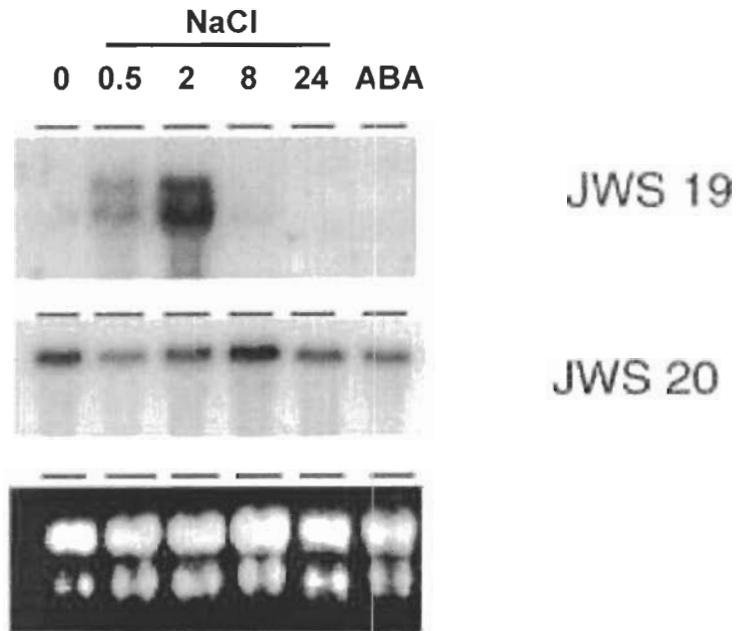


Figure 2-14 Expression of genes corresponding to JWS19 and JWS20 in salt-treated *ric* roots.

RNA was isolated from roots of plants exposed to MS nutrient medium with no NaCl for 0h (0) or with NaCl for 0.5h (NaCl, 0.5), 2 h (NaCl, 2), 8 h (NaCl, 8), 24 h (NaCl, 24), and with ABA (ABA) for 24 h. A representative ethidium bromide-stained agarose gel is shown in order to indicate the approximate equal loading of RNA samples. Plants were grown and stressed by both Dr. Jun-Zhi Wei and Ananchanok Tirajoh. RNA extraction and blots were generated by Ananchanok Tirajoh and Jollanda Effendy, and hybridization was performed by Ananchanok Tirajoh. This experiment was independently performed for two times.

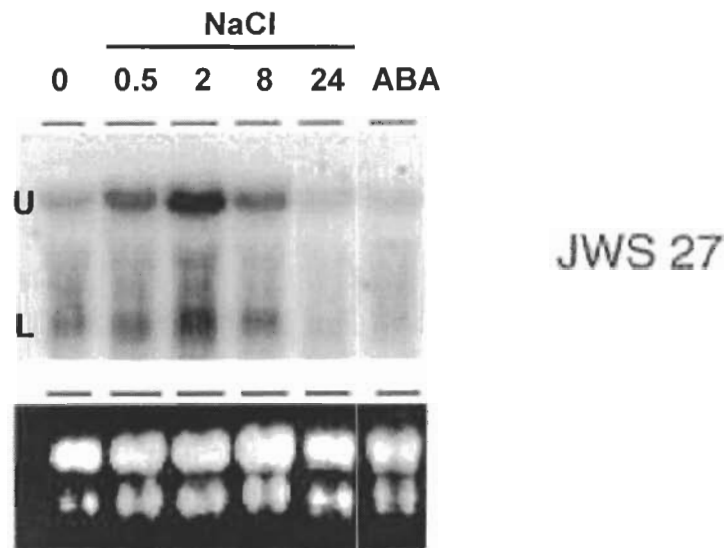


Figure 2-15 Expression of the genes corresponding to JWS27 in salt-treated *flc* roots. RNA was isolated from roots of plants exposed to MS nutrient medium with no NaCl for 0h (0) or with NaCl for 0.5h (NaCl, 0.5), 2 h (NaCl 2), 8 h (NaCl, 8), 24 h (NaCl, 24), and with ABA (ABA) for 24 h. A representative ethidium bromide-stained agarose gel is shown in order to indicate the approximate equal loading of RNA samples. RNA extraction and blot were generated by Dr. Jun-Zhi Wei and hybridization was performed by Ananchanok Tirajoh. This experiment was independently performed for two times.

2.4 Discussion

mRNA differential display clearly demonstrated that changes of gene expression occurred in salt-treated tomato roots and allowed for the isolation of several salt responsive partial cDNAs from a subset of the mRNA population. These salt responsive partial cDNAs were subsequently sequenced in order to gain more information about the corresponding genes. The expression of the genes corresponding to the identified salt responsive partial cDNAs was examined in salt-treated roots using northern hybridization analyses in order to confirm that they correspond to genes that are expressed in a salt dependent manner.

In tomato roots, it has been demonstrated previously that a salt treatment elicits altered synthesis and accumulation of a number of polypeptides (Chen and Plant, 1999; Jin et al., 2000). In agreement with those findings, this study demonstrated that salt-induced changes also occur at the mRNA level. Taken together these results suggest that following salinity stress, changes of gene expression occur at both the protein and mRNA level.

In tomato, salt stress resulted in lowered water content in both root and shoot tissues (Figure 2-2), an indication that salt stress led to a subsequent reduction in the osmotic potential. This study demonstrated that the accumulation of a number of different RNAs was clearly affected by the applied salt treatment (Figure 2-3 A, B, C, D and E). A total of eight salt responsive partial cDNAs were identified (Table 2-2), of which one (CC4-3, Figure 2-3E) was salt-induced, three were salt-up regulated (CG5-1 and CG5-2, Figure 2-3A and CG2-1, Figure 2-

3C), and four were salt-down regulated (CC1-5 & CC1-7, Figure 2-3D; CG1-1, Figure 2-3B; CG2-2, Figure 2-3C). Inspection of DD-PCR cDNA profiles revealed that in most cases, changes of gene expression occurred rapidly following the applied salt treatment (Table 2-2 and Figure 2-3). This result is in accordance with the salt-enhanced accumulation of root polypeptides (Chen and Plant, 1999) in which the earliest changes were detected within 30 min of a salt treatment. The rapid induction of gene expression in salt treated roots has also been demonstrated by others (Gulick and Dvorak, 1992; Botella et al., 1994; Galvez et al., 1993; Moons et al., 1997b; Kawasaki et al., 2001).

This study did not distinguish whether the changes in the RNA levels are due to the osmotic or ionic toxicity components of the salt treatment. It has been suggested that most changes of gene expression at the earlier time points such as 0.5h, 2h and possibly 8h after salt application are, in part, due to the osmotic component of salt stress (Galvez et al., 1993). This was also supported by the fact that one of the salt responsive partial cDNAs (JWS20) isolated from this study was identical to a DD-PCR product isolated in this lab from water-deficit stressed tomato roots (Jin Shu, 1999).

In order to confirm the differential expression obtained using the DD-PCR technique, it is imperative to do northern hybridization analyses. In this study, northern analyses confirmed that the genes corresponding to JWS19, JWS20 and JWS27 were expressed in a salt-dependent manner (Figures 2-12, 2-13). This however is not always the case since several salt responsive partial cDNAs isolated in parallel gave no signal on northern analyses (Wei et al., 2000). The

lack of a positive signal with northern hybridization analyses may be the outcome of a very low expression level or cloning artifact. The time dependent and transient accumulation of both JWS19 and JWS27 transcripts detected by northern hybridization analyses corresponds to the initial pattern observed for the partial cDNAs on the differential display gels. However, this was not the case for JWS20. JWS20 was initially identified as a salt down regulated partial cDNA, and northern hybridization analyses revealed that the gene(s) corresponding to JWS20 was up regulated following salt treatment. This may be caused by the fact that JWS20 belongs to a small gene family (see Chapter 4). Therefore, it is likely that, when using JWS20 as a probe, it also hybridized to other isoforms that may be up-regulated by salt.

Many salt-responsive genes in roots are also ABA responsive; these include several early-salt-induced genes of wheat (*ESI2*, *ESI18* and *ESI48*, Galvez et al., 1993), S-adenosylmethionine synthetase (*SAM1* and *SAM3*, Amitai-Zeigerson et al., 1995), and late embryogenesis abundant³ of rice (*oslea3*, Moons et al., 1997a). The ABA responsiveness of gene expression suggests a role for ABA in mediating the expression of these genes, but it is not sufficient to prove that accumulated ABA is actually required for stress-responsive expression. In this study, the gene(s) correspond to JWS20 was ABA responsive, however, in salt-stressed *flc* roots, the expression level was comparable to that detected in salt-stressed roots of the wild type. This suggests that JWS20 expression may not depend on an elevated level of ABA. However, since *flc* has been demonstrated to accumulate ABA following salt stress (Chen

and Plant, 1999), further studies are needed to address the role of ABA in regulating the expression of gene(s) corresponding to JWS20 during salt stress. Interestingly, the expression of the gene(s) corresponding to JWS20 in control roots of *flc* was higher than that of wild type (see Chapter 4).

In plants, a number of genes have been demonstrated to be induced or up regulated by salinity stress. Sequence analyse of these genes has revealed that their gene products might function in stress tolerance and response (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Wang et al., 2003; Seki et al 2002a; Rabbani et al., 2003). The gene corresponding to JWS19 shares identity to a tomato auxin-regulated protein. The significance of tomato auxin-regulated protein with respect to salt stress is further discussed in Chapter 3. BLASTN result also showed that JWS19 shares high similarity to several ESTs that were derived from tomato ovary, and one from tomato leaves (Table 2-3), all of which shares 100% sequence identities with JWS19. In addition, BLASTN result also demonstrated that there is no root-derived EST that shares similarity with JWS19 (Table 2-3). Therefore, together this data indicated that JWS19 may be expressed constitutively in both ovaries and non-stressed leaves. In the roots, however, the expression of gene(s) corresponding to JWS19 appear not to be constitutive but can be induced by salt stress.

JWS20 shares high sequence identity with alpha-dioxygenases, which were initially identified as pathogen-induced in tobacco (Sanz et al., 1998). In tobacco leaves, α -DOX expression was undetected in control leaves and induced following both bacterial infection and wounding treatments. Furthermore, its

expression in leaves is also up regulated by salicylic acid, jasmonic acid, and chemicals that generate ROS such as paraquat and 3-amino-1,2,4-triazole. This study demonstrated that, in tomato roots, α -DOX expression is both ABA and salt responsive. Studies in the lab also indicated that α -DOX was responsive to water deficit stress (Jin Shu, 1999). Since α -DOX expression is responsive to both abiotic and biotic treatments, it is possible that α -DOX is a general stress response intermediate. Further discussion of salt-induction of α -DOX is presented in Chapter 4.

BLASTN results for JWS27 showed that it shares similarities to a variety of tomato ESTs that were generated from flower, seedling and shoot. This result indicates that the expression of gene(s) corresponding to JWS27 may not be restricted to roots. In addition, JWS27 also share high similarity to EST derived from mixed potato tissues (shoot and roots) that were exposed to salt, drought and cold, respectively; this result substantiated the salt responsive nature of JWS27 (Table 2-7). JWS27 also shares high similarity with a hydrogen-peroxide induced tobacco cDNA-AFLP fragment. In living organisms, reactive oxygen species (ROS) such as hydrogen peroxide are generated during several metabolic processes including photosynthesis and respiration. ROS are highly reactive and toxic and therefore can lead to the oxidative destruction of the cell. Consequently, the evolution of all aerobic organisms has been dependent on the development of an efficient ROS-scavenging mechanism (Mittler et al., 2002). During salt stress, owing to stomatal closure, carbon dioxide supply is limited. On the other hand, light supply is unaffected, and the excess energy is readily to

transfer to reactive molecules such as singlet oxygen ($^1\text{O}_2$) which is produced in chloroplasts at high light intensities (Greene, 2002). Simultaneously, the hyperreduction of the photosynthetic electron carrier favours the direct reduction of O_2 by photosystem I, and the subsequent production of superoxide, H_2O_2 and the hydroxyl radical (Foyer and Noctor, 2003). Salt stress can thus alter the metabolic balance of cells resulting in increased ROS production. Since JWS27 cDNA identity with a tobacco hydrogen peroxide-induced cDNA, it is possible that its induction relates to salt stress-induced oxidative stress.

3 ISOLATION AND CHARACTERIZATION OF FULL-LENGTH cDNA CLONES CORRESPONDING TO JWS19 AND JWS27

3.1 Introduction

Changes in gene expression as reflected in altered mRNA profiles were demonstrated in salinity-stressed tomato roots using mRNA differential display (Chapter II). As a result, a number of salt responsive partial cDNAs, JWS19, JWS20, and JWS27 were identified and isolated. This chapter describes the isolation and characterization of full-length cDNAs corresponding to JWS19, and JWS27. It also describes further investigation into the regulation of gene expression by ABA. Isolating full-length cDNAs that corresponds to JWS19 and JWS27 allows us to gain a better understanding of their encoded gene products and their potential function during salinity stress.

The objectives of this chapter are:

1. To isolate full-length cDNAs by screening a cDNA library using the JWS19 and JWS27 partial cDNAs as probes.
2. To determine the nucleotide and predicted amino acid sequences of the positive clones identified via the cDNA library screening in order to gain information about their encoded polypeptides.
3. To determine the role of ABA in regulating the expression of genes corresponding to JWS19 and JWS27 in salt-stressed roots.

3.2 Methods

3.2.1 Materials

All plants were grown as described in section 2.2.1.

3.2.2 Experimental Treatment

Both salt and ABA treatments were performed as described in section 2.2.2. Fluridone (FLU) treatments were applied by exposing plants to 50 μ M FLU (SePRO Corporation, Carmel, IN) in 2/3 MS nutrient solution for 24h prior to exposure to 2/3 MS nutrient solution containing 170 mM NaCl for varying periods of time ranging from 0 to 24h in the absence of FLU. Control plants were maintained in 2/3 MS solution containing 50 μ M FLU for 24h before transfer to 2/3 MS solution lacking FLU for 0h (conducted by soaking the plants briefly in fresh 2/3 MS solution) and 24h. Following each treatment, roots were harvested and frozen in liquid nitrogen before storing at -80°C until needed. All treatments were independently performed for three times.

3.2.3 Screening a Salt-treated Root cDNA Library

A tomato root cDNA library constructed by Dr. Jun-Zhi Wei from poly(A)⁺RNA isolated from six-week-old tomato roots exposed to 170 mM NaCl for 2h (Stratagene, ZAP-cDNA Library Construction Kit, La Jolla, CA, USA) was screened. For primary screening, the amplified cDNA library was plated at a density of 50,000 plaques per 150 mm NZY (8 mM NaCl, 8 mM MgSO₄·7H₂O, 0.5% yeast extract, 1% w/v casein hydrolysate, 1.5% agar, pH 7.5) agar plate on a XL1-Blue MRF['] bacterial lawn (total 600,000 pfu were used). Phage from the

plaques was transferred to nitrocellulose membranes (Schleider and Schuell Inc., Roche Diagnostic, Basel, Switzerland) by placing the membrane onto each agar plate for 2 min. For orientation, these membranes were marked with an 18 gauge needle in an asymmetrical pattern. Phage DNA was released from the phage head and denatured by placing the nitrocellulose membranes on saturated Whatman 3MM paper containing denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 min, followed by placing them on neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min. The membranes were then transferred for 30 sec to the surface of Whatman 3 MM™ paper saturated with 0.2 M Tris-HCl (pH 7.5) and 2X SSC. Finally, the membranes were blotted briefly on Whatman 3 MM paper and the DNA was crosslinked to the membranes using the autocrosslink setting on the UV Stratalinker™ 2400 (Stratagene) for approximately 30 sec followed by baking at 80°C for 1h. Prior to hybridization, the membranes were soaked in 2X SSC for 5 min and incubated in pre-washing solution (5X SSC, 0.5% SDS, 1mM EDTA pH 8.0) for 1h at 42°C. After 1h, the washing solution was replaced with hybridization solution (6X SSPE - 0.15 M NaCl, 0.010 M NaH₂PO₄, 0.001 M EDTA, pH 7.4- and 0.05X BLOTTO [1X BLOTTO: 5% w/v nonfat dried milk dissolved in water]) and incubated at 65°C for 1.5h. The membranes were hybridized at 65°C overnight in 15 ml hybridization solution containing six pooled probes: JWS15, JWS19, JWS21, JWS23, JWS26 and JWS27, each with a total activity of 1.5×10^7 cpm, respectively. Following hybridization, the membranes were washed three times, each for 5 min in 500 ml 2X SSC, 0.1% SDS at room temperature. Subsequently, the membranes were

washed two times at 65°C for 1 h in 500 ml 1X SSC, 0.1% SDS. After the final wash, the membranes were covered with saran wrap and exposed to autoradiography film for 3 days at 4°C. Plaques that gave dark signals on the autoradiography film were picked from the agar plates and transferred to a 15 ml Falcon tube containing 1 ml SM buffer (10 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) and one drop of chloroform, gently mixed and left sit at room temperature for 1-2h. This phage stock was stored at 4°C until further use.

Prior to the secondary screen, the titre of the positive phage from the primary screening was determined by plating 1000- and 100-fold dilutions. The 100-fold dilution gave approximately 100-200 plaques per plate, and this dilution was used for the secondary screen. Duplicate plaque lifts were performed from the same plate, and the first set of membranes was hybridized with a pooled JWS21 and JWS26 probe, whereas the second set of membranes was hybridized with a pooled JWS19 and JWS27 probe.

Third round screening was performed by plating a 10,000 fold dilution of the positive phage recovered from the second round screening using the pooled JWS19 and JWS27 probe. This dilution gave approximately 20-100 pfu/ 90 mm NZY plate. Duplicate membrane lifts were performed and one set of membranes was hybridized with JWS19, whereas the second set was hybridized with JWS-27. A negative control derived from a non-hybridizing plaque was included for this screening. Fourth round screening was performed as described for the

tertiary screening; positives corresponding to either JWS19 or JWS27 were plated to an approximate density of 20-50 pfu/90 mm NZY plate to allow isolation of a single plaque.

3.2.4 Preparation of Radiolabelled DNA Probes

Radiolabelled partial cDNA probes were prepared as described in Chapter II section 2.2.8.

3.2.5 *In vivo* Excision of pBluescript Phagemid

In vivo excision of the lambda phage from positive clones was performed using the *in vivo* excision protocol as described by the manufacturer (Stratagene). A 37°C overnight culture of XL1-Blue MRF' cells was centrifuged for 10 min at 3000 rpm in a microfuge (Sorvall® MC 12V) and resuspended in 10 mM MgSO₄ to an OD₆₀₀ of 1.0. The following components were then combined in a 15 ml Falcon Tube: 200 µl XL1-Blue MRF' cells, 250 µl phage stock derived from the positive plaque identified after the final screening step, and 1 µl of ExAssist helper phage (with a titre greater than 1x10⁶ pfu/µl). The mixture was incubated at 37°C for 15 min, and three ml of Luria-Bertani broth (LB) (17 mM NaCl, 1% tryptone, 0.5% yeast extract, pH 7.0) was added and the incubation was continued at 37°C for 3 h with shaking. After the 3 h incubation, the mixture was heated at 70°C for 20 min to inactivate the parent lambda and to kill the bacteria. The tube was then centrifuged for 5 min at 1000g and the supernatant was decanted into a sterile tube and stored at 4°C until further use. To recover the excised phagemid from this stock, 200 µl SOLR™ cells (resuspended in 10

mM MgSO₄ to an OD₆₀₀ =1.0) and 100 µl of a 10,000-fold diluted phagemid stock containing the excised pBluescript phagemids were combined, gently mixed and incubated for 15 min at 37°C. Two hundred µl of this mixture was plated onto LB plates containing ampicillin (50µg/ml) and incubated at 37°C overnight. Ten white colonies of phagemids containing potential cDNAs corresponding to JWS19 and JWS27 were selected and cultured in 5 ml LB broth containing ampicillin (50 µg/ml), at 37°C, overnight. Plasmid minipreparations were performed using the alkaline lysis method (Sambrook et al., 1989). The cultured cells were centrifuged for 30 sec at 12,000 rpm, and the pellet was resuspended in 100 µl cold solution I (50 mM glucose, 25 mM TrisHCl pH 8.0, 10 mM EDTA pH 8.0). Subsequently, 200 µl ice-cold solution II (0.2 M NaOH, 1% SDS) was added, the tube was inverted five times and kept on ice for 5 min until the solution turned clear. One-hundred fifty µl ice cold solution III (3M KoA, pH 6.0) was added and the tube was vortexed briefly at low speed, followed by an incubation on ice for 5 min. The sample was then centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was transferred to a new tube. Two volumes 99% ethanol was added, vortexed and incubated for 2 min at room temperature. This was followed by a five min centrifugation at maximum speed at 4°C. The supernatant was decanted, and the pellet was rinsed with ice-cold 70% ethanol and centrifuged as before for 5 min. Following centrifugation, the pellet was air-dried and resuspended in 100 µl TE (pH 8.0) containing DNase-free pancreatic RNase (20 µg/ml).

In order to release the insert, recovered plasmids were digested with both *EcoRI* and *XhoI*, and the size of the inserts was determined by running the digested products on a 1.2% agarose gel in 1X TBE buffer (Sambrook et al., 1989). Confirmation of the plasmids containing cDNA inserts was performed with PCR using the same primer combinations used to generate the original partial cDNA. Each PCR reaction contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 μM random primer and 0.5 μM anchor primer, 2.5U Taq DNA polymerase (Gibco BRL® Life Technologies) and template DNA (10 ng). PCR was performed in a Mini Cycler (MiniCycler™, MJ Research, Watertown, Massachusetts, USA) for 36 cycles of 94°C for 30 s, 43°C for 2 min and 72°C for 30 s; the final cycle was followed by a 10 min elongation at 72°C.

Further confirmation was also performed with southern blot hybridization analyses. Selected clones containing the largest insert size were digested with *EcoRI* and *XhoI* or subjected to PCR as described above, except the primers used were the T3 and T7 universal primers and the annealing temperature was changed to 55°C. The PCR amplicons of the selected clones together with their corresponding partial cDNA were separated on a 1.2% agarose gel in 1X TBE buffer at 60V for 3h. Following electrophoresis, the DNA in the gel was denatured in 1.5 M NaCl, 0.5 N NaOH for 45 min, followed by a rinse in sterile deionized water. DNA was neutralized twice in 1 M Tris-HCl (pH 7.4), 1.5 M NaCl each for 30 min and 15 min, respectively. DNA was capillary transferred (Sambrook et al., 1989) onto positively charged nylon membrane. DNA was fixed to the membrane by exposing to the UV light using the auto crosslink setting on the UV

Stratalinker™ 2400 (Stratagene) for 30 seconds followed by baking for 1h at 80°C. Prior to hybridization, the membrane was wet in 5X SSC for 5 min and hybridized with the respective partial cDNA probe. Both prehybridization and hybridization for the southern blot analyses was conducted according to that described in the library screening (see section 3.2.1).

3.2.6 Determination of the Nucleotide Sequence of JWL19 and JWL27

Plasmid minipreparations of selected clones were performed using the Qiagen miniprep (Qiagen, Mississauga, Canada) kit following the manufacturer's instruction. Using the T3 and T7 universal primers, the nucleotide sequence of the cloned cDNAs was determined with an ABI 311 automatic sequencer (Perkin Elmer, Foster City, CA, USA) at the NAPS unit (Biotechnology Laboratory, University of British Columbia, BC, Canada). Gene specific primers were designed using the computer software OLIGO™ Version 4.0 (National Biosciences Plymouth, MN, USA), and were PCR-tested with the respective positive clones prior to use in automatic sequencing.

3.2.7 Nucleotide and Deduced Amino Acid Sequence Analyses

Similarity searches were conducted as described in section 2.2.7. Hydrophobicity plots (Kyte and Doolittle, 1982) were generated using the hydrophobicity program available at <http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html>. Cleavage sites and signal peptide predictions for signal peptidase I were performed based on Signal IP (Signal IP3.0) prediction (<http://www.cbs.dtu.dk/services/SignalP>). The transmembrane prediction

(Hoffman and Stoffel, 1993) was performed using <http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html>). Analyses of Arabidopsis microarray data were conducted using the GENEVESTIGATOR (<https://www.genevestigator.ethz.ch>) toolbox.

3.2.8 Northern Hybridization Analyses

Northern hybridization analyses were performed as described in section 2.2.9. To assess RNA loading, a tomato 18S rRNA clone (made by Adam Foster) was used as a constitutive probe. The insert of the 18S rRNA clone was amplified using the T3 (5' AAT TAA CCC TCA CTA AAG GG 3') and T7 (5' TAA TAC GAC TCA CTA TAG GG 3') universal primers. PCR conditions were similar to that described in section 2.2.6, with the exception of the annealing temperature, which was changed to 55°C. The PCR product was size separated at 80V for 1.5h in a 1.2% agarose gel in 1X TBE. The band of interest was excised from the gel using a gel extraction kit (Qiagen, Mississauga, Canada) according to the manufacturer's instruction. For quantification, an aliquot of the excised product was size separated in a 1.2% agarose gel in 1X TBE at 80V for 1.5h together with a DNA mass ladder.

Band intensity from autoradiography films was determined with the Scion Image Beta software version 1.62c using macrofunction gel plot 2 (Scion Corporation, Frederick, MD, USA) and subsequently normalized by dividing the hybridization signal obtained for a salt responsive cDNA probe by that obtained for the 18S rRNA probe. An appropriate exposure was carefully chosen for blots that were analyzed by Scion image in order to avoid saturation of bands.

3.3 Results

3.3.1 Isolation of Positive cDNA Clones Corresponding to the JWS19 and JWS27 Partial cDNAs

As part of an effort to isolate full-length cDNAs corresponding to several salt-responsive cDNAs, the primary screening of the 2h salt-treated tomato root cDNA library was initiated using a pooled probe derived from six partial salt-responsive cDNAs (JWS15, JWS19, JWS21, JWS23, JWS26 and JWS27), which included several that had been isolated by Dr. Jun-Zhi Wei (JWS15, JWS21, JWS23 and JWS26). Eighty nine positives plaques were identified and picked. Of these, twelve plaques corresponding to the strongest signals were chosen and subjected to a second round of screening using a pooled JWS19 and JWS27 probe. Following the second round of screening, twelve positives plaques were isolated and subjected to a third round. Duplicate lifts were performed, and one set of membranes was hybridized against JWS19 whereas the other set was hybridized against JWS27. After the fourth round screen, ten well-isolated plaques were picked for JWS19 and JWS27, respectively and subjected to *in vivo* excision. The positive cDNA clones corresponding to JWS19 were named as JWL19-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, whereas those that correspond to JWS27 were named as JWL27-1, -2, -3, -4, -5, -6, -7, -8, -9, -10. To release the inserts from putative JWL19 cDNA clones, a double restriction enzyme digestion was performed using *EcoRI* and *XhoI*. Following digestion, all ten clones contained an insert of similar size of approximately 1.8 kb (data not shown), which corresponds to the predicted size of the smaller transcript that hybridized to JWS19 (Chapter II, Fig. 2-9). Two positive clones were chosen for further

analysis: JWL19-3 and JWL19-9. Following digestion with *EcoRI* and *XhoI*, both JWL19-3 and JWL19-9 gave an insert of approximately 1.8 kb (Figure 3-1). To confirm their identity, both JWL 19-3 and JWL 19-9 were subjected to PCR amplification using the same primer combination originally used for DD-PCR. PCR amplification of both JWL19-3 and JWL19-9 resulted in an amplicon of approximately 340 bp, which corresponds to the size of the JWS19 partial cDNA (data not shown). In addition to PCR confirmation, southern blot hybridization analysis was performed (Figure 3-2). The JWS19 partial cDNA insert, undigested and *EcoRI/XhoI* digested JWL19-3 and JWL19-9 were separated in a 1.2% agarose gel (Figure 3-2A) and a southern blot was generated and probed with JWS19 (Figure 3-2B). The JWS19 probe strongly hybridized to the insert of both JWL19-3 (Figure 3-2B, lane 3) and JWL19-9 (Figure 3-2B, lane 7), JWS19, and to the the undigested JWL19-3 (Figure 3-2B, lane 4) and JWL19-9 plasmid DNA (Figure 3-2B, lane 8). This result indicates that both JWL19-3 and JWL19-9 contain an insert that is related to JWS19. To gain more information about the putative JWL-19 clone, JWL 19-9 was selected for nucleotide sequence analyses.

Ten positive cDNA clones (JWL27-1 to JWL27-10) corresponding to JWS27 were identified. To release the inserts, all ten clones were digested with both *EcoRI* and *XhoI*. Following digestion, all ten clones gave multiple fragments that combined to an insert size of 2.8 kb (data not shown). Two positive clones were chosen for further analyses: JWL 27-7 and JWL27-8. When digested with

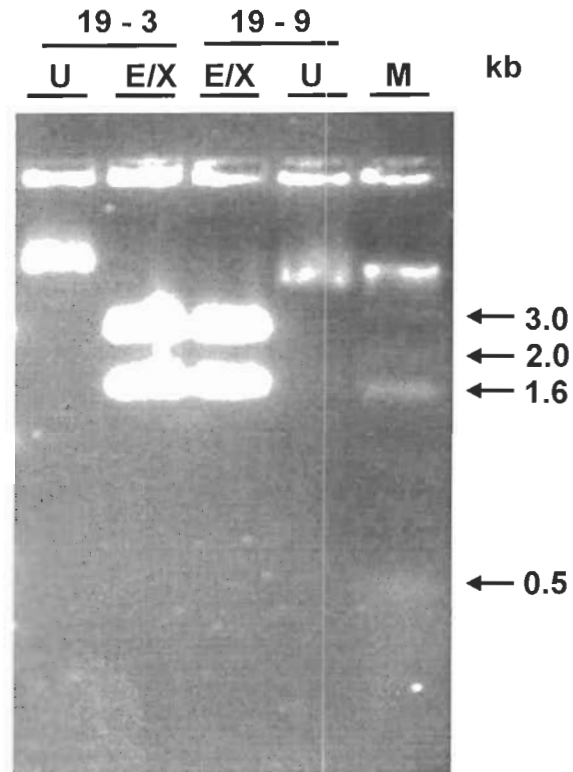


Figure 3-1 Restriction enzyme digestion of putative JW19 clones.

Plasmid DNA was digested with both *EcoRI* and *XhoI* (E/X) to release the insert, and separated on a 1.2% agarose gel in 1X TBE buffer. U represents undigested plasmid DNA. Marker shown is a DNA 1 kb ladder.

Figure 3-2 Southern blot hybridization analyses of JWL19-3 and JWL19-9.

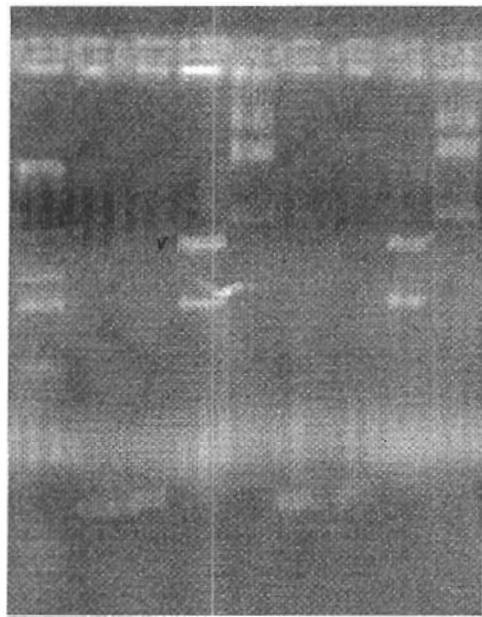
A. The JWS19 cDNA (lanes 1, 2, 5, 6), *EcoRI* and *XhoI* digested JWL19-3 (lane 3) and JWL19-9 (lane 7), undigested JWL19-3 (lane 4) and undigested JWL 19-9 (lane 8) were separated in a 1.2% agarose gel in 1X TBE buffer. M represents a DNA 1 kb ladder.

B. Southern blot generated from the gel shown in (A) and probed with the JWS19 cDNA.

A.

JWL19-3 JWL19-9
M 1 2 3 4 5 6 7 8

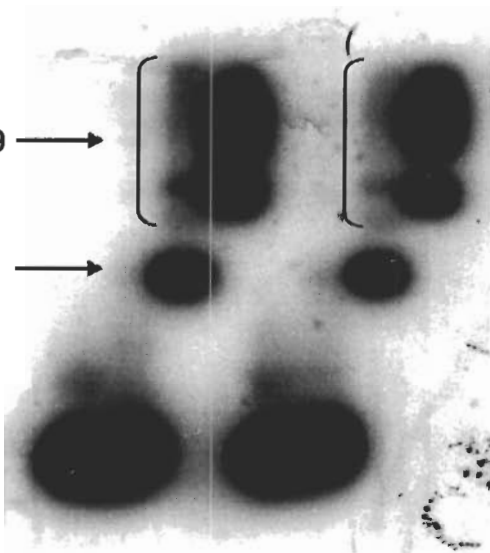
2.0 →
1.6 →



B.

JWL19-3 JWL19-9
1 2 3 4 5 6 7 8

Undigested JWL19 →
JWL19 insert →
JWS19 →



EcoRI alone, both JWL 27-7 and JWL 27-8 gave two fragments of approximate size 1.4 and 0.6 kb, respectively (Figure 3-3). Following digestion with both *EcoRI* and *XhoI*, both clones gave four fragments, each with an approximate size of 1.4, 0.6, 0.5, and 0.3 kb (Figure 3-3). In order to confirm the identity of JWL27-7 and JWL27-8, southern blot hybridization analysis was performed. Undigested and *EcoRI/XhoI* digested JWL27-7 and JWL27-8 DNA, and the JWS27 cDNA were separated in a 1.2% agarose 1X TBE gel (Figure 3-4A). A southern blot was generated and probed with JWS27 (Figure 3-4B). JWS27 strongly hybridized to the 0.6 kb fragment generated by digestion with *EcoRI* and *XhoI* of JWL27-7 (Figure 3-4B, lane 2) and JWL27-8 clones (Figure 3-4B lane 5), indicating that both cDNA clones contain sequences that are related to JWS27. The JWS27 probe also hybridized to JWS27 DNA and to the undigested DNA of JWL27-7 (Figure 3-4B, lane 3) and JWL27-8 (Figure 3-4B, lane 6). To gain more information about JWL27, JWL27-7 was chosen for nucleotide sequence analysis.

3.3.2 Nucleotide Sequence Analyses of JWL19

The JWL19-9 clone is renamed hereafter as JWL19 and was sequenced in the forward and reverse directions using the T3 and T7 universal primers. The nucleotide sequences obtained were submitted to the NCBI server to search against the EST database using the BLASTN program (Altschul, 1997). The BLASTN search with the nucleotide sequence obtained with the T7 primer showed that it contained the JWS19 partial cDNA (score 698 bits; E value:0). In addition, the nucleotide sequence obtained with the T7 primer revealed the

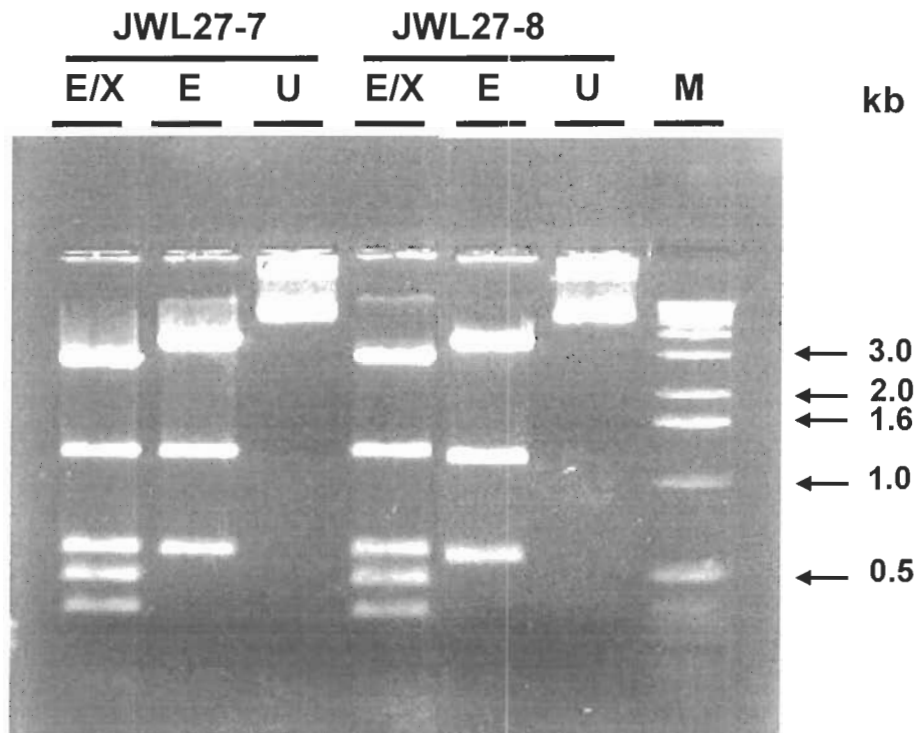


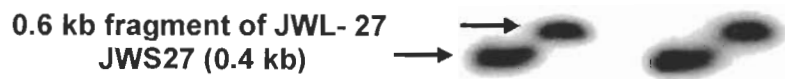
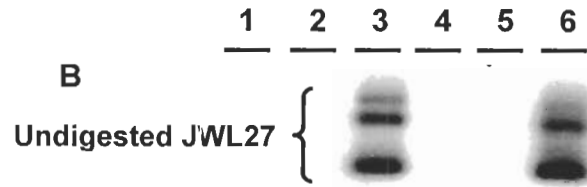
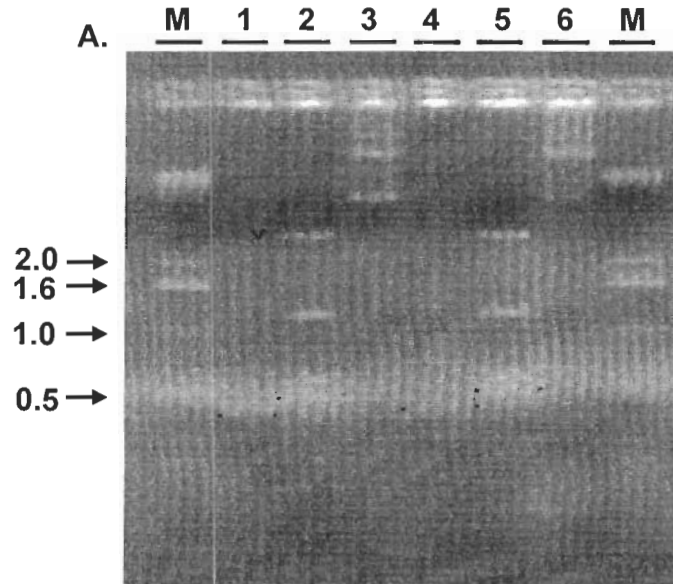
Figure 3-3 Restriction enzyme digestion products of putative JWL27 clones.

JWL27-7 and JWL27-8 plasmid DNA was digested with *EcoRI* (E) alone or together with *XhoI* (E/X) to drop the insert. U represents undigested plasmid DNA. All samples were size separated on a 1.2% agarose gel in 1X TBE buffer. Marker shown is a DNA 1 kb ladder.

Figure 3-4 Southern blot analysis of JWL27 cDNA.

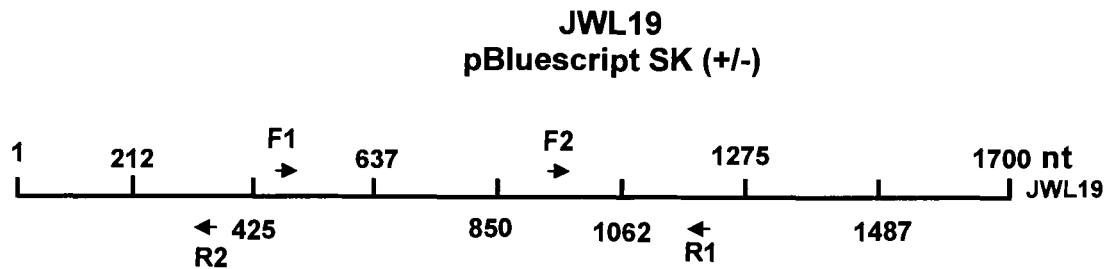
A. The JWS27 cDNA (lanes 1 and 4), *EcoRI* and *XhoI* digested JWL27-7 (lane 2) and JWL27-8 (lane 5), undigested JWL27-7 (lane 3) and JWL27-8 (lane 6) plasmids were separated on a 1.2% agarose gel in 1X TBE. M represents a DNA 1 kb ladder.

B. Southern blot generated from the gel shown in (A) and probed with the JWS27 cDNA.



presence of the poly A tract that was also present within the JWS19 partial cDNA nucleotide sequence (Chapter II, Figure 2-6). In order to obtain the entire nucleotide sequence of the JWL19 clone, nested forward JWS19F1 (5' CAC GAT TTG ACA GAG AAC 3') and reverse primers JWS19R1 (5' CTC TCT ACA TCC TTC ACC 3') were designed. Following sequencing with JWS19F1 and JWS19R1, two more nested primers (JWS19F2 and JWS19R2) were designed in order to completely sequence the JWL19 insert (Figure 3-5). The nucleotide sequences obtained using all primers were used to generate a contig for JWL19.

The complete nucleotide and deduced amino acid sequence of JWL19 is presented in Figure 3-6. JWL19 has 140 nucleotides in the 5' untranslated region (UTR) and 266 nucleotides in the 3' UTR including the stop codon. The JWL19 open reading frame starts at nucleotide 140 and ends at nucleotide 1442. The JWS19 partial cDNA corresponds to nucleotides 1392-1708 of JWL19 (Figure 3-6). The JWL19 nucleotide sequence was submitted for a BLASTN search against both the EST and nr databases. Against the EST database, JWL19 shares high similarities to various ESTs derived from cDNA libraries made using RNA isolated from tomato shoots, callus, ovary, green mature fruit, as well as to JWS19 (Table 3-1). A BLASTN search using the *Lycopersicon esculentum* database of The Institute of Genomic Research (TIGR) (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato) showed that the nucleotide sequence of JWL19 corresponds to TC162697 (score bits: 8114, E value: 0), which represents a tomato auxin-regulated protein (LE-ARP). The sequence of TC162697 was built from a number of ESTs derived from tomato callus, fruit,



Primer sequences:

Forward primers for sequencing (+) strand of JWL19:

JWS19F1: 5' CAC GAT TTG ACA GAG AAC 3' (442 nt)

JWS19F2: 5' CCC GAG AAG AAG AGA CAG 3' (927 nt)

Reverse primers for sequencing (-) strand of JWL19:

JWS19R1: 5' CTC TCT ACA TCC TTC ACC 3' (1183 nt)

JWS19R2: 5' CAC GGG AGG AAA GAG GCA 3' (327 nt)

Figure 3-5 Sequencing strategy of JWL19

Nested forward and reverse primers used to sequence the JWL19 clone are indicated within the JWL19 sequencing map. Annealing site of each primer is indicated within parenthesis following the primer sequences.

Figure 3-6 JWL19 nucleotide and deduced amino acid sequence

The italicized bold sequence designates the location of the JWS19 partial cDNA within JWL19. The deduced single-letter amino acid sequence is given below the center nucleotide of each codon. A putative Kozak sequence (RCCATGR, in which R is a purine (A or G), Kozak, M., 1986) is both italicized and bolded. The initiating ATG is underlined, and the stop codon is indicated in bold and underlined.

1GC TGG AGC TCC CCG CGG TGG CGG CCG CTC TAG AAC TAG TGG ATC CCC CGG GCT
54 GCA GGA ATT CGG CAC GAG GCT TTC AAA TTT CAA ATA TCG AGG TTC CAT TTC
105 TTC TAA CTT TCT CAA ATT AAG CGG TTG GGA AGA **ACA ATG** TCG AGG ACG ACG
M S R T T
156 GAG CTC CAA ATG CAC AAG AAA TGG AAA GGT AGA GAA ACA AGC CCT GAA CGG
E L Q M H K K W K G R E T S P E R
207 ACC AAA GTT TGG ACT GAA CCT CCT AAC CAT AAG CTC AGC AAA GTA CCC GTT
T K V W T E P P N H K L S K V P V
258 GTT TAC TAT CTC TCC AGA AAT GGC CAA CTT GAG CAT CCT CAT TTC ATG GAA
V Y Y L S R N G Q L E H P H F M E
309 GTG CCT CTT TCC TCC CGT GAT GGT CTG TAT CTC AGA GAT GTG ATC AAC CGC
V P L S S R D G L Y L R D V I N R
360 TTG AAT TGT CTT CGT GGA AAA GGG ATG GCC TCT ATG TAC TCC TGG TCT GCT
L N C L R G K G M A S M Y S W S A
411 AAA AGA AGC TAT AGG AAT GGA TTC GTG TGG CAC GAT TTG ACA GAG AAC GAT
K R S Y R N G F V W H D L T E N D
462 TTT ATA TAC CCA GCA CAT GGT CAA GAG TAT GTT CTG AAA GGA TCA GAG CTT
F I Y P A H G Q E Y V L K G S E L
513 CTC GAT AGC GCA TTG CCT TCA CAA CCC GAT GAA ATC GCT TGT TCT AAT TCT
L D S A L P S Q P D E I A C S N S
564 AGA AAT ACG GTG CCG GAA AAA CAG AAA TTG AGT GGA AAG ATC GCC GAG TTT
R N T V P E K Q K L S G K I A E F
615 CCT GCA GTG GCA AGG CGC CGA AAT CAA TCC TGG AGT TCC TCC GAT TTC CAC
P A V A R R R N Q S W S S S D F H
666 GAA TAC CTA GTT TAC AAG GCC GAA TCA CCC GGC GAA ATA CTC GGA AGA ATT
E Y L V Y K A E S P G E I L G R I
717 GGA GCC GAC GCT TCA ACT CAA ACA GAG GAC AGA CGA CGC CGG CGA GGA GGA
G A D A S T Q T E D R R R R R G G
768 ATG CGA ATC GTT GAA GAA GAA GAA GAG TTG AGT GAA AAC CGG ATT ATT GAA
M R I V E E E E E L S E N R I I E
819 TCT GAC TGT AAA GAA CCG GAC GAG GTT GAA CAT AGT CCG AAC CAG TCA ACG
S D C K E P D E V E H S P N Q S T
870 GAG CTG AAC AGA GGT GAA ATT TCA CCA CCA CTA TCC GAT TCA AGC TCT GAA
E L N R G E I S P P L S D S S S E
921 ACG CTA GAA ACG TTG ATG AAG GCG GAC GGA AAA TTA ATC CTA CGG CCA GAT
T L E T L M K A D G K L I L R P D
972 ACA ATC AGC GAA GAT CCA ACG GCT AAT ACT CAT TCA AGC GGA AAG AGC AAA
T I S E D P T A N T H S S G K S K
1023 GCA GCT TCC GTT TTG ATG CAA TTA CTC TAT TGC GGT TCA ATG TCC TTC AAG
A A S V L M Q L L Y C G S M S F K
1074 CAA TGC GGA CCC GGT TAT GGG AAG GAA AAT GGA TTT TCC TTG ATT TCA CAG
Q C G P G Y G K E N G F S L I S Q

1125 TAT AAA AAC CGT CTG TCC CAT GGC AGG TGT ACC AAT CAA GCG GTG AAG GAT
Y K N R L S H G R C T N Q A V K D
1176 GTA GAG AGT CCA ATT GTG GAA TAT CAC GGA AGT GAA GAG AGA ATT AAG CTG
V E S P I V E Y H G S E E R I K L
1227 GAA GAT AAG GAA TAC TTC AGT GGG AGT TTA ATA GAG ATG AAG AAG AAG GAG
E D K E Y F S G S L I E M K K K E
1278 AAA TTT CCA GCT CTA AAG AGG TCT TCT TCA TAC AAC GTA GAA CGG AGT ACA
K F P A L K R S S S Y N V E R S T
1329 AAA TTG GAG CTG ACT GAG AAG CAA GAA GAA GGG AAA ACA AAA TGT TAT CTG
K L E L T E K Q E E G K T K C Y L
1380 CGA AAG CAA AAG **AAC CAA TCG ACC CGA GAA GAA GGA GAC AGT GAC TTG TCT**
R K Q K N Q S T R E E G D S D L S
1431 **AAT GCC CTG CAG TAG CAT TTG CTA TTA TTT CTA GTA TTA CTA GCT AGT TAT**
N A L Q *
1482 **CAA AAT GGT AGC AAG ATG TTA TCA AAA TGG TAG CAA GAT GAG TAG ATG ATA**
1533 **GTG GTT AAG CAG TAG ATT AGG GAA ATG GTA GGA AAA ATT GTG GAA ACA ATT**
1584 **GCT CCT GAT CAT GAT CTC AGT TGT AGT TAT TGT TTG TGT GGA TTT CGC AGT**
1635 **AAA AAT AAC TAA ATT ATC TGT ATC AAG TGT TTG CAT CAA AGA AAT GAT ATG**
1686 **CGA GTG CGA CAT ACT ATT GAG CCAAAAAAAAAA**

Table 3-1 BLASTN search results for JWL19 (February, 2005) against the EST data base.

BLAST result*	GenBank Accession Number	Bit Score	E value
cDNA library			
Tomato shoot/meristem cDNA	BG124116	1455	0
Tomato callus cDNA	AW035057	1152	0
Tomato breaker fruit cDNA	BM536435	1110	0
Tomato callus cDNA	AW032594	1102	0
Tomato callus cDNA	AW216980	1096	0
Tomato callus cDNA	AI896653	1094	0
Tomato callus cDNA	AW031333	1057	0
Tomato callus cDNA	AW035361	1043	0
Tomato callus cDNA	BI921286	1023	0
Tomato ovary cDNA	AI485573	1001	0
Tomato callus cDNA	AW216946	991	0
Tomato ovary cDNA	AI487302	989	0
Tomato breaker fruit cDNA	BF113392	985	0

Table 3-1. continued.

BLAST result*	GenBank Accession Number	Bit Score	E value
cDNA library			
Tomato ovary cDNA	AI899400	954	0
Tomato green fruit cDNA	AW933237	950	0
Tomato ovary cDNA	AI488576	944	0
Tomato ovary cDNA	AI771852	906	0
Tomato ovary cDNA	AI486194	906	0
Tomato ovary cDNA	AI771919	902	0
Tomato ovary cDNA	AI487359	898	0
Tomato susceptible cDNA	AI899669	823	0
Tomato JWS-19 salt-stressed roots cDNA	AW062237	617	e-173

*only ESTs with an E value equal to 0 are presented, with the exception of JWS19.

shoot, ovary and root (the JWS19 partial cDNA). An amino acid sequence alignment of the deduced JWL19 protein and LE-ARP is presented in Figure 3-7. tBLASTX searches against the non-redundant database showed that the JWL19 protein shares high similarity with a variety of plant proteins (Table 3-2). JWL19 protein contains a Domain of Unknown Function (DUF966) (score bits: 410, E value: 1e-115) present in a family of plant proteins of unknown function (Marchler-Bauer et al., 2005). Proteins containing DUF966 includes the *A. thaliana* protein encoded by At5g10150 (Gen Bank: AAT70472), *A. thaliana* expressed protein At3g46110 (NP974389), *A. thaliana* protein encoded by At5g59790 (Gen Bank: AAP37875), *A. thaliana* hypothetical protein encoded by At2g28150 (Gen Bank: AAC98451). *O. sativa* protein (Gen Bank: NP915409), *O. sativa* protein (Gen Bank: NP914381), and *L. esculentum* auxin-regulated protein (Gen Bank: AAL08561). The alignment of the JWL19 protein with other plant proteins containing DUF966 is presented in Figure 3-8.

To gain further information about JWL19, a search using GENEVESTIGATOR (<http://www.genevestigator.ethz.ch>), a gene expression database for *Arabidopsis thaliana* was performed. GENEVESTIGATOR contains a data set for *A. thaliana* that was generated from a common microarray hybridization platform (Affymetrix chip), which provides a standardized system with a high degree of reproducibility (Zimmermann, 2004). Among all the *A. thaliana* clones that share high similarity with the JWL19 protein, *At3g46110* was chosen to be a target gene against the GENEVESTIGATOR database. A search using the GENEVESTIGATOR digital northern tool, with *At3g46110* as the target

JWL-19	MSRTTELQMHKKWKGRETS PERTKVWTEPPNHKLSKVPVVYYLSRNGQLEHPHFMEVPLS	60
LE-ARP	MSRTTELQMHKKWKGRETS PERTKVWTEPPNHKLSKVPVVYYLSRNGQLEHPHFMEVPLS	60
JWL-19	SRDGLYLRDV INRLNCLRGKGMASMYSWSAKRSYRNGFVWHDLTENDFIYPAHGQEYVLK	120
LE-ARP	SRDGLYLRDV INRLNCLRGKGMASMYSWSAKRSYRNGFVWHDLTENDFIYPAHGQEYVLK	120
JWL-19	GSELLDSALPSQPDEIACSNRNTVPEKQKLSGKIAEFPAVARRRNQSWSSSDFHEYLVY	180
LE-ARP	GSELLDSALPSQPDEIACSNRNTVPEKQKLS-EDREFPAVARRRNQSWSSSDFHEYLVY	179
JWL-19	KAESPGEILGRIGADASTQTEDRRRRRGGMRIVEEEEEELSENRIIESDCKEPDEVEHSPN	240
LE-ARP	KAESPGEILGRIGADASTQTEDRRRRRGGMRIVEEEEEELSENRIIESDCKEPDEVEHSPN	239
JWL-19	QSTELNRGEISPPLSDSSSETLETLMKADGKLILRPDTISEDPTANTHSSGKSKAASVLM	300
LE-ARP	QSTELNRGEISPPLSDSSSETLETLMKADGKLILRPDTISEDPTANTHSSGKSKAASVLM	299
JWL-19	QLLYCGSMSFKQCGPGYGKENGFSLSISQYKNRLSHGRCTNQAVKDVESPIVEYHGSEERI	360
LE-ARP	QLLYCGSMSFKQCGPGYGKENGFSLSISQYKNRLSHGRCTNQAVKDVESPIVEYHGSEERI	359
JWL-19	KLEDKEYFSGSLIEMKKKEKFPALKRSSSYNVERSTKLELTEKQEEGKTKCYLRKQKNQS	420
LE-ARP	KLEDKEYFSGSLIEMKKKEKFPALKRSSSYNVERSTKLELTEKQEEGKTKCYLRKQKNQS	419
JWL-19	TREEGSDLSNALQ	434
LE-ARP	TREEGSDLSNALQ	433

Figure 3-7 Amino acid sequence alignment of the JWL19 deduced amino acid sequence and the *Lycopersicon esculentum* auxin regulated protein (LE-ARP).

Amino acids that are identical are shaded in black, unshaded residues represented non-identical amino acids. A dash was introduced to maximize the alignment.

Table 3-2 TBLASTX search results for JWL19 (February, 2005) against the Non-redundant database.

tBLASTX result	GenBank Accession Number	Bit Score	E value
<i>Lycopersicon esculentum</i> auxin-regulated protein	AAL08561	776	0
<i>Arabidopsis thaliana</i> unknown protein (At5g59790)	AP37875	341	2e-92
<i>Arabidopsis thaliana</i> expressed protein (At3g46110)	BAC42479	264	3e-69
<i>Arabidopsis thaliana</i> unknown protein (At2g28150)	AAP04115	218	3e-55
<i>Oryza sativa</i> hypothetical protein	NP915409	211	2e-53
<i>Oryza sativa</i> expressed protein	AAT76997	188	2e-46
<i>Oryza sativa</i> putative auxin-regulated protein	XP483788	152	2e-35
<i>Oryza sativa</i> hypothetical protein	BAD46604	102	2e-20
<i>Arabidopsis thaliana</i> mRNA (At5g10150)	BT015021	149	4e-36
<i>Arabidopsis thaliana</i> hypothetical protein (At1g05577)	AAV68800	100	6e-20
<i>Oryza sativa</i> hypothetical protein	NP914381	91	6e-17

Figure 3-8 Alignment of the JWL19 protein with plant proteins containing the DUF966.
The consensus sequence within the DUF966 family (JWL19, *L. esculentum* auxin-regulated protein, Gen Bank: AAL08561; *A. thaliana* expressed protein At3g46110, Gen Bank: NP974389; *A. thaliana* protein At5g59790, Gen Bank: AAP37875; *A. thaliana* hypothetical protein, At2g28150, Gen Bank: AAC98451; *O. sativa* proteins, Gen Bank: NP915409 and NP914381; and *A. thaliana* protein, At5g10150, Gen Bank: AAT70472) is indicated on the top of the alignment. The amino acid residues that match the consensus are shaded in black.

Majority MS-RV---RATE---L-PHRSKDGRETSPERAKVQSE-PPLGQVRRKQVPAVYLSR-NGQLERPHMEVTLSSHDG-----LVLRDVIMRLNDLRG

10 20 30 40 50 60 70 80 90 100

Jw119.pro [consensus] 79
 Le-arp.pro [consensus] 79
 AT7646-1.PRO [consensus] 69
 AT5659-1.PRO [consensus] 69
 AT2628-1.PRO [consensus] 76
 OZNP91-1.PRO [consensus] 67
 OZNP91-2.PRO [consensus] 66
 AT5610-1.PRO [consensus] 66

Majority KGMASMYQSSKRSYRN--GFVQHDLSEDDIYPAQG--QKVLK;SELLDSA-----LISMP--LLDTSAFSSSSRVLPEKQK-

110 120 130 140 150 160 170 180 190 200

Jw119.pro [consensus] 151
 Le-arp.pro [consensus] 151
 AT7646-1.PRO [consensus] 165
 AT5659-1.PRO [consensus] 165
 AT2628-1.PRO [consensus] 172
 OZNP91-1.PRO [consensus] 127
 OZNP91-2.PRO [consensus] 160
 AT5610-1.PRO [consensus] 170

Majority SDD-----I---AGVARRRQSSSSD--LSEYKVKYA---ESSAEILGLAADAQTEDRRRRR-G-RIVEEIEE-SEM---D-KSPAS-

210 220 230 240 250 260 270 280 290 300

Jw119.pro [consensus] 225
 Le-arp.pro [consensus] 224
 AT7646-1.PRO [consensus] 225
 AT5659-1.PRO [consensus] 225
 AT2628-1.PRO [consensus] 267
 OZNP91-1.PRO [consensus] 161
 OZNP91-2.PRO [consensus] 220
 AT5610-1.PRO [consensus] 226

Majority E---QSTELSRGEISPPPSDSSP---ETLETLIKADGRLILR-DSITEDRTAATLSSGMRASVVLMLQLISCGMSFKEC--GVLGXKMGFALI

310 320 330 340 350 360 370 380 390 400

Jw119.pro [consensus] 226
 Le-arp.pro [consensus] 225
 AT7646-1.PRO [consensus] 224
 AT5659-1.PRO [consensus] 224
 AT2628-1.PRO [consensus] 265
 OZNP91-1.PRO [consensus] 226
 OZNP91-2.PRO [consensus] 200
 AT5610-1.PRO [consensus] 206

Majority GQXKRLSRGA-----GVNDVESLVSF-S--PG--RIQLDEKEYTSGSLIE-TKQ-----ELVPALKRSSSYNADRSXVPELTKDEEG--KAK--

410 420 430 440 450 460 470 480 490 500

Jw119.pro [consensus] 411
 Le-arp.pro [consensus] 410
 AT7646-1.PRO [consensus] 406
 AT5659-1.PRO [consensus] 406
 AT2628-1.PRO [consensus] 452
 OZNP91-1.PRO [consensus] 202
 OZNP91-2.PRO [consensus] 261
 AT5610-1.PRO [consensus] 266

Majority -----CIPPKKRVKX-----RE---EGDS--DLSMALQ-----

510 520 530 540 550 560 570 580 590

Jw119.pro [consensus] 424
 Le-arp.pro [consensus] 422
 AT7646-1.PRO [consensus] 422
 AT5659-1.PRO [consensus] 422
 AT2628-1.PRO [consensus] 540
 OZNP91-1.PRO [consensus] 292
 OZNP91-2.PRO [consensus] 411
 AT5610-1.PRO [consensus] 414

Decoration 'Decoration #1': Shade (with solid black) residue: that match the Consensus exactly.

gene showed that *At3g46110* is expressed in both roots and shoots in response to various stresses including salt, drought, wounding, heat, ultra violet (UV B) and oxidative stress. *At3g46110* is also expressed in response to ABA, ethylene and *Pseudomonas syringae* treatments, and during programmed cell death. Furthermore, *At3g46110* is expressed in cotyledons, hypocotyl, roots, shoot apex, leaves, stem, cauline leaves and senescing leaves.

3.3.2.1 Analysis of the JWL19 cDNA and Its Deduced Protein

The deduced open reading frame (ORF) of JWL19 encodes a protein of 434 amino acids with a calculated molar mass of 49.4 kDa and an isoelectric point of 7.17. A Kyte and Doolittle hydrophathy profile was generated (Kyte and Doolittle, 1982) and showed that the JWL19 deduced protein is mostly hydrophilic, with the exception of region 295 to 314 (Figure 3-9). The transmembrane (TM) topology (Hoffmann and Stoffel, 1993) prediction suggests that the JWL19 deduced protein has one transmembrane helix (amino acid 295 to 314), which corresponds to the hydrophobic domain predicted by the Kyte and Doolittle hydrophathy profile. The N-terminus of JWL19 deduced protein is predicted to be located in the cytosol (Figure 3-10). Signal IP prediction (Signal IP 3.0, Bendtsen et al., 2004) showed that the JWL19 deduced protein contains no signal peptide.

3.3.3 Nucleotide Sequence Analyses of JWL27

The JWL27-7 clone is renamed hereafter as JWL27. The JWL27 clone contains an insert with an approximate size of 2.8 kb (Figure 3-3), and was

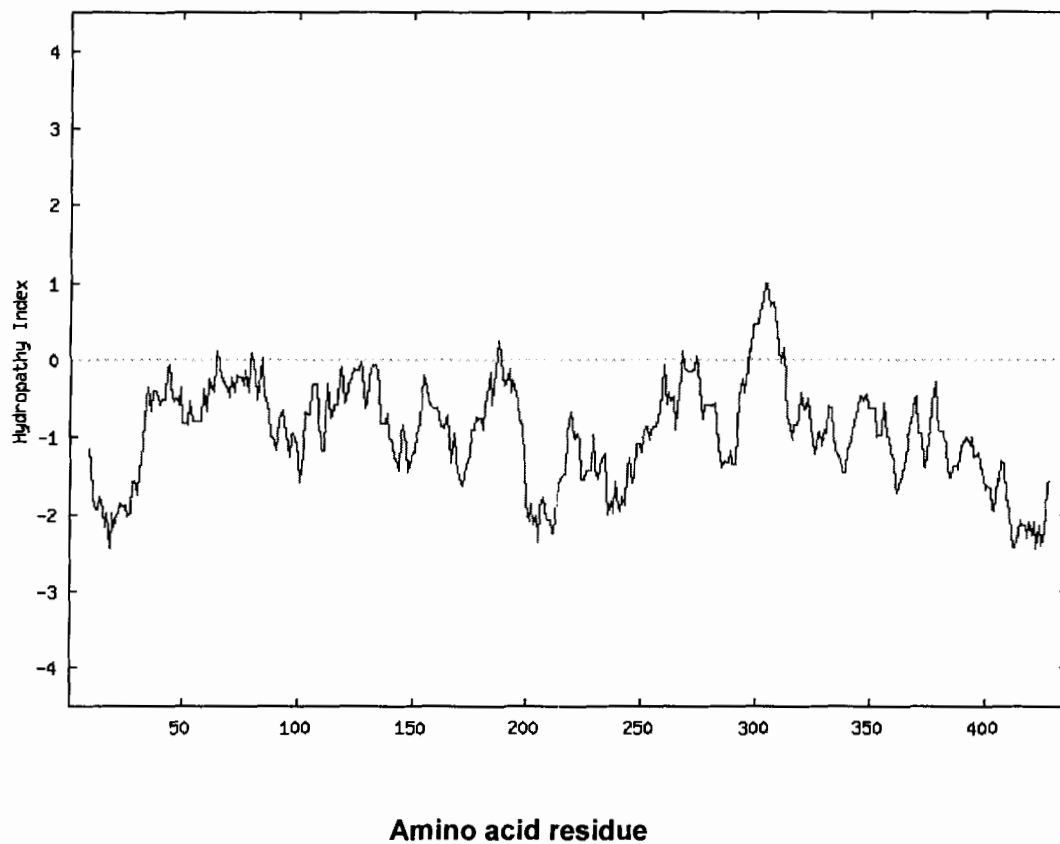


Figure 3-9 Hydropathy profile of JWL19 deduced protein.

Hydropathy profile of the JWL19 deduced protein calculated using the Kyte and Doolittle (1982) method with a window length of 17 amino acid residues. The x axis indicates amino acid residue number and the y axis indicates hydropathy value (positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions).

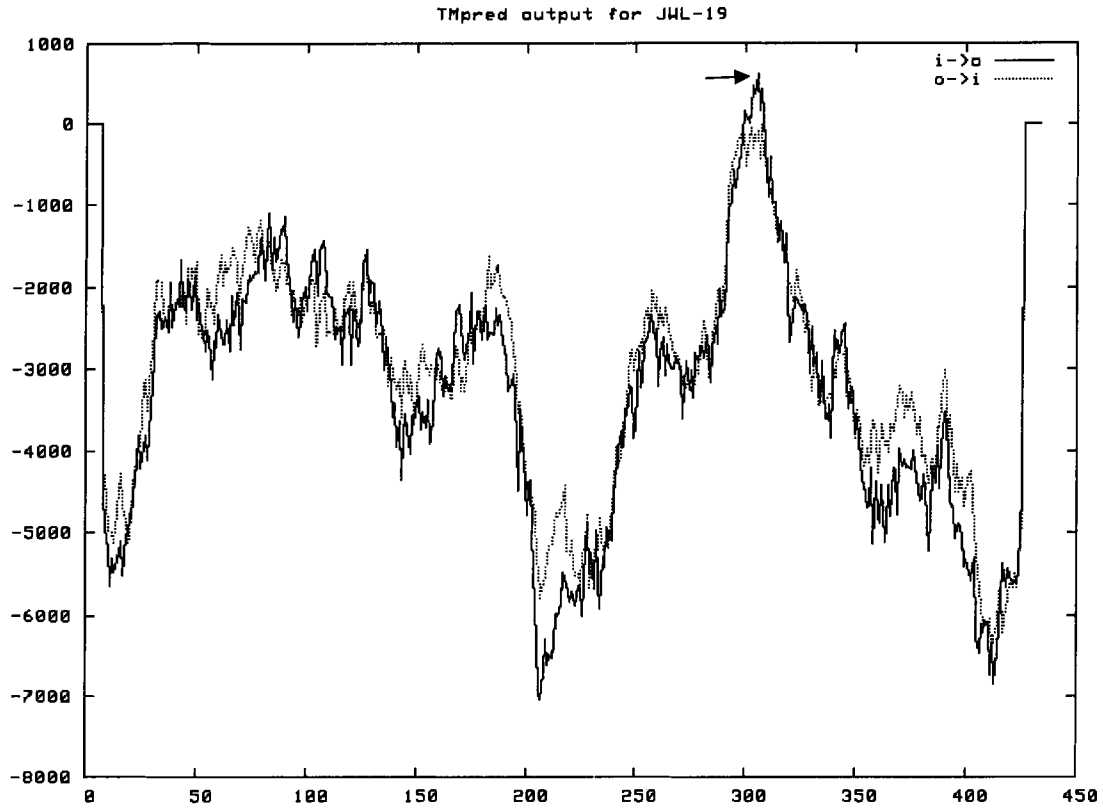


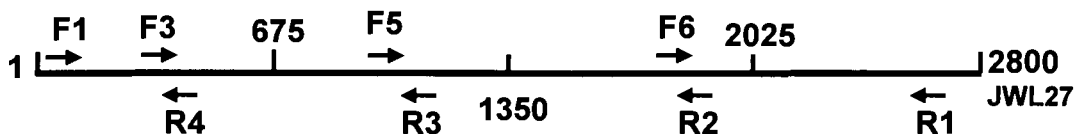
Figure 3-10 Transmembrane prediction of JWL19 deduced protein.

The x axis indicates the amino acid residue number, and the y axis indicates the predicted TM value (a value larger than 500 is considered significant for a transmembrane region) . The JWL19 deduced protein contains one potential transmembrane helice (amino acid residue 295 to 314) with a score of 621 (Hoffmann and Stoffel, 1993).

sequenced in both the forward and reverse directions using the T3 and T7 universal primers. Sequencing with the T7 primer did not reveal the poly A tail in the JWL27 clone, therefore another universal primer that annealed upstream of the T7 annealing site (-21 M13) was used. The nucleotide sequence obtained with the -21 M13 primer contained a poly A tract. Based on the sequence information obtained from the T3, T7, and -21 M13 universal primers, nested forward (JWL27F3: 5' CGC TCT TGT TGC CTG ATA 3') and reverse (JWL27R2: 5' GAA GGT GAA GGT GAA GTA 3') primers were designed (Figure 3-11). Based on the nucleotide sequence obtained using the JWL27F3 and JWL27R2 primers, two nested forward primers (JWL27F5: 5' CCT GCT GTC TCT CTG TT 3' and JWL27F6: 5' GCA CCC AGA ACA GTA ATA A 3') and two nested reverse primers (JWL27R3: 5' GGA AAG AAA CAG AGA GAC 3' and JWL27R4: 5' GGC AAC AAG AGC GTA AAC AC 3') were designed in order to complete the nucleotide sequence of the JWL27 insert (Figure 3-11).

The cDNA insert of JWL27 is 2.7kb (Figure 3-12). A BLASTN search with the JWL27 nucleotide sequence against the EST database showed that the JWL27 cDNA shares high nucleotide sequence similarity with a variety of ESTs derived from various tissues including fruit, callus, trichome, root, ovary, seedling, flower, *P. infestans*-treated tissues and salt-treated roots (Table 3-3). In addition, BLASTN searches against the EST database indicated that JWL27 contains two potential introns located at nucleotide 138 – 166 and 1631 – 1699 (Figure 3-12). A BLASTN search with the nucleotide sequence of JWL27 against the nr database showed that JWL27 shares similarities with a hydrogen peroxide-

JWL27
pBluescript SK (+/-)



Primer sequences (5' to 3'):

Forward primer for sequencing + strand of JWL27-7:

JWL27F1: 5' ATA TTC TGT GAG AGA CGC CG 3' (16 nt)

JWL27F3: 5' CGC TCT TGT TGC CTG ATA 3' (349 nt)

JWL27F5: 5' CCT GCT GTC TCT CTG TT 3' (1097 nt)

JWL27F6: 5' GCA CCC AGA ACA GTA ATA A 3' (1855 nt)

Reverse primer for sequencing – strand JWL27-7:

JWL27R1: 5' TCA TTG ATG TAG AAC AAT GG 3' (2630 nt)

JWL27R2: 5' GAA GGT GAA GGT GAA GTA 3' (1903 nt)

JWL27R3: 5' GGA AAG AAA CAG AGA GAC 3' (1120 nt)

JWL27R4: 5' GGC AAC AAG AGC GTA AAC AC 3' (361 nt)

Figure 3-11 Sequencing strategy of JWL27.

Nested forward and reverse primers used to sequence the JWL27 clone are indicated below the JWL27 sequencing map. Annealing site of each primer is indicated within parenthesis following the primer sequences.

Figure 3-12 JWL27 nucleotide and its deduced amino acid sequence

The deduced JWL27 amino acid sequence is given below the center nucleotide of each codon. The bold nucleotide sequence designates the location of the JWS27 partial cDNA within JWL27, whereas the italicized nucleotide sequence designates the location of RA 121 within JWL27. The deduced amino acid sequence of the two potential ORFs that are present within JWL27 (ORF1: 230-559 nt) and (ORF2: 1576-2445 nt) are bolded and italicized. The location of two potential introns (138 – 165 and 1631 – 1699) as indicated by the presence of (A/C)GU at the donor site and AGG at the acceptor site (Abelson et al., 1998) are bolded and underlined.

1
GCA CGA GCT CTT TCA ATA TTC TGT GAG AGA CGC CGT AAC CTA ATT CTA GGG TTT CCA GAA
A R A L S I F C E R R R N L I L G F P E
H E L F Q Y S V R D A V T * F * G F Q K
T S S F N I L * E T P * P N S R V S R N
61
ATT TTT GAT TCT GCG ATT CAA TCA CAA GTA ATC GTT TGT ATC GCG CCA AAT CGA TCT GAA
I F D S A I Q S Q V I V C I A P N R S E
F L I L R F N H K * S F V S R Q I D L K
F * F C D S I T S N R L Y R A K S I * N
121
ATC TGA ACC ATA GTG TTG **TAT TTT TTG CGA TTT ATT TTT GTT TTT TGG TAA AGA AAG GGA**
I * T I V L Y F L R F I F V F W * R K G
S E P * C C I F C D L F L F F G K E R E
L N H S V V F F A I Y F C F L V K K G R
181
GGG AAG TCT CAG ATC TGA AAT CGA AAA GGG ATT TGT CTG TGT ATT GAG GAT GTC TCG GTG
G K S Q I * N R K G I C L C I E D V S V
G S L R S E I E K G F V C V L R M S R C
E V S D L K S K R D L S V Y * G C L G A
241
CTT CCC ATA CCC ACC ACC TGG CTA TTC GCT GAA TAG ATC CAG CAA TGA AGC CTT GAT CGA
L P I P T T W L F A E * I Q Q * S L D R
F P Y P P P G Y S L N R S S N E A L I E
S H T H H L A I R * I D P A M K P * S N
301
ATC GAT TAA GGT TTG TTG AAA CAA AGA GCA CTT GAA TTT ATG TGT TTA CGC TCT TGT TGC
I D * G L L K Q R A L E F M C L R S C C
S I K V C * N K E H L N L C V Y A L V A
R L R F V E T K S T * I Y V F T L L L P
361
CTG ATA TAA TCT AGC AGG GAT CTA TAC TCT ATG TAA ATC TGT AAG GAA AAT TTG TGG TTT
L I * S S R D L Y S M * I C K E N L W F
* Y N L A G I Y T L C K S V R K I C G F
D I I * Q G S I L Y V N L * G K F V V F
421
TCG TGG GTT GTG CAT TGG TTG TCA ACT TGG ATT TAG GGT TTG TAG GAT TTG ATT TGC GCT
S W V V H W L S T W I * G L * D L I C A
R G L C I G C Q L G F R V C R I * F A L
V G C A L V V N L D L G F V G F D L R W
481
GGG ATA AAT TGA CGT TTT TGT AAG CCT TTT GTG TGG ATT TAT TAA TTA TAC CTG GTT AGA
G I N * R F C K P F V W I Y * L Y L V R
G * I D V F V S L C G F I N Y T W L E
D K L T F L * A F C V D L L I I P G * K
541
AAT GAG GAT TAG TAT ATA CTT TTT TGT GCT CAC CTT GTG GAT TTA ACA GTA TTT AAT GAA
N E D * Y I L F C A H L V D L T V F N E
M R I S I Y F F V L T L W I * Q Y L M N
* G L V Y T F L C S P C G F N S I * * I
601
TTT TAC GAG AAT AGG TTA TGC GAA ATC TGA GCA ATT TGA TAT CAT TCT AAC CTT TGG TAA
F Y E N R L C E I * A I * Y H S N L W *
S T R I G Y A K S E Q F D I I L T F G K
L R E * V M R N L S N L I S F * P L V K
661
AGG AGA GGT GAT AAG TTT AGG CAA CTT TTA GGC TTT CAA TTT GTT GTT TGT GGA GGA AAT
R R G D K F R Q L L G G F Q F V V C G G N
G E V I S L G N F * A F N L L F V E E I
E R * * V * A T F R L S I C C L W R K S

721 751
CGT AAT GAG AAG TTA GAA GTG TAT GCA AAC ATT GAG GTG TTA ATG TAT TGT TTG AGG AAG
R N E K L E V Y A N I E V L M Y C L R K
V M R S * K C M Q T L R C * C I V * G R
* * E V R S V C K H * G V N V L F E E E

781 811
AAG TCC AGC AGA AAT TGT AGG CAA AAA CCT GTG CTT GAA TAG ATT TTC TAA CTT GTA ATA
K S S R N C R Q K P V L E * I F * L V I
S P A E I V G K N L C L N R F S N L * Y
V Q Q K L * A K T C A * I D F L T C N I

841 871
TAC TTC TTT TTA AAA AGT AAT CAC TAT ACA TCA AAG ATG TTT GAG AAA AGA TTT TTG GGT
Y F F L K S N H Y T S K M F E K R F L G
T S F * K V I T I H Q R C L R K D F W V
L L F K K * S L Y I K D V * E K I F G *

901 931
AAA ACG AGG TGC TCT TGG AAA GTT ATA TTT TGG CAG ATT GCT CAT ACA AGA ACT AAC CAT
K T R C S W K V I F W Q I A H T R T N H
K R G A L G K L Y F G R L L I Q E L T I
N E V L L E S Y I L A D C S Y K N * P L

961 991
TGT GCA TAA TTG AAA AGC CTT TCG AAA AAA TTG TAT TTC ACA CTA CTA GTC GCA GCT ATA
C A * L K S L S K K L Y F T L L V A A I
V H N * K A F R K N C I S H Y * S Q L *
C I I E K P F E K I V F H T T S R S Y N

1021 1051
ACT ACT AAT TGA AGC TGT GTA GCA TTA TGA ATA TGG ATG CCC TCT GCT GAA TTT TGG GTC
T T N * S C V A L * I W M P S A E F W V
L L I E A V * H Y E Y G C P L L N F G S
Y * L K L C S I M N M D A L C * I L G P

1081 1111
CAT ACC ATG TTC CTT TCC TGC TGT CTC TCT GTT TCT TTC CAT GGT AGA AGT TTT AAA TGA
H T M F L S C C L S V S F H G R S F K *
I P C S F P A V S L F L S M V E V L N E
Y H V P F L L S L C F F P W * K F * M R

1141 1171
GGT TCC GTC TGT GAA ATT GGT TTT CTT AAG ATT CCC CTG TAC CCT GTC ACA ACT ATA GAA
G S V C E I G F L K I P L Y P V T I E
V P S V K L V F L R F P C T L S Q L * K
F R L * N W F S * D S P V P C H N Y R K

1201 1231
AGT TAC TAT TCG GTT TAG TCC ACA ATG TGA GTG TTC TGT AAT ATG TTA TTC TTT TCT ACC
S Y Y S V * S T M * V F C N M L F F S T
V T I R F S P Q C E C S V I C Y S F L P
L L F G L V H N V S V L * Y V I L F Y L

1261 1291
TGC ATA ATT AGT GTT CGC ATC ACT AAT ACT TTG TTT GTA AGT CTT GCT TGA TTT AGC TTG
C I I S V R I T N T L F V S L A * F S L
A * L V F A S L I L C L * V L L D L A W
H N * C S H H * Y F V C K S C L I * L G

1321 1351
GTT ACC ACT TTT ATG CAA CTT CAT CTA TAG ACC AGG GGC CAG TTC TTT GTG TTA AGT AGA
V T T F M Q L H L * T R G Q F F V L S R
L P L L C N F I Y R F G A S S L C * V E
Y H F Y A T S S I D Q G P V L C V K * N

1381 1411
ATT AGG TGA CTG AGA TTG TGC ATT CTG TCA TAA TGA TGG AAG TAT ATG TTG TGA ACC CAA
I R * L R L C I L S * * W K Y M L * T Q
L G D * D C A F C H N D G S I C C E P N
* V T E I V H S V I M M E V Y V V N P T

1441
CAC ATG TTT GGT GCC TAC ACC CAT GTC TGT TTG ATA TGA TAA AAG TTC AGT GAA TGA TCC
H M F G A Y T H V C L I * * K F S E * S
T C L V P T P M S V * Y D K S S V N D P
H V W C L H P C L F D M I K V Q * M I L
1501
TCA GAA TCT GAG GGA TCT TAA TTG TTC GTT ATC CTT CTG GTC ATG CTG TAT TAG TCT TCT
S E S E G S * L F V I L L V M L Y * S S
Q N L R D L N C S L S F W S C C I S L L
R I * G I L I V R Y P S G H A V L V F Y
1561
ATC TCC CTC TAT TCT GAA CTT GTT ATC CTG GTG CTC TAA AGC TCC AAA AGG AGA GGG AGG
I S L Y S E L V I L V L * S S K R R G R
S P S I L N L L S W C S K A P K G E G G
L P L F * T C Y P G A L K L Q K E R E V
1621
TAG CTA AAG CAG AAA AGA AGG ACA GGA AGA GGG AAA AGA AAG AAA AGA AAA GAG AAG AGA
* L K Q K R R T G R G K R K K R K E K R
S * S R K E G Q E E G K E R K E K R R E
A K A E K K D R K R E K K E K K R E E K
1681
AGA AGG CTA AAA AGG AGA AGA GTA ATC TTG GAT TTG GCA AGG CCA CTC ATG AGT CCA AAG
R R L K R R R V I L D L A R P L M S P K
E G * K G E E * S W I W Q G H S * V Q R
K A K K E K S N L G F G K A T H E S K G
1741
GGA AGT ACT TGT TCA AAT GTT TCG AAG ATG AAC CTG AGC AGT TGG AGA GGA GCA ATC TCA
G S T C S N V S K M N L S S W R G A I S
E V L V Q M F R R * T * A V G E E Q S H
K Y L F K C F E D E P E Q L E R S N L T
1801
CTG AGG AAC ATG AGC CTG CTG TGT GTT CAC AGA ATT CCA GCT GCT CAT CCG ACA GCA CCC
L R N M S L L C V H R I P A A H P T A P
* G T * A C C V F T E F Q L L I R Q H P
E E H E P A V C S Q N S S C S S D S T Q
1861
AGA ACA GTA ATA AAA GGA AGA GGC CTA CTT CAC CTT CAC CTT CAC GAG GTG GCA TAC AGG
R T V I K G R G L L H L H L H E V A Y R
E Q * * K E E A Y F T F T F T R W H T G
N S N K R K R P T S P S P S R G G I Q A
1921
CGC ATG GTA GCA TTA TCA GAA TTA GAT TGT CTA AAA AGG GTG TGC AAG GTG AAA TAT CAG
R M V A L S E L D C L K R V C K V K Y Q
A W * H Y Q N * I V * K G C A R * N I S
H G S I I R I R L S K K G V Q G E I S V
1981
TAT CCA AAG AGA AGC ACT TGC CAA AAC CTG CTC AAC AAG TTG CGG AAG TTA CTG TCA GGA
Y P K R S T C Q N L L N K L R K L L S G
I Q R E A L A K T C S T S C G S Y C Q D
S K E K H L P K P A Q Q V A E V T V R T
2041
CAT CTG CCG AAA GAG CCA ACC CTT TGT TGA AAA CTA CAA ACA AAC GAA GTT GTC CTC CTC
H L P K E P T L C * K L Q T N E V V L L
I C R K S Q P F V E N Y K Q T K L S S S
S A E R A N P L L K T T N K R S C P P P
2101
CTG TAG CAG TTT CAG AGC CAA GTA CAT CCA ATT GTG GGT GGG TTG ACC GTG TGG CTG AAG
L * Q F Q S V H P I V G G L T V W L K
C S S F R A K Y I Q L W V G * P C G * R
V A V S E P S T S N C G W V D R V A E D

2161
 ACA ACG CAA CTC CAT CTT GTT CCA AAG TGC 2191
 T T Q L H L V P K C T R T A * S F S T R
 Q R N S I L F Q S A R E Q H R V S V Q E
N A T P S C S K V H E N S I E F Q Y K N
 2221
 ATT TGA TTG AGA ACT GGC TTC CAC CAA GTC 2251
 I * L R T G F H Q V C Q V I I * I W R M
 F D * E L A S T K S A K * * S R F G G *
L I E N W L P P S L P S D N L D L E D D
 2281
 ATC AAT CAT GGC TGT TTC AAA GAA AAC CTA 2311
 I N H G C F K E N L S K L E L R K K T *
 S I M A V S K K T * A S S S * G K K L R
Q S W L F Q R K P K Q A R V E E K N L G
 2341
 GCG GCG GTG ACA AAA CTT GTG GAA GCT GCA 2371
 A A V T K L V E A A V H C G S S H G H N
 R R * Q N L W K L Q F I V A A A T G T I
G G D K T C G S C S S L W Q Q P R A Q Y
 2401
 ACC TTC CTG ATG TTG AGT TGT ATG CAT TGC 2431
 T F L M L S C M H C P T Q F L F E S H N
 P S * C * V V C I A L H S S F L N H I T
L P D V E L Y A L P Y T V P F * I T * P
 2461
 CGT GTG TAT AGA AGA TGT TTT AGC TGG ATA 2491
 R V Y R R C F S W I Y C S G N F V A L L
 V C I E D V L A G Y T A Q G I L * L F S
 C V * K M F * L D I L L R E F C S S S L
 2521
 TGG GAA CTG ATG TAG TTT GAA CAG GAT TTA 2551
 W E L M * F E Q D L G A M T K M F F I
 G N * C S L N R I * A R * Q K C S F L F
 G T D V V * T G F R R D D K N V L F Y F
 2581
 TTT TAG CTG CCA GTT GCT ATC TTC AGA AAT 2611
 F * L P V A I F R N P L F Y I N E N D Q
 F S C Q L L S S E I H C S T S M K M I N
 L A A S C Y L Q K S I V L H Q * K * S I
 2641
 TAT AGT ATT GGA TAT TTT CTT TCT CAA AAA 2671
 Y S I G Y F L S Q K K K K K
 I V L D I F F L K K K K K K
 * Y W I F S F S K K K K K

Table 3-3 BLASTN search result for JWL27 (February, 2005) against both the EST and non-redundant databases.

BLAST result*	GenBank Accession Number	Bit Score	E value
cDNA library			
<i>L. esculentum</i> breaker fruit	BM411204	1382	0
<i>L. esculentum</i> cotyledon-derived callus	BI421809	1302	0
<i>L. esculentum</i> breaker fruit	BE461946	1233	0
<i>L. pennellii</i> trichome	AW399221	1158	0
<i>L. esculentum</i> root during/after fruit set	AW218750	1156	0
<i>L. esculentum</i> root during/after fruit set	AW218749	1156	0
<i>L. esculentum</i> breaker fruit	BE461293	1102	0
<i>L. esculentum</i> ovary	AI487609	1078	0
<i>L. esculentum</i> ovary	AI487609	1063	0
<i>L. esculentum</i> root	BE449746	1057	0
<i>L. esculentum</i> germinating seedling	AW647743	1053	0
<i>L. esculentum</i> flower	BI935181	1051	0
<i>L. esculentum</i> root	BE451047	1037	0
<i>L. esculentum</i> ovary	AI489152	1029	0

Table 3-3. Continued

BLAST result	GenBank Accession Number	Bit Score	E value
cDNA library			
<i>L. esculentum</i> ovary	AI488924	1027	0
<i>L. esculentum</i> flower	BI935258	1017	0
<i>L. esculentum</i> immature green fruit	BE458346	993	0
<i>L. esculentum</i> flower	BI932323	985	0
<i>L. esculentum</i> ovary	AI771345	969	0
<i>L. esculentum</i> ovary	AI486512	961	0
<i>L. esculentum</i> callus	AW035956	959	0
<i>L. esculentum</i> germinating seedling	AW649586	948	0
<i>L. esculentum</i> flower buds	BE463307	942	0
<i>L. esculentum</i> crown gall	BG132708	940	0
<i>L. esculentum</i> germinating seedling	AW647792	882	0
<i>L. esculentum</i> fruit red ripe	AW222760	874	0
<i>L. esculentum</i> breaker fruit	BM409848	864	0
<i>L. esculentum</i> callus	BI421766	854	0
<i>Phytophthora infestans</i> - treated and untreated potato tissue	BQ116384	819	0
<i>L. esculentum</i> root deficiency	AW979397	779	0

Table 3-3. Continued

BLAST result	GenBank Accession Number	Bit Score	E value
cDNA library			
<i>L. esculentum</i> immature green fruit	BE458940	775	0
<i>L. esculentum</i> flower	AW929600	759	0
<i>L. esculentum</i> salt-stressed roots JWS27	AW062242	759	0
<i>L. esculentum</i> root	BE449768	757	0
<i>L. esculentum</i> callus	AW031912	726	0
<i>L. esculentum</i> seedlings treated with 0.2mM CdCl ₂	CK714948	710	0
<i>Solanum tuberosum</i> mixed tissues	BQ518625	694	0
<i>L. esculentum</i> nutrient deficient root	BF096341	678	0
<i>Solanum tuberosum</i> mixed of abiotic (salt, water-deficit, cold, heat)-treated tissues	CK2777534	662	0
<i>L. esculentum</i> callus	BI421817	654	0
Non-redundant database			
<i>Nicotiana tabacum</i> hydrogen peroxide -induced cDNA	AJ538962	84	2e-12

* only ESTs with an E value equal to 0 are presented in this table.

induced tobacco (*Nicotiana tabacum*) cDNA (bit score: 84; E value 2e-12, Table 3-3).

JWL27 contains two potential ORFs, the first at position 230 – 317 nt and the second at position 1576 – 2445 nt (Figure 3-12). The amino acid sequences of the two ORFs in the JWL27 sequence were submitted to the BLAST server for comparison to protein sequences residing in the Gen Bank database using the BLASTP search engine, however, no significant similarity was found. A BLASTN search against the TIGR tomato Gene Index database showed that JWL27 shared 98% identity with TC162290 that was built from a variety of tomato ESTs including JWS27 (Appendix 1). An alignment of the nucleotide sequence of JWL27 and TC162290 is presented in Appendix-2.

To determine whether a transcript corresponding to JWL27 is present in salt-treated roots, RT-PCR was performed. Forward (JWL27F1: 5' ATA TTC TGT GAG AGA CGC CG 3') and reverse (JWL27R1: 5' TCA TTG ATG TAG AAC AAT GG 3') primers were designed (Figure 3-11) to amplify the entire JWL27 cDNA. In addition, the nested forward (JWS27F3) and reverse primers (JWS27R2) were included in the PCR reaction. The JWL27F1 and JWL27R1 primer combination with JWL27 cDNA as a template gave a product of approximately 2.7 kb which is close to the expected size of the JWL27 cDNA insert (data not shown). RNA isolated from 2h salt-treated roots was used as a template for cDNA synthesis, and PCR with the 60°C annealing temperature was performed using the JWL27F1 and JWL27R1 and the JWL27F3 and JWL27R2

primer combinations; however, no amplified product was observed with the JWL27F1 and JWL27R1 combination, whereas the JWL27F3 and JWL27R2 combination gave the expected product of 1.6 kb. The 1.6 kb product was cloned to produce RA121, and its nucleotide sequence was determined. The nucleotide sequence of RA121 corresponds to nts 349 – 1903 of the JWL27 cDNA (Figure 3-12). Using the JWL27F1/JWL27R1 primer combination all further attempts to amplify a full-length JWL27 cDNA by RT-PCR failed. Potential explanations for this are possibly due to the quality of both the RNA and the primers used for RT-PCR.

3.3.4 Expression Analyses of Genes Corresponding to JWL19 and JWS27 in Salt-treated Roots

It has been demonstrated by northern hybridization analyses that genes corresponding to both the JWS19 and JWS27 partial cDNAs are induced or up regulated in response to an applied salt treatment (Chapter II, Figures 2-12, 2-13). Previously, the salt treatment was conducted with only two control time points: 0 and 24h. This time, control treatments were performed in parallel with all salt treatments to confirm the salt-responsive expression of genes corresponding to JWL19 and JWS27.

In salt-treated roots, the JWS19 partial cDNA probe hybridized to two transcripts: an upper (JWS19U) and a lower transcript (JWS19L), with an approximate size of 1.8 and 2.1 kb, respectively (Figure 2- 12, Chapter II). This result was confirmed using JWL19 as a probe (Figure 3-13), and hereafter

JWS19U and JWS19L will be referred to as JWL19U and JWL19L, respectively. In control roots, both JWL19U and JWL19L were absent at 2h. At 0.5h, only JWL19L was present (Figure 3-13). Following a salt treatment, the gene corresponding to JWL19 was transiently expressed (Figure 3-13). At 0.5h following the application of salt, both JWL19U and JWL19L were present, but the level of JWL19L was lower than that in control roots. At 2h following the application of salt, the level of both JWL19U and JWL19L was dramatically elevated. Transcripts corresponding to JWL19 were not detected at any other time point.

In salt-treated roots, at least two transcripts hybridized to the JWS27 probe, the upper transcript (JWS27U) and the lower transcript (JWS27L), with approximate sizes of 4.5 and 2 kb, respectively (Figure 3-14, and Chapter II, Figure 2-13). In control roots, at the 0h time point JWS27U is present, albeit at a low level. Thereafter, JWS27U levels increased at 0.5h before declining by 2h. In control roots, at all times, the level of JWS27L was higher than that of JWS27U. Following salt treatment, the JWS27U levels was increased at all times, with the highest level obtained at 2h (Figure 3-14). The JWS27L level was up-regulated by salt at 2h and 8h, whereas at 0.5h and 24h, it was down-regulated (Figure 3-14A). To determine whether the RA 121 clone detected a similar expression pattern to that obtained with JWS27, which is derived from the 5' end of JWL27, the same blot was probed with RA 121 (Figure 3-14B). When RA121 was used as a probe, only JWS27U was detected.

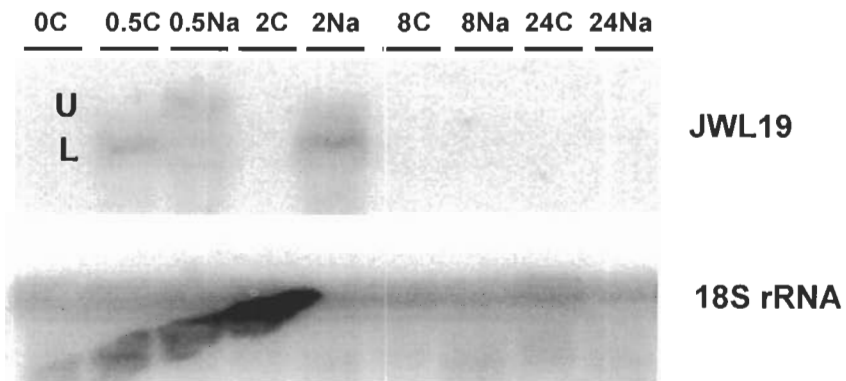
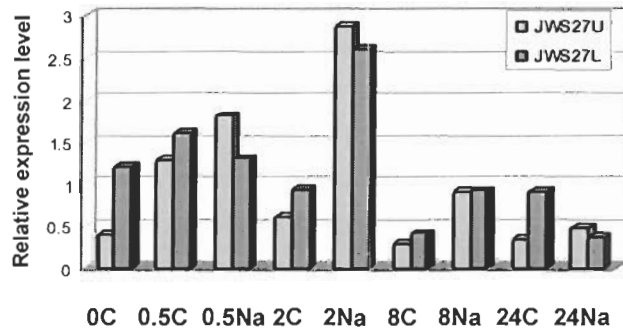
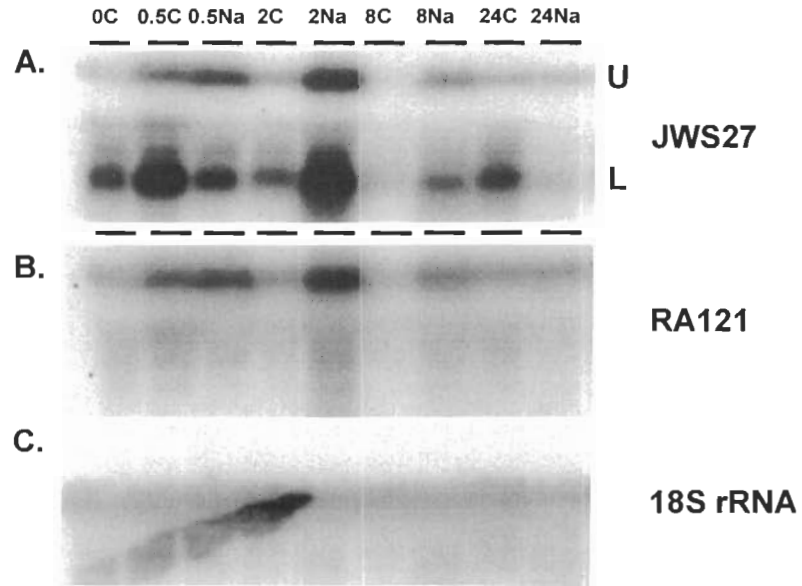


Figure 3-13 Expression of gene(s) corresponding to JWL19 in salt- treated Ailsa Craig roots.

RNA was isolated from roots of AC plants exposed to MS nutrient medium containing NaCl for 0.5h (0.5 Na), 2h (2 Na), 8h (8Na), 24h (24Na) or to MS nutrient medium with no NaCl for 0h (0C), 0.5h (0.5C), 2h (2C), 8h (8C), and 24h (24C)The blot was probed with JWL19 and then with an 18S rRNA probe. This experiment was independently performed for three times.

Figure 3-14 Expression of genes corresponding to JWS27 in salt-treated Ailsa Craig roots. RNA was isolated from roots of AC plants that were exposed to nutrient medium contains no NaCl for 0h (0C), 0.5h (0.5C), 2h, (2C), 8h (8C), and 24h (24C) or to nutrient medium with NaCl for 0.5h (0.5Na), 2h, (2Na), 8h (8Na), and 24h (24Na). The blot was probed with JWS27 (A), the RA 121 clone (B) and then with an 18S rRNA probe (C). This experiment was independently performed for three times. The plot shows normalized JWS27U and JWS27L levels as detected by JWS27 probe. Map showing the location of JWS27 (black shaded box), RA121 cDNA (black shaded box) and the two potential ORFs (unshaded boxes) is presented (D). Numbers within the map correspond to the nucleotide sequence of the JWS27 cDNA.



3.3.5 The Role of ABA in Regulating the Expression of Genes Corresponding to JWL19 and JWS27

Abscisic acid has been proposed to regulate changes of gene expression in salt-treated roots (Galvez et al., 1993; Moons et al., 1995). In order to gain insight into the role of ABA, the expression of gene(s) corresponding to JWL19 and JWS27 was investigated in ABA-treated Ailsa Craig roots. The expression of gene(s) corresponding to JWL19 and JWS27 was also investigated in the ABA deficient mutant, *flc* following salt treatment. *Flacca* is blocked at the final step of ABA biosynthesis- the conversion of ABA-aldehyde to ABA (Taylor et al., 1998, Marin and Marion-Poll, 1997). However, following salt stress, the endogenous ABA level in *flc* roots increased 14 fold to 45 pg/mg FW (Chen and Plant, 1999), which is about half the ABA content of salt-treated AC roots (103 pg/mg FW). Therefore, since salt-induced expression in *flc* alone may not be sufficient to establish a role for ABA in regulating changes of gene expression, fluridone was used to reduce ABA content further. In salt-treated roots of tomato seedlings, FLU has been demonstrated to reduce endogenous ABA to a level that is lower (5.3 pg/mg FW) than that observed in *flc* (45 pg/ mg FW) (Chen and Plant, 1999). Fluridone affects phytoene desaturase (Kowalczyk-Schroder and Sandman, 1992), an enzyme that blocks carotenoid biosynthesis at the conversion of phytoene to phytofluene. Since carotenoids provide the ABA precursor, FLU also blocks ABA biosynthesis.

The level of transcripts corresponding to JWL19 and JWS27 were not affected by a 24h ABA treatment (Chapter II, Figures 2-12, 2-13). However,

transcripts corresponding to these genes are not present at 24h; therefore it is unlikely that they would be ABA responsive at that time point. Thus, an ABA time course experiment was performed. At 0.5h following the ABA application, the JWL19L level increased (Figure 3-15); however, no expression was detected at any other times. JWL19U was not detected at any time following ABA application.

The level of JWS27U was similar in both ABA- and non-treated roots at 0.5h (Figure 3-16). At both 2h and 8h following ABA treatment, the level of JWS27U was higher than that in the non-treated roots; whereas at 24h following ABA treatment the level of JWS27U was slightly lower than that in the non-treated roots. The level of JWS27L was lower than that in the non-treated roots at 24h following ABA treatment, but was not affected by ABA at 0.5h and 2h (Figure 3-16). At 8h following the ABA treatment, the level of JWS27L was higher than that of the non-treated roots.

In *flc* control roots, both JWL19U and JWL19L were not present at 0h, 2h, and 24h (Figure 3-17). JWL19L was present at 0.5h. At 0.5h following salt treatment, the JWL19L was present at a low level. The level of JWL19U was similar to that in non-treated roots at 0.5h. Both JWL19U and JWL19L were present at 8h control roots, however this result was not consistently observed. At 2h following salt treatment, the level of both JWL19U and JWL19L was noticeably elevated, and their expression level was similar to that found in salt-treated wild type roots (Figures 3-13, 3-17). Previous experiments demonstrated that the level of JWL19U and JWL19L in salt-treated *flc* roots was similar to that

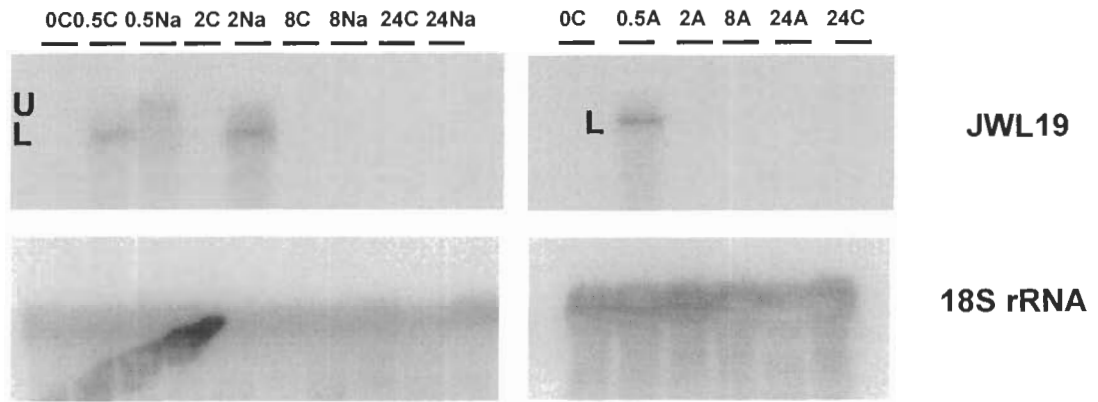
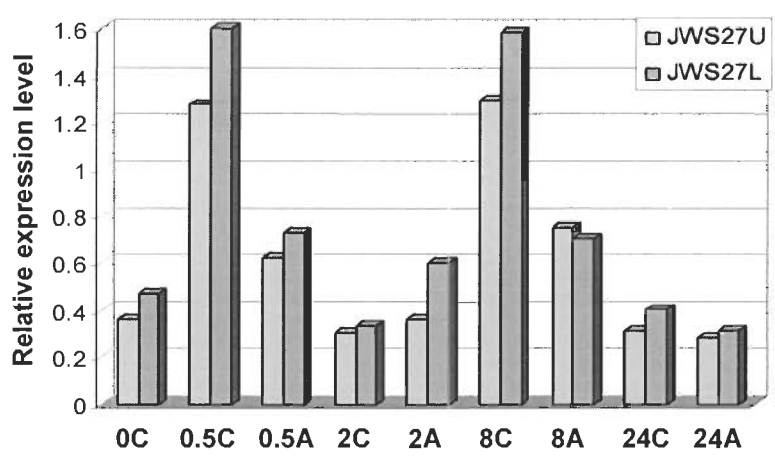
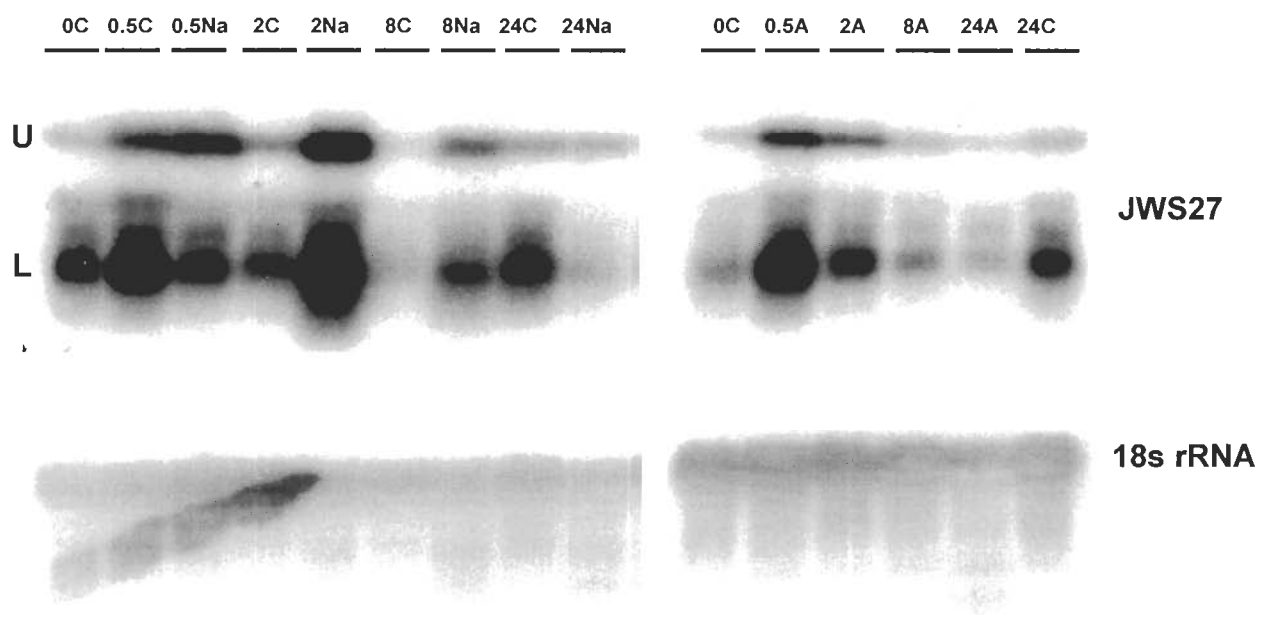


Figure 3-15 Expression of genes corresponding to JWL19 in ABA-treated AC roots. RNA was isolated from root of plants exposed to MS nutrient medium contains no NaCl for 0h (0C), 0.5h (0.5C), 2h, (2C), 8h (8C), and 24h (24C) or to MS nutrient medium with NaCl for 0.5h (0.5Na), 2h, (2Na), 8h (8Na), and 24h (24Na), and to MS nutrient medium with 100 μ M ABA (mixed isomers, +/- *cis/trans*) for 0.5h (ABA, 0.5), 2h (ABA, 2), 8h (ABA, 8) and 24h (ABA, 24) or to MS nutrient medium with no ABA for 0h (0C) and 24h (24C). The blot was probed with JWL19 and then with an 18S rRNA probe. This experiment was independently performed for at least three times.

Figure 3-16 Expression of genes corresponding to JWS27 in salt and ABA-treated AC roots.

RNA was isolated from root of plants exposed to MS nutrient medium contains no NaCl for 0h (0C), 0.5h (0.5C), 2h, (2C), 8h (8C), and 24h (24C) or to MS nutrient medium with NaCl for 0.5h (0.5Na), 2h, (2Na), 8h (8Na), and 24h (24Na), and to MS nutrient medium contains 100 μ M ABA (mixed isomers, +/- *cis/trans*) for 0.5h (ABA, 0.5), 2h (ABA, 2), 8h (ABA, 8) and 24h (ABA, 24) or to MS nutrient medium contains no ABA for 0h (0C), and 24h (24C). The blot was probed with JWS27 and then with an 18S rRNA probe. The plot shows the normalized level of JWS27U and JWS27L. This experiment was independently performed for three times.



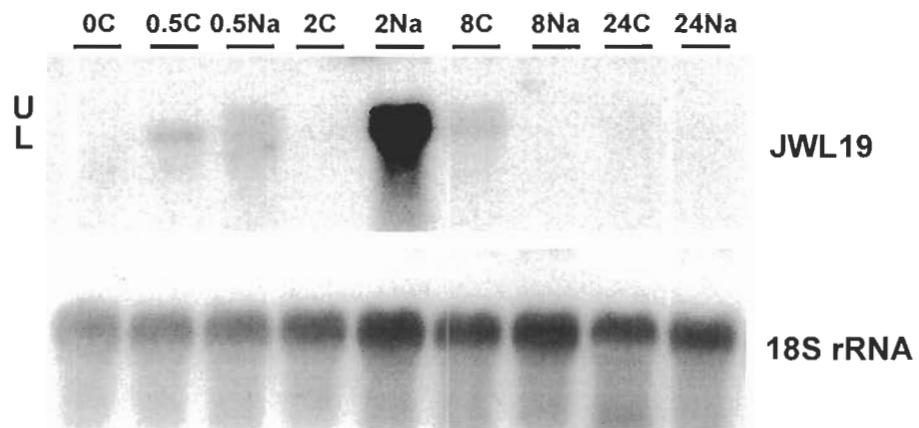


Figure 3-17 Expression of genes corresponding to JWL19 in salt-treated roots of *flacca*. RNA was isolated from roots of *flc* plants exposed to nutrient medium containing NaCl for 0.5h (0.5 Na), 2h (2 Na), 8h (8 Na), 24h (24 Na) or to nutrient medium containing no NaCl for 0h (0C), 0.5h (0.5C), 2h (2C), 8h (8C), 24h (24C). The blot was probed with JWL19 and then with an 18S rRNA probe. This experiment was independently performed for three times.

observed in salt-treated wild type roots (Chapter II, Figure 2-14), and this result was confirmed when JWL19 was used as a probe (Figure 3-17).

In control roots of *flc*, JWS27U was present at all time points tested, with the exception of 8h (Figure 3-18). Following a salt treatment, the level of JWS27U increased 0.5, 2h, and 8h, which is similar to the pattern in salt-treated AC roots (Figures 3-14, 3-18). At 24h, the level of JWS27U was unaffected by the applied salt treatment; this was also observed in salt-treated AC roots. In control roots, JWS27L was also present at all time points tested with the exception of 8h. The JWS27L level increased following a salt treatment at both 2h and 8h, whereas at 0.5h, the JWS27L level decreased in response to salt. These expression patterns are similar to those obtained in salt-treated AC roots (Figures 3-14, 3-18).

To further reduce ABA level, FLU was applied as a pretreatment for 24h prior to the application of a salt treatment in the absence of FLU. Relative to a salt treatment without a FLU pretreatment, FLU pretreatment followed by a subsequent salt treatment did not have a major effect on the level of JWL19L (Figure 3-19).

At 0h, the level of both JWS27U and JWS27L in control roots that did not receive a FLU pre-treatment was higher than in control roots with a FLU pre-treatment. At 24h, the level of both JWS27U and JWS27L was similar in both control roots with no FLU pre-treatment and in control roots with a FLU pre-treatment (Figure 3-20). Following a salt treatment, the level of both JWS27U

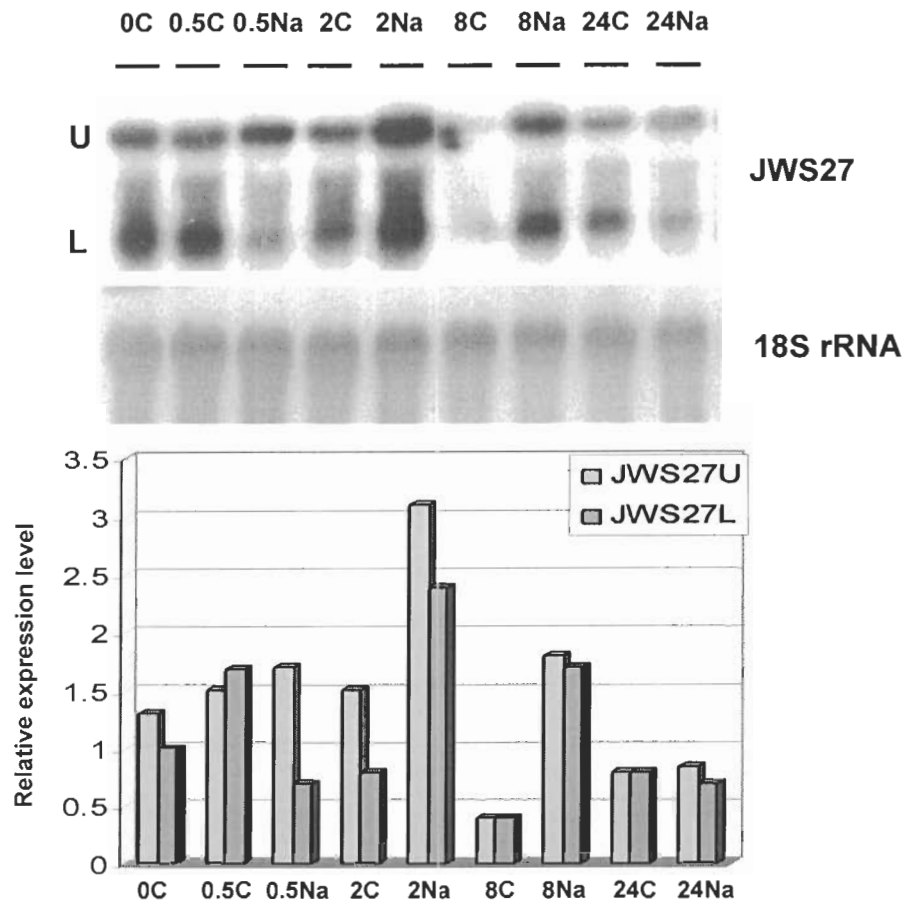


Figure 3-18 Expression of genes corresponding to JWS27 in salt-treated *flc* roots. RNA was isolated from root of *flc* plants that were exposed to nutrient medium containing no NaCl for 0h (0C), 0.5h (0.5C), 2h, (2C), 8h (8C), and 24h (24C) or to nutrient medium with 170 mM NaCl for 0.5h (0.5Na), 2h, (2Na), 8h (8Na), and 24h (24Na). The blot was probed with JWS27 and then with an 18S rRNA probe. The plot shows the normalized levels of JWS27U and JWS27L. This experiment was independently performed for three times.

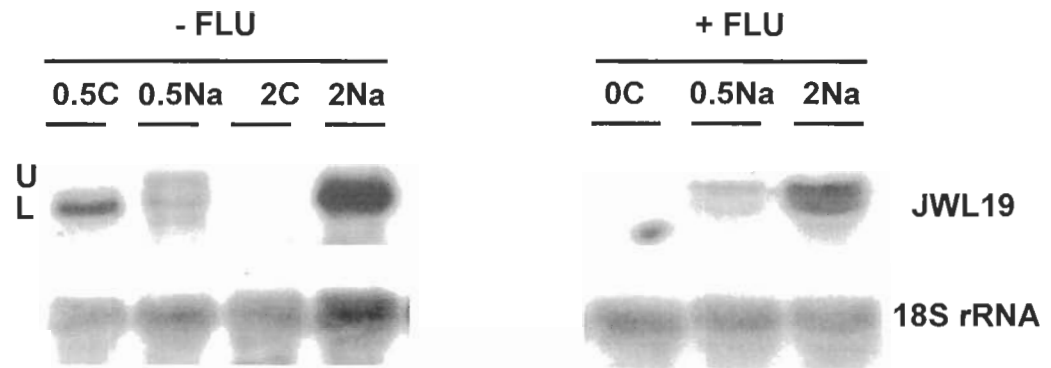
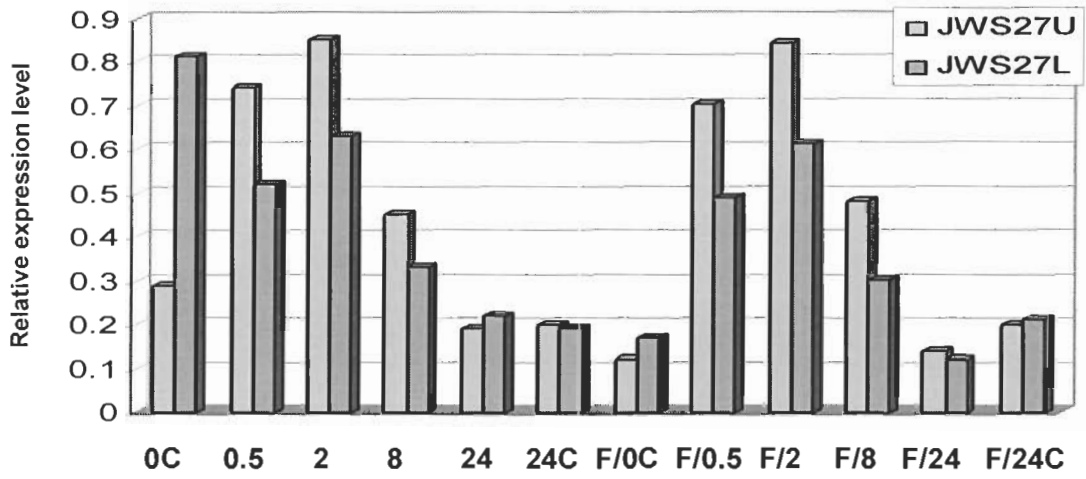
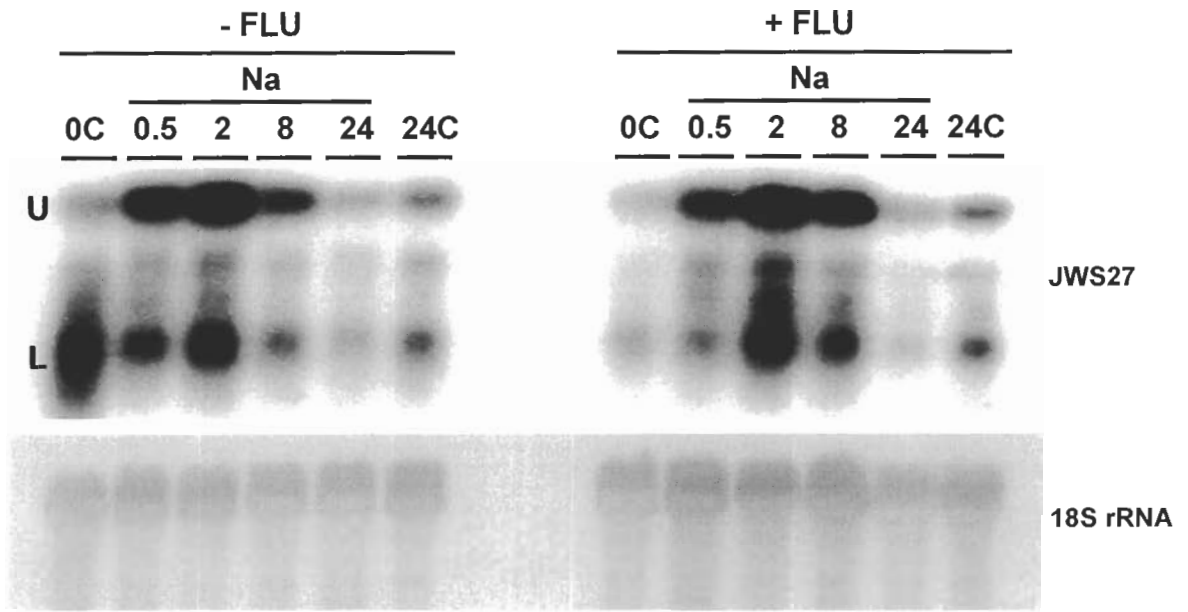


Figure 3-19 Expression of genes corresponding to JWL19 in salt-treated roots of Ailsa Craig without (-FLU) or with (+FLU) a 24h Fluridone pre-treatment.

RNA was isolated from root of AC plants exposed to MS nutrient medium containing no NaCl for 0.5h (0.5C), 2h (2C), or to MS nutrient medium containing NaCl for 0.5h (0.5Na) and 2h (2Na) with a (+FLU) or without (-FLU) pre-treatment. The blot was probed with JWL19 and then with an 18S rRNA probe. This experiment was independently performed for three times.

Figure 3-20 Expression of genes corresponding to JWS27 in salt-treated roots of Ailsa Craig without (-FLU) or with (+FLU) a 24h FLU pre-treatment.

RNA was isolated from salt-treated root of AC plants that were exposed to MS nutrient medium containing no NaCl for 0h (0C), and 24h (24C) or to nutrient medium with NaCl for 0.5h (Na 0.5), 2h, (Na 2), 8h (Na 8), 24h (Na 24) with (+FLU) or without (-FLU) a FLU pre-treatment. The blot was probed with JWS27 and then with an 18S rRNA. The plot shows the normalized level of JWS27U and JWS27L. This experiment was independently performed for three times.



and JWS27L in FLU pre-treated roots was similar to that in roots which did not receive a FLU pre-treatment (Figure 3-20).

3.4 Discussion

3.4.1 JWL27 Full-length cDNA

Screening a two hour salt-treated cDNA library using the JWS27 partial cDNA as a probe resulted in the identification of the JWL27 clone. BLASTN searches with JWL27 against both the EST and non-redundant databases showed that the nucleotide sequence of JWL27 shares high similarity with a variety of *Lycopersicon esculentum* ESTs, and to a *N. tabacum* hydrogen peroxide-induced cDNA (Table 3-3). In addition, the nucleotide sequence of JWL27 shared 98% identity with TC 162290 in the TIGR tomato gene index (Table 3-4). TC 162290 was built from a variety of ESTs that were generated from various tissue sources ranging from immature fruits, trichomes, ovaries, germinating seedlings, flowers, flower buds, crown gall, mature fruits, nutrient deficient roots, and salt-treated roots (JWS27). This result indicates that the transcripts corresponding to JWL27 were not limited to root tissues. The JWL27 cDNA was initially identified as a salt responsive partial cDNA, and the BLASTN result showed that several ESTs with sequence similarities to JWL27 were derived from *L. esculentum* nutrient deficient roots, abiotic stressed *Solanum tuberosum* tissues and hydrogen peroxide-treated *N. tabacum* leaves, which substantiates the stress responsive nature of JWL27.

The JWL27 clone contains an insert of 2683 nt, in which two ORFs are present (Figure 3-12). The distribution of all ESTs that share high nucleotide

sequence similarity to JWL27 extends from the beginning of the JWL27 insert to its end (Table 3-3), suggesting that the JWL27 cDNA is not an artifact generated during the process of making the cDNA library. Inspection of the nucleotide sequence of JWL27 revealed that it contains two introns that were also present in the same location in ESTs that mapped to JWL27. Amplification of the full-length JWL27 cDNA by RT-PCR using RNA isolated from 2h salt-treated roots was unsuccessful, although cDNA corresponding to nucleotide sequences 349 – 1903 of JWL27 was obtained, and then cloned to generate RA121 (Figure 3-12). RA121 also contains an intron, which corresponds to the second intron of JWL27 (Figure 3-12). Together, these data suggest that JWL27 is genuine, but may be derived from either an unprocessed transcript(s) or a pseudogene.

Detection of partially and fully processed transcripts has been reported in plants such as cabbage (Kumar and Trick, 1994), maize (Li et al., 1996) and tomato (Olson et al., 1995). In tomato, the expression of 1-aminocyclopropane-1-carboxylic acid synthase-3 (*LE-ACS3*) gene is induced in roots in response to flooding (Olson et al., 1995). A probe derived from the coding region of *LE-ACS3* hybridizes to two transcripts, whose sizes coincide with the size of the *LE-ACS3* RNA before and after processing. However, an intron-derived *LE-ACS3* probe only hybridized to the larger unprocessed transcript.

Pseudogenes are DNA sequences characterized by their close similarity to known functional genes, but which contain important defects that make them incapable of producing proteins at either the gene transcription and/or mRNA

translational stages (Vanin, 1985; Mighell et al., 2000). Non functionality in pseudogenes is often caused by the lack of functional promoters or other regulatory elements; hence these sequences are released from selection pressure. It is believed that pseudogenes arise in two ways: duplication and retrotransposition. Duplications are modifications (mutations, insertions, deletions, frame shifts) to the DNA sequence of gene(s). This type of pseudogene often retains the original exon-intron structure of the functional genes. Copies of genes that are disabled in this manner are called non-processed or duplicated pseudogenes. Retrotransposition is reverse transcription of an mRNA transcript followed by subsequent re-integration of the cDNA into the genome presumably in the germ line. Copies of genes that are disabled in this manner are called processed pseudogenes (Maestre et al., 1995; Esnault et al., 2000; Goncalves et al., 2000). Since both RA121 and JWL27 contain introns, it is possible that they may have originated from a non-processed or duplicated pseudogene. In plants, pseudogenes have been identified from several species including the *L. esculentum Aldehyde dehydrogenase2* (Longhurst et al., 1994) and *Aldehyde oxidase* (Min et al., 2000), and *Picea engelmannii cyclin-dependent protein kinases* (Kvarnheden et al., 1998).

3.4.2 JWL19 Full-length cDNA

cDNA library screening was performed in order to identify and isolate the full-length cDNA corresponding to JWS19. JWL19 was identified via screening a two hour salt-treated cDNA library with JWS19 as a probe. JWL19 contains an insert of 1708 nt which consists of 140 nucleotides of 5'UTR, 1302 nucleotides of

ORF and 266 nucleotides of 3'UTR (Figure 3-6). Hydropathy analysis of the deduced protein of JWL19 indicated that it contains one trans-membrane domain (Figure 3-9, 3-10). Generally, hydrophobic trans-membrane segments function as the sorting signals into the endoplasmic reticulum (ER) membrane; therefore it is possible that the protein corresponding to JWL19 is anchored to the ER membrane. Comparison with the nr database showed that the JWL19 deduced protein shares high similarity (bit score: 3023, E value: 0) with an auxin regulated protein of tomato, LE-ARP (Figure 3.10). Attempts to establish whether gene(s) corresponding to JWL19 is regulated by auxin was made by treating tomato roots with indole acetic acid (IAA) at various concentrations (1, 10 and 100 μ M). Preliminary results however showed that gene(s) corresponding to JWL19 was not auxin responsive (data not shown).

The JWL19 protein contains the DUF966 consensus domain. DUF966 is present in a family of plant proteins including LE-ARP, several unknown proteins of Arabidopsis (At3g46110, At5g59790, At2g28150, At5g10150, At1g05577) and two rice proteins (Figure 3-8, Table 3-2). Results from the GENEVESTIGATOR search with *At3g46110* shows that *At3g46110* is up-regulated following various stresses including salt, drought, wounding, heat, UVB, oxidative stress, as well as to a *Pseudomonas syringae* treatment. Thus, it is tempting to speculate that the gene(s) corresponding to JWL19 maybe involved in general stress responses. During the course of this study, it was not determined whether the expression of gene(s) corresponding to JWL19 is restricted solely to the root. BLASTN results against the EST database showed that the expression of genes corresponding to

JWL19 is not restricted to roots. In fact, the expression of gene(s) corresponding to JWL19 in non-stressed tissues is also detected in fruits, shoots, and ovaries, but not in roots (Table 3-1). Therefore, it is possible that the expression of genes corresponding to JWL19 in roots is stress induced, in this case by salt.

To my knowledge, no known function has been assigned to either *LE-ARP* or to *At3g46110* or to other proteins with DUF966. *LE-ARP* is auxin-responsive and a number of auxin responsive genes with unknown functions have also been reported (Gee et al., 1991; Guilfoyle, 1986; Takahashi and Nagata, 1992; van der Zaal et al., 1987). The plant hormone auxin plays a key role in a wide variety of growth and developmental processes, which include embryogenesis, lateral root development, vascular differentiation, apical dominance, tropic response and flower development (Bartel, 1997). In order to tolerate salinity stress, a plant must maintain its growth, albeit at a slower pace. Maintaining growth, in particular root growth, is an important defense mechanism in response to salinity stress since it will allow roots to explore soils that have less or no salt. Auxin is an important regulator of differential growth, which involves an asymmetric auxin distribution that causes cells on one side of the organ to elongate faster than cells on the other side (Philippar et al., 1999; Friml and Palme, 2002). Auxin activates plasma membrane H⁺-ATPases leading to apoplast acidification and expansin activation (Cosgrove, 2000). Expansins are cell wall proteins that loosen the wall in a pH-dependent manner, thereby allowing turgor-driven cell enlargement (McQueen-Mason and Cosgrove, 1994). In a study addressing root growth maintenance during water deficit stress in maize, both expansin activities

and their protein level increased substantially (Wu et al., 1994). A subsequent study showed that four expansin genes are expressed specifically in the growth zone of well-watered maize roots, and three of these are rapidly up-regulated in response to water deficit stress (Wu et al., 1996; Wu et al., 2001).

Salinity stress causes both osmotic and ionic stress, which individually or together will affect the physiological status of plants. It is believed that osmotic stress plays the dominant role in the inhibition of plant growth during the initial phase of salt stress (Munns, 2002; Lefevre et al., 2001; Ueda et al., 2003). The gene(s) corresponding to JWL19 is expressed transiently and rapidly after the applied salt treatment. Therefore, it is tempting to speculate that the gene(s) corresponding to JWL19 may be expressed in response to the osmotic component of salt stress. In *Arabidopsis*, an auxin responsive gene that encodes an epoxide hydrolase was isolated in an effort to address the molecular response of plants to dehydration (Kiyosue et al., 1994). Recently, using cDNA microarray technology, several auxin regulated genes including *IAA18* and the auxin responsive GH3-like protein have been identified as water deficit stress responsive, suggesting a possible link between auxin and the osmotic-stress responses (Seki et al., 2002a). These results, together with the identification of JWL19 suggest that auxin may play a role during the initial response phase to osmotic stress.

3.4.3 The Role of ABA in Regulating the Expression of Gene(s) Corresponding to JWS27 during Salinity Stress

The JWS27 probe detects two transcripts, JWS27U and JWS27L, which are transiently accumulated following a salt treatment (Figure 3-14). Exogenous ABA did not have a major effect on the level of either JWS27U or JWS27L, although a slightly upregulation of JWS27U at 2h and JWS27L at 8h after ABA application was observed (Figure 3-16). The level of both JWS27U and JWS27L was similar in salt-treated *flc* roots and AC roots (Figure 3-14, 3-18).

Furthermore, the level of these transcripts in FLU/salt-treated AC roots was similar to that obtained in salt treated AC roots which were not exposed to FLU (Figure 3-20). Together, these results suggest that ABA is not involved in regulating the expression of gene(s) corresponding to JWS27. Previous studies in our lab demonstrated that at the polypeptide level the synthesis of most salt responsive polypeptides is not dependent on an elevated level of ABA (Chen and Plant, 1999). This study demonstrated that, at the mRNA level, an elevated level of ABA is not necessary for the expression of gene(s) corresponding to JWS27. Together, these results signify the presence of an ABA-independent pathway that operates during salinity stress in tomato roots.

3.4.4 The Role of ABA in Regulating the Expression of Gene(s) Corresponding to JWL19 during Salinity Stress

The gene(s) corresponding to JWL19 was transiently expressed in response to salt at 0.5h and 2h after the applied salt treatment (Figure 3-16). Transient expression of salt-responsive genes has been reported previously (Galvez et al., 1993; Moons et al., 1997c), and is believed due to the

acclimatization of plants to the osmotic component of salinity stress. The rapid induction of genes corresponding to JWL19 is also in agreement with the salt-induced accumulation of a number of polypeptides in tomato roots for which the earliest changes were observed within 30 min following salt treatment (Chen and Plant, 1999).

The level of both JWL19U and JWL19L was not drastically affected by exogenous ABA (Figure 3-15) indicating that their expression during salt stress may not be regulated by ABA. Results obtained from salt-treated *flc* roots, showed that the level of both JWL19U and JWL19L was similar to that obtained in salt-treated AC roots (Figure 3-17). Furthermore, the level of JWL19U and JWL19L was similar in salt-treated AC roots that were or were not treated with FLU (Figure 3-19). Together, these results suggest that the expression of gene(s) corresponding to JWL19 following a salt treatment is not dependent on an elevated level of endogenous ABA. Previous studies in our lab have demonstrated that the changes in ABA level in roots of tomato seedlings are not apparent until 2h after the imposition of salt (Chen and Plant, 1999). Furthermore, other studies in the lab demonstrated that the accumulation of most salt-responsive polypeptides following salt stress was not dependent on an elevated level of ABA (Jin et al., 2000). Taken together, with the ABA-independent nature of JWS27 expression, these results for JWL19 indicate that an ABA independent pathway may play a substantial role in regulating the changes of gene expression during salinity stress. An ABA independent component regulating salt stress responsive gene expression has been

demonstrated by others including the expression of osmotin in salt-treated leaves of *flc* (Grillo et al., 1995) and *osr40c1* and *oslea3* in *Oryza sativa* (Moons et al., 1997b;c).

4 STRESS RESPONSIVE ALPHA-DIOXYGENASES

4.1 Introduction

The abiotic stresses — salinity, low temperature and drought in particular — are the most common environmental stresses that influence plant growth and development and thereby place major limits on plant productivity. Under salinity stress, the focus of this thesis, plants experience two types of stresses: 1) ionic, resulting from high amount of salts primarily sodium in the soil, and 2) osmotic, resulting from an increased amount of dissolved ions that reduce water availability by decreasing the osmotic potential of the soil solution. Furthermore, salinity stress can also cause oxidative stress (Asada, 1994), and it was recently demonstrated that salt ions can induce programmed cell death (PCD) in roots (Katsuhara and Shibasaki, 2000; Huh et al., 2002).

In order to understand how plants adapt and survive within environments that contain high salt, responses of plants to salinity stress have been actively studied (Hasegawa et al., 2000; Shinozaki and Yamaguchi, 2000; Zhu, 2001; Zhu, 2002). The identification of novel genes, determination of their expression patterns in response to salt stress, and understanding their functions in stress adaptation or tolerance will facilitate the generation of effective approaches to improve stress tolerance in plants (Cushman and Bohnert, 2000). In an effort to characterize molecular responses of tomato roots to salinity stress, a number of salt responsive partial cDNAs were identified and isolated using mRNA

differential display (Chapter II, Wei et al., 2000). One of the isolated salt responsive partial cDNAs was JWS20. The nucleotide sequence of JWS20 shares high similarity with the DNA sequence of α -dioxygenases isolated from various plants including *N. tabacum*, *N. attenuata*, *C. anuum*, *P. balsamifera* subspecies *trichocarpa*, *A. thaliana* and *C. arietinum* (Chapter II, Table 2-5).

Alpha-dioxygenase enzymes were first identified in tobacco, and it was initially named as pathogen induced oxxygenase (*PIOX*). In tobacco, *PIOX* is expressed in the leaves following applications of *Erwinia amylovora*, caterpillar herbivory, wounding, jasmonic acid (JA), salicylic acid (SA) and chemicals that elicit the production of ROS (Sanz et al., 1998; Hermsmeier et al., 2001). In *Arabidopsis*, *PIOX* expression is also responsive to pathogen treatment, SA, and chemicals that elicit ROS; but is not JA responsive (de Leon Ponce et al., 2002). The *PIOX* polypeptide shares significant identity with cyclooxygenases of animals that are responsible for prostaglandin biosynthesis. It was subsequently demonstrated that *PIOX* is an α -dioxygenase that catalyzes the first step of α -oxidation of linolenic (18:3) acid to a 2-*R*-hydroperoxide derivative (Hamberg et al., 1999). It is proposed that in plants *PIOX* maybe involved in generating lipid-derived signals following pathogen attack and wounding. With respect to its enzymatic activity, the *PIOX* protein was recently renamed as α -dioxygenase (α -DOX) (de Leon Ponce et al., 2002).

The research objectives of this chapter are:

1. To isolate full-length cDNA clones corresponding to the JWS20 partial cDNA.

2. To determine the expression of gene(s) corresponding to JWS20 in salt-treated and wounded tomato roots.
3. To determine the role of ABA in regulating the expression of gene(s) corresponding to JWS20 in salt-treated roots.

4.2 Materials and Methods

4.2.1 Materials

Seeds of tomato (*Lycopersicon esculentum* Mill cv Ailsa Craig) and the near-isogenic mutant *flacca* (*flc*) were grown and maintained as described in section 2.2.1. Mature plants were maintained in a green house under conditions that are typical for a spring and summer season in Burnaby, B.C. Canada

4.2.2 Experimental Treatments

Six-week-old plants were used for all experiments. Salt treatments were imposed as described in section 2.2.2. Wounding treatments were performed by crushing the roots with forceps. The whole roots were pooled together, and crushed with forceps along their length with an approximately one cm spacing from one crushing to the next. Pathogen challenge was conducted by adding mycelial fragments (400,000/L) of *Pythium aphanidermatum* (Edson) Fitzp to the nutrient medium. ABA (mixed isomers, +/- cis/trans ABA; Sigma), ethephon (Sigma), 1-aminoethoxyvinylglycine (AVG, Sigma), or 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma) were added to the nutrient media either alone or in combination with a salt treatment. Silver thiosulfate (STS) was prepared by combining sodium thiosulfate ($\text{Na}_2\text{O}_3\text{S}_2$) (Sigma-Aldrich) to silver nitrate (AgNO_3)

with a molar ratio 2:1. To avoid precipitation, the silver nitrate solution was added to the sodium thiosulfate solution while stirring continuously. STS was then added to the nutrient media either alone or in combination with salt. Control plants were transferred to fresh nutrient solution for the duration of the experimental period. Fluridone treatments were applied as described in section 3.2.2. Following each treatment, root tissues were harvested and frozen in liquid N₂ before storing at -80°C until needed. Each treatment was performed independently at least three times.

4.2.3 RNA Isolation

Total RNA for both northern blot and RT-PCR analyses was extracted using the LiCl-phenol method (Prescott and Martin, 1987), as described in Chapter II, section 2.2.4.

4.2.4 Northern Hybridization Analyses

Northern hybridization analyses were performed as described in Chapter II, section 2.2.9. Band intensity was determined using Scion image version 1.62c (macrofunction gel plot 2) and subsequently normalized by dividing the hybridization signal obtained for the α -DOX probe by that of the rRNA probe. Full-length *LE α -DOX1*, *LE α -DOX2*, and *LE α -DOX3* probes were generated using the PCR conditions described in Chapter II, section 2.2.5, with the exception that an annealing temperature of 55°C and the T3 and T7 universal primers were used. Template DNA for PCR were cLEW8G12, cLEW26H11 and cTOD20F16 corresponding to

LE α -DOX1, *LE α -DOX2*, and *LE α -DOX3*, respectively. 5' probes for *LE α -DOX1* and *LE α -DOX2* were generated utilizing the following forward and reverse primers: DOX1-F: 5' TAT CTT GGA GCA CGG CGG AG 3' and DOX1-R: 5' CTA AAG GAC TTG AGT GGG 3' or DOX2 F: 5' CAA AAT GAA TCT CCG CGA CA 3' and DOX 2 R: 5' TCC GGT AGG AGT TTC TTT TGA T 3', and the PCR conditions described in Chapter II, section 2.2.5, with modifications for the annealing temperatures (58°C for 5' *LE α -DOX1* and 55°C for 5' *LE α -DOX2*). Following PCR amplification, DNA purification and quantification of each probe was performed as described in Chapter II, section 2.2.7.

4.2.5 Nucleotide and Deduced Amino Acid Sequencing Analyses

Nucleotide sequence determination was carried out by the NAPS Unit (Biotechnology Laboratory, University of British Columbia, Canada) on a Perkin Elmer 377 (ABI Prism) DNA analyzer. Nucleotide sequences were submitted to the NCBI BLAST server for BLASTN and BLASTX searches against the non-redundant and EST databases (Altschul *et al.*, 1997). Multiple alignments were performed using CLUSTALW (<http://www2.ebi.ac.uk/clustalw/>) and MacVector 7.1.1 using a 10.0 open gap penalty, 40% delay divergent and Blosum similarity matrix. The hydrophobicity plot (Kyte and Doolittle, 1982) was generated using program available in <http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html>. Cleavage sites and a signal peptide/non-signal peptide prediction were performed based on Signal IP prediction (<http://www.cbs.dtu.dk/services/SignalP>). Analysis of Arabidopsis microarray

database was conducted using the GENEVESTIGATOR (<https://www.genevestigator.ethz.ch>) toolbox.

4.2.6 Genomic Southern Hybridization

Genomic DNA was extracted according to Dellaporta *et al.*, (1983). Frozen tissue of six-week-old tomato shoots (5 g) was grind to a fine powder in the presence of liquid nitrogen. The frozen powder was subsequently transferred to a 50 ml polypropylene tube (nalgene), followed by the addition of 10 mL extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, and 7% (v/v)- β mercaptoethanol). The mixture was vortexed and incubated on ice for 20 min which was followed by a centrifugation at 11,951 x g for 20 min. Following centrifugation, the mixture was poured through eight layers of cheese cloth and was collected in a 50 ml polypropylene tube containing 10 ml ice-cold isopropanol. The mixture was gently mixed and incubated at -20°C for 30 min. The DNA was collected by centrifugation at 11,951 x g for 15 min at 4°C. The resulting pellet was dried and resuspended over night in 50 mM Tris-HCl pH 8.0, 0.5M EDTA pH 8.0. The next day, the DNA solution was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 12,000 rpm. Following centrifugation, the supernatant was transferred to a new tube, and 0.1 volume 3M NaOAc pH 6.0 and 0.7 volume isopropanol was added and gently mixed. The DNA was recovered by centrifugation for 10 min at 12,000 rpm and the pellet was further washed with 70% ethanol. After air dried, the pellet was resuspended in 100 μ l 10 mM Tris-HCl pH 7.5, 0.5 M EDTA pH 8.0 overnight. The following day, DNase-free RNase was added to the DNA and incubated for 30 min at room

temperature followed by extraction with an equal volume of phenol:chloroform (50:50) and then with an equal volume chloroform:isoamylalcohol (24:1). The DNA was precipitated with 2 volumes 99% ethanol and 0.05 volume 3 M NaOAc pH 6.0 at -20°C for at least 1h; followed by centrifugation as described previously. DNA concentration was determined in a spectrophotometer (Biorad SmartSpec 3000, Mississauga, Ontario) at 260 nm. To determine the quality, DNA was separated on a 0.7% agarose gel in 1X TBE running buffer (Sambrook et al., 1989) followed by ethidium bromide staining. The genomic DNA (20 µg) was digested with *EcoRI*, *HindIII*, *XhoI*, or *BamHI* overnight and analysed by southern hybridization as described in Sambrook et al. (1989). The membrane was washed twice each in: 2X SSC, 0.1% SDS, 1X SSC, 0.1% SDS, and 0.5 X SSC, 0.1% SDS at 65°C, and finally in 0.1 X SSC, 0.1% SDS at 68°C. Following the washes the membrane was exposed to X-ray film at -80°C with an intensifying screen.

4.2.7 DNA Dot-blot Analysis

DNA was suspended in 0.4 M NaOH and 25 mM Na₂EDTA and denatured at 94°C for 10 min, followed by a quick chill on ice. Subsequently, 10X SSC was added to bring the volume to 200 µl and the samples were applied to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) that was previously equilibrated in 10X SSC. Vacuum was applied to draw the DNA solution through the membrane. The membrane was air dried and the DNA was fixed to the membrane by UV radiance using the autocrosslink function of

the UV Stratalinker (UV Stratalinker™ 2400, Stratagene) for 30 seconds followed by baking at 80°C for 1 h. The membrane was stored at 4°C until needed. DNA dot blot hybridizations were performed according to the hybridization procedure described in Chapter II, section 2.2.8. The washing steps for DNA dot blots were conducted as follows: following hybridization, the membrane was washed 3X for 5 min each in 10 ml 2X SSC, 0.1% SDS at room temperature, then the membrane was washed two times in 1X SSC, 0.1% SDS at 65°C and at 68°C, each for a period of 45 min. A final wash was performed in 0.5 XSSC, 0.1% SDS at 65°C for 45 min. and in 0.1XSSC, 0.1% SDS at 65°C for two times. Following the final washing, the membrane was dried briefly, covered with Saran wrap before exposure to autoradiography film (Kodak Scientific Imaging Film X-Omat Blue XB1, NEN™ Life Science, Boston, MA, USA), without an intensifying screen at -80°C.

4.3 Results

4.3.1 Isolation of the Full-length cDNA Corresponding to JWS20.

Efforts to obtain a full-length cDNA corresponding to JWS20 by screening a cDNA library constructed from 2h salt-treated tomato roots (Constructed by Dr. Jun-Zhi Wei) and a tomato cell library derived from tomato leaves 5h after they were treated with *Cladosporium fulvum* (provided by Dr. Eduardo Blumwaldo) failed. A BLASTN search against the non redundant database using the complete nucleotide sequence of

the tobacco α -*DOX* gene (Gen Bank Acc: AJ007630) revealed that several tomato ESTs with high similarity to the tobacco α -*DOX* were present. Closer inspection of all tomato ESTs revealed that in tomato, α -*DOX* is represented by a family of three genes. cDNA clones corresponding to three ESTs (341283, cLEW8G12/AW979675; 428893, cLEW26H11/BF098372; and 55463, cTOD20F16/BI935764) that aligned with the 5' end of the tobacco α -*DOX* nucleotide sequence were obtained from Clemson University Genomics Institute (CUGI).

The cLEW8G12 cDNA clone was initially sequenced using the T3 and T7 universal primer combinations. To obtain the complete sequence of cLEW8G12, internal forward (5' AAG TTG CTA ATGAAT GCC 3') and reverse primers (5' ACA CTT CTT TCT CTA TCC 3') were designed. The cLEW26H11 cDNA clone was sequenced using both the T3 and T7 universal primers. The complete sequence of cLEW26H11 was generated by combining the nucleotide sequence data obtained with the T3 and T7 universal primers with the nucleotide sequences of TC127979, TC122317 and BF098372 available from the TIGR Tomato Gene Index database. The nucleotide sequence of the third tomato α -*DOX* gene was constructed by aligning ESTs 355349, 554655, 247591, 470139, 261869, and the nucleotide sequence of the Feebly gene (U35643; van der Biezen et al., 1996). For a complete sequence, portion of the cTOD20F16 EST clone was sequenced using the T3 and T7 universal primers.

The cLEW8G12 clone encodes an α -DOX-like polypeptide, and hereafter will be referred to as *LE α -DOX1* (GenBank accession AY344539). *LE α -DOX1* contains an insert of 2115 nucleotides with an ORF that encodes a polypeptide of 639 amino acids and a predicted molecular weight of 86 kDa (Figure 4-1). In addition, cLEW8G12 has 35 nucleotides in the 5'UTR and 163 nucleotides in the 3' UTR including the stop codon and the poly A tract. BLASTN searches against the EST database shows that the nucleotide sequence of *LE α -DOX1* shares high similarity to the nucleotide sequences of tomato and potato-derived ESTs (Table 4-1). Against the non-redundant database, the nucleotide sequence of *LE α -DOX1* shares high sequence similarity to α -DOX from various plants (Table 4-2). A Kyte and Doolittle hydrophathy profile (Kyte and Doolittle, 1982) revealed that the protein encoded by *LE α -DOX1* is mostly hydrophilic, except for the regions that correspond to amino acids 22-45 and 542-560 (Figure 4-2). The TM topology (Hoffmann and Stoffel, 1993) prediction suggests that the the protein encoded by *LE α -DOX1* has two transmembrane helices, and that both its N- and C-terminus are located outside the cell (Figure 4-3). Signal IP prediction showed that the *LE α -DOX1*-encoded protein contains no signal peptide (Bendtsen et al., 2004).

The cLEW26H11 clone contained an insert of 2078 nucleotides, and its 3'end was identical to the JWS20 partial cDNA. However, the ORF

Figure 4-1 The nucleotide and deduced amino acid sequences of *LE α -DOX1*.

The deduced single-letter amino acid sequence is given below the center nucleotide of each codon. A putative Kozak sequence (RCCATGR, in which R is a purine (A or G), Kozak, M., 1986) is both italicized and bolded. The initiating ATG, stop codon, forward and reverse sequencing primers are bolded and underlined.

1 GC ACG AGG AAG TGA TAT TAA TTT CTC GTA TAA **ACA**
 36 **ATG** TCT TTT GTT ATG CTC AAG AAT CTC TTG CTA TCC TCT CTC CGT
 M S F V M L K N L L L S S L R
 81 AAA TTC ATC CAC AAA GAT TTC CAT GAG ATC TTT GAC AAA ATG ACT
 K F I H K D F H E I F D K M T
 126 CTC ATC GAT AAA TTA TTT TTT TTG ATT GTT CAT TTT ATT GAT AAA
 L I D K L F F L I V H F I D K
 171 CAT AAC TTT TGG CAC CGG CTA CCG GTA TTC TTC GGG TTA CTT TAT
 H N F W H R L P V F F G L L Y
 216 CTT GGA GCA CGG CGG AGT CTT CAC CAG CAA TAT AAT TTG ATC AAC
 L G A R R S L H Q Q Y N L I N
 261 GTC GGT AGA ACA CCT ACC GGA GTT CGA TCA AAT CCG GCA GAT TAC
 V G R T P T G V R S N P A D Y
 306 CCT CAC AGA ACT GCT GAT GGA AAA TTC AAT GAC CCT TTT AAT GAA
 P H R T A D G K F N D P F N E
 351 GGA ACA GGC AGT CAA TTT TCT TTC TTT GGC AGG AAT ATG ATG CCT
 G T G S Q F S F F G R N M M P
 396 CTT CAT CAG AAT AAT AAG TTA AAA AAG CCA GAT CCA ATG GTA GTA
 L H Q N N K L K K P D P M V V
 441 GCA ACC AAG CTT CTA GCA CGA AGA AAA TTC ATA GAC ACT GGA AAA
 A T K L L A R R K F I D T G K
 486 CAA TTC AAT ATG ATA GCT GCT TCT TGG ATA CAG TTT ATG GTT CAT
 Q F N M I A A S W I Q F M V H
 531 GAT TGG ATC GAT CAT TTG GAA GAT ACT CAA CAG GTT GAG CTA AGG
 D W I D H L E D T Q Q V E L R
 576 GCA CCA AAA **GAA GTT GCT AAT GAA TGC** CCA CTC AAG TCC TTT AGG
 A P K E V A N E C P L K S F R
 621 TTT AAC AAA TCC AAA GAA ACT CCT ACA GAT TTT TAT GAA ATC AAA
 F N K S K E T P T D F Y E I K
 666 ACC GGT CAC TTG AAC AGC CGT ACT CCC TGG TGG GAC GGA AGT GTA
 T G H L N S R T P W W D G S V
 711 ATT TAT GGA AGT AAC GAG GAT GTT TTG AAG AAA GTG AGA ACA TTT
 I Y G S N E D V L K K V R T F
 756 AGA GAC GGA AAA CTG AAA TTA GGT GAA AAT GGA CTC ATC CAA CAA
 R D G K L K L G E N G L I Q Q
 801 GAT GAA AAT GGA AAA ATT ATC TCT GGT GAT GTT CGT AAC ACT TGG
 D E N G K I I S G D V R N T W
 846 GCT GGA CTT TTA ACG CTT CAA GCT CTC TTT GTT CAA GAG CAC AAT
 A G L L T L Q A L F V Q E H N
 891 GCT GTT TGT GAC ACT TTG AAG AAA GAA TAT CCA GAA TTA GAG GAT
 A V C D T L K K E Y P E L E D
 936 GAA GAG TTG TAT CGT CAT GCA AGG CTA GTC ACT TCA GCT GTA ATT
 E E L Y R H A R L V T S A V I
 981 GCA AAA GTT CAC ACC ATA GAT TGG ACT GTT CAG CTT CTG AAA ACC
 A K V H T I D W T V Q L L K T
 1026 GAT ACT ATG CTT GCA GGA ATG CGT GCC AAT TGG TAT GGA TTA CTA
 D T M L A G M R A N W Y G L L
 1071 GGA AAG AAG TTC AAG GAT ACA TTT GGT CAT GTT GGT TCC ATT TTA
 G K K F K D T F G H V G S I L

1116 AGT GGT GTT GTT GGA ATG AAG AAA CCT GAG AAT CAT GGA GTG CCT
S G V V G M K K P E N H G V P
1161 TAT TCC TTA ACT GAA GAA TTT ACG AGT GTT TAT AGA ATG CAT CAA
Y S L T E E F T S V Y R M H Q
1206 CTG TTA CCT GAT ACA CTT CAG CTA AGA AAT ATA GAT GCC ACG CCT
L L P D T L Q L R N I D A T P
1251 GGG CCA AAC AAA TCT CTT CCT TTA ACT AAT GAA ATT CCC ATG GAA
G P N K S L P L T N E I P M E
1296 GAA GTA GTT GGG AGT AAA GGA AAA GAG AAT TTA TCA AGA ATT GGG
E V V G S K G K E N L S R I G
1341 TTT ACT AAG CAA ATG GTT TCA ATG GGG CAT CAA GCT AGT GGA GCT
F T K Q M V S M G H Q A S G A
1386 CTT GAG CTT TGG AAT TAT CCA GTG TGG ATG AGA GAT CTT ATT GCC
L E L W N Y P V W M R D L I A
1431 CAA GAT GTT GAT GGA ACA GAC AGG CCA GAT CCT ATT GAC CTT GCA
Q D V D G T D R P D P I D L A
1476 GCT CTT GAA ATT TAT **AGG GAT AGA GAA AGA AGT GTT** CCT AGG TAC
A L E I Y R D R E R S V P R Y
1521 AAT GAC TTT AGA AGA GGA ATG CTT CAA ATT CCT ATT TCG AAA TGG
N D F R R G M L Q I P I S K W
1566 GAA GAT TTG ACA GAT GAT GAA GAA GCA ATC AAA ACA CTT GGT GAA
E D L T D D E E A I K T L G E
1611 GTA TAT GAT GAT GAT ATA CAA GAG TTG GAT TTA TTA GTG GGA CTC
V Y D D D I Q E L D L L V G L
1656 ATG GCG GAG AAA AAA ATT AAA GGA TTT GCC ATT TCA GAA ACA GCC
M A E K K I K G F A I S E T A
1701 TTC AAC ATA TTC CTT CTC ATG GCT ATA AGG AGG TTA GAG GCA GAT
F N I F L L M A I R R L E A D
1746 AGA TTT TTC ACA AGC AAT TAC AAC GAT GAG ACA TAC ACA AAG AAA
R F F T S N Y N D E T Y T K K
1791 GGA TTA GAA TGG GTG AAT ACT ACT GAG AGT TTA AAA GAT GTG TTA
G L E W V N T T E S L K D V L
1836 GAT CGT CAT TAT CCA GAA ATG ACT GAT AAA TGG ATG AAT TCA AAC
D R H Y P E M T D K W M N S N
1881 AGT GCC TTC TCT GTT TGG GAT TCT TCT CCA CAA CCT CAT AAT CCT
S A F S V W D S S P Q P H N P
1926 ATT CCA CTC TAT TTT CGT GTT CCT CAG **TAG** TAG ACC TCA AAA TCA
I P L Y F R V P Q *
1971 TGT GTG TAA CTA TTT ATT AAT ACC GTA ATA CGT ACA AGT TTG TAT
2016 TTA AGG TGT CTT GTT CTA TTT CAT CAA AAT AAT ATA TTG TAT CTT
2061 GGT CTG TGA AAA TAA CTA TTA GTT AGT AAT TCG TAT TAA AAA AAA
2106 AAA AAA AAA A

Table 4-1 Results of *LE α -DOX1* BLASTN search against the EST database (February, 2005).

BLAST result*	GenBank Accession Number	Bit score	E value
cDNA library			
Tomato callus	BI422699	1475	0
Tomato callus	AW034453	1354	0
Mixed of untreated and <i>P.infestans</i> -treated	BQ515396	1243	0
Potato tissues			
<i>P. infestans</i> -challenged potato leaves	BI432698	1237	0
Mixed of untreated and <i>P.infestans</i> -treated	BQ515397	1174	0
Potato tissues			
Tomato root deficiency	AW979675	1162	0
Tomato callus	AW217264	1130	0
Tomato callus	BI921854	1125	0
Tomato germinating seedling	AW647841	1088	0
Mixed of untreated and <i>P.infestans</i> -treated	BQ120526	918	0
Potato tissues			
<i>P. infestans</i> -challenged potato leaves	BG589935	910	0
Tomato callus	AW032281	819	0
Callus-derived suspension culture of potato	CN215878	684	0
JWS20 tomato salt-stressed roots	AW062238	456	e-124

*ESTs presented in this table are those with an E value equal to 0, with the exception of JWS20.

Table 4-2 Results of *LE α -DOX1* tBLASTX search against the NR database (February, 2005).

tBLASTX result*	GenBank Accession Number	Bit score	E value
cDNA library			
<i>N. attenuata</i> pathogen-inducible α -DOX	AF229926	1183	0
<i>N. tabacum</i> mRNA for oxygenase	AJ007630	1172	0
<i>C. annuum</i> cyclooxygenase-like protein	AY040869	1104	0
<i>P. sativum</i> mRNA for α -DOX1	AJ784963	178	3e-41
<i>P. balsamifera</i> DNA	AC149545	168	4e-38
<i>C. arietinum</i> partial mRNA for α -DOX	AJ487467	113	2e-21
<i>M. truncatula</i> clone mth2-9b23	AC139526	94	2e-15
<i>A. thaliana</i> feebly like protein	AY064666	92	7e-15
<i>A. thaliana</i> α -DOX1	AF334402	76	4e-10
<i>O. sativa</i> α -DOX	AF229813	66	4e-07

*cDNA clones presented in this table are those with an E value equal to or less than 4e-07.

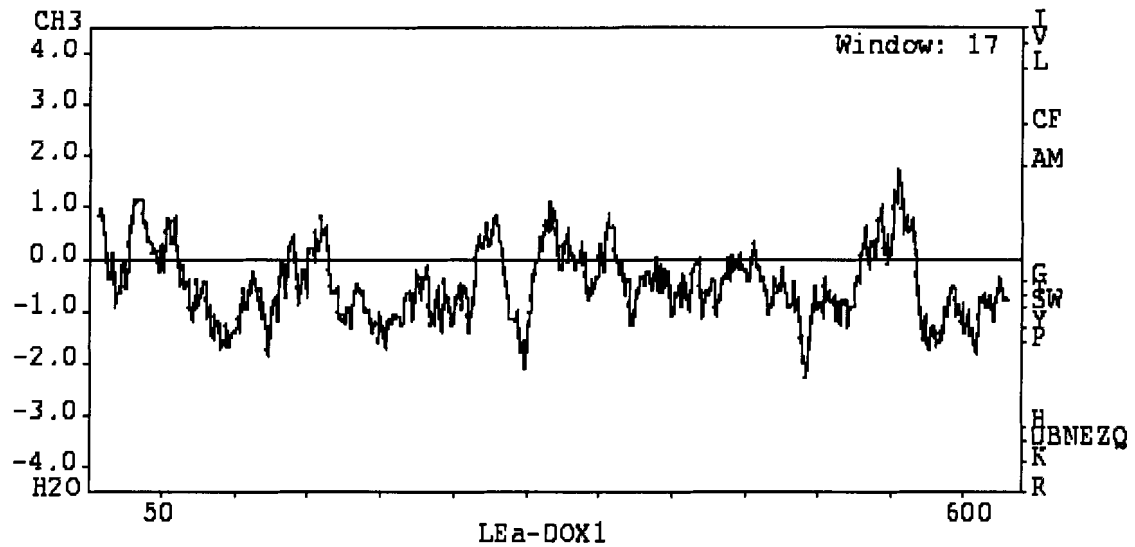


Figure 4-2 The hydropathy plot of *LEα-DOX1*.

The hydropathy profile of the *LEα-DOX1* deduced protein was calculated using the Kyte and Doolittle (1982) method with a window length of 17 amino acid residues. The x axis shows the amino acid residue number and the y axis shows the hydropathy value. Positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions. A hydropathy index greater than 1.6 is considered to be a significant transmembrane domain.

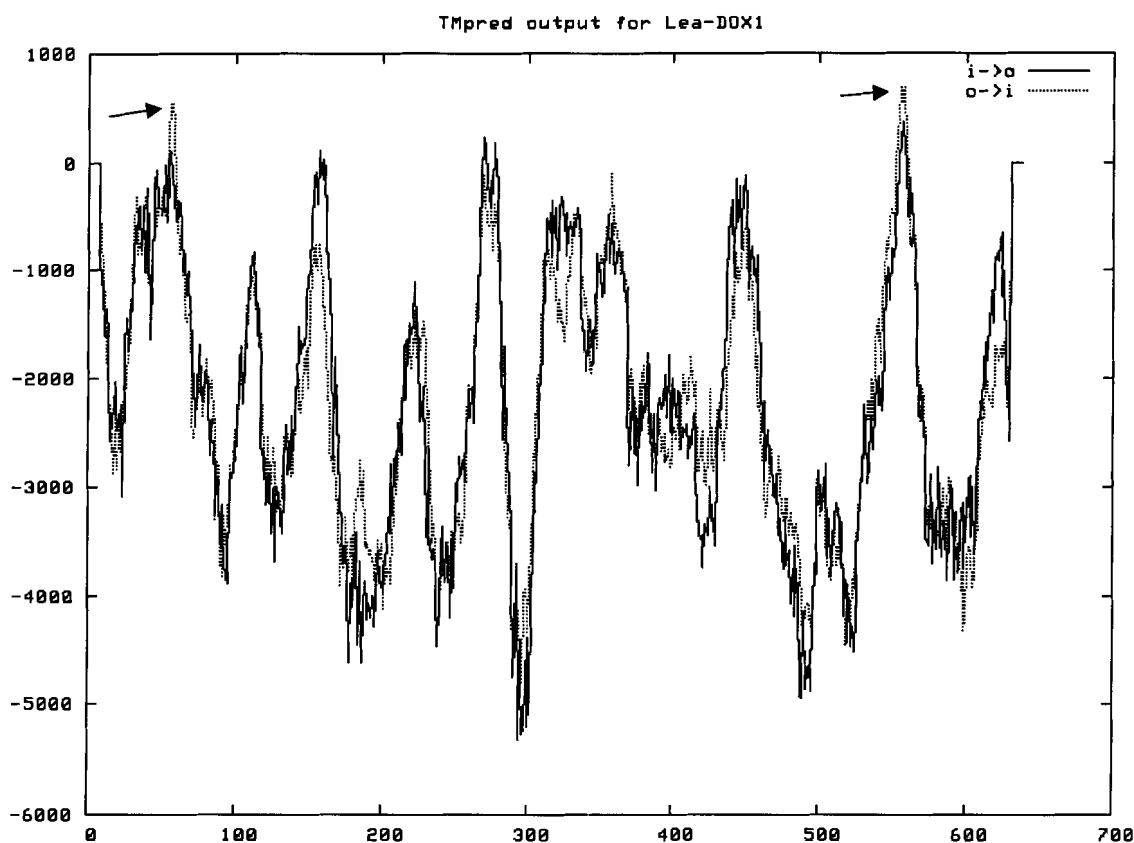


Figure 4-3 Transmembrane prediction of *LE α -DOX1*-encoded protein

The x axis indicates the amino acid residue number, and the y axis indicates the predicted TM value (a value larger than 500 is considered significant for a transmembrane region) . The *LE α -DOX1*-encoded protein contains two potential transmembrane helices: amino acid residue 47-67 and 548-564, with a score of 560 and 703, respectively (Hoffmann and Stoffel, 1993).

has a 40 amino acid deletion at the N terminus, and further examination of the nucleotide sequence revealed the presence of an intron. A pair of primers, (JWS20 control 5' AAA ATG AAT CTC CGC GAC A, and JWS20 outer 5' AAG AGC GCT TGC AGT GTT CA 3') were designed to determine whether the cLEW26H11 clone corresponded to a viable transcript (Figure 4-4). Using cDNA made from RNA isolated from 24h pathogen-treated roots with a FLU pretreatment, RT-PCR was performed to generate a product that covered both the deletion and intron. This RT-PCR product was sequenced, and the nucleotide sequence obtained contained no deletion or intron, indicating that cLEW26H11 corresponds to a viable transcript. The corrected nucleotide sequence of cLEW26H11 is shown in Figure 4-4. It encodes an α -DOX-like polypeptide, and hereafter will be referred to as *LE α -DOX2* (GenBank accession AY344540). The ORF of *LE α -DOX2* consists of 642 amino acids with a predicted molecular weight of 87 kDa. In addition, it contains 48 nucleotides in the 5' UTR and 154 nucleotides in the 3' UTR including the stop codon and the poly A tail. BLASTN searches against the EST database shows that the nucleotide sequence of *LE α -DOX2* shares high similarity with the nucleotide sequences of tomato and potato-derived ESTs (Table 4-3). Against the non-redundant database, the nucleotide sequence of *LE α -DOX2* shares high sequence similarity to α -DOX from various plants (Table 4-4). A Kyte and Doolittle hydrophathy profile (Kyte and Doolittle, 1982) revealed that the protein encoded by *LE α -DOX2* is mostly hydrophilic, except for the region that corresponds to amino acids 544-563 (Figure 4-5). The TM topology (Hoffmann and Stoffel, 1993) prediction

Figure 4-4 The nucleotide and deduced amino acid sequences of *LE α -DOX2*.

The deduced single-letter amino acid sequence is given below the center nucleotide of each codon. The location of the JWS20 nucleotide sequences within the *LE α -DOX2* sequence is italicized. A putative Kozak sequence (RCCATGR, in which R is a purine (A or G), Kozak, M., 1986) is both italicized and bolded. The initiating ATG, stop codon, JWS20 control and JWS20 outer primers are bolded and underlined.

1 CAC GAG GAT TTT AAT TAC TAT CAT ATA TAT ATA TAT AAA GTA GGA **AAC ATG** ACT ATG ATT
M T M I
61 ATG CTG AAG AAT CTC TTG TTC TCC CCT CTT CGT GGT TTC ATC CAC AAA GAT TTT CAT CAG
M L K N L L F S P L R G F I H K D F H Q
121 ATA CTT GAC **AAA ATG AAT CTC CGC GAC** AAA CTT TCA TTT CTG ATA GTT CAT ATC ATT GAT
I L D K M N L R D K L S F L I V H I I D
181 AAA CAT AAC TTG TGG CAC CGG GTA CCG GTG TTT TTA GGG CTA GTT TAT CTT GCA CTT CGT
K H N L W H R V P V F L G L V Y L A L R
241 CGA CAT CTT AAT CAG GAA TAT AAT TTG ATC AAC GTG GGT AGA ACA CCG AGC GGA GTC CGA
R H L N Q E Y N L I N V G R T P S G V R
301 TCG AAT CCG GGC GAC TTC CCT TAT AGA ACA GCA GAT GGA AAA TAT AAT GAT CCT TTC AAT
S N P G D F P Y R T A D G K Y N D P F N
361 GAA GGA GCA GGA AGT GAA TTT TCT TTC TTT GGG AGG AAT ATG ATG CCT GTT GAG CAA CAT
E G A G S E F S F F G R N M M P V E Q H
421 GAC AAG TTA AAG AAT CCA GAT CCA ATG GTA GTG GCA ACA AAG CTG CTA GCA CGG AGA GAA
D K L K N P D P M V V A T K L L A R R E
481 TTC ATA GAC ACT GGA AAA CAA TTT AAC ATG ATA GCT GCA TCA TGG ATA CAG TTT ATG ATT
F I D T G K Q F N M I A A S W I Q F M I
541 CAT GAT TGG ATC GAT CAT TTG GAA GAT ACT CAA CAG ATT GAG GAG CTT AGG GCA CCT GAA
H D W I D H L E D T Q Q I E E L R A P E
601 GAA GTT GCA AGT CAA TGT CCT CTC AAG TCC TTT AAG TTT TAC AAA TCA AAA GAA ACT CCT
E V A S Q C P L K S F K F Y K S K E T P
661 ACC GGA TTT TAC GAA ATT AAA ACC GGT CAT TTG AAT AGG CGT ACC CCT TGG TGG GAT GGA
T G F Y E I K T G H L N R R T P W W D G
721 AGT GTT ATT TAT GGG AGC AAC GTA GAG ATC TTG AAG AAA GTA AGG ACA TTT AAA GAC GGA
S V I Y G S N V E I L K K V R T F K D G
781 AAA TTG AAA CTA TCA GAA AAT GGA CTC CTT GAA CAA GAT GAA AAT GGA AAA ATT ATA TCT
K L K L S E N G L L E Q D E N G K I I S
841 GGT GAT GTT CGC AAT ACT TGG GCT GGA TTT **GTA ACA CTG CAA GCG CTC TTT GTA** CAA GAG
G D V R N T W A G F V T L Q A L F V Q E
901 CAT AAT CTT GTT TGT GAT GTC TTG AAG AAA GAA TAT CCA GAA TTG GAA GAT GAA GAG TTG
H N L V C D V L K K E Y P E L E D E E L
961 TAT CGT CAT GCA AGA CTG GTG ACT TCT GCT GTT ATT GCA AAA GTT CAC ACA ATA GAT TGG
Y R H A R L V T S A V I A K V H T I D W
1021 ACT GTC GAG CTT CTT AAA ACA GAC ACC TTA TTT GCA GCA ATG CGA ACC AAT TGG TAT GGA
T V E L L K T D T L F A A M R T N W Y G
1081 TTG CTA GGG AAG AAA TTT AAA GAT ACA TTT GGG CAC GTT GGA GGT GCA ATT TTG GGT GGT
L L G K K F K D T F G H V G G A I L G G
1141 TTA GTA GGA CTA AAA AAA CCC GAA AAT CAT GGA GTG CCT TAT TCC TTA ACT GAA GAA TTT
L V G L K K P E N H G V P Y S L T E E F
1201 GTG AGT GTG TAT CGA ATG CAT CAA CTC TTA CCT GAT AAA CTT CAG TTA AGA AAT ATA GAT
V S V Y R M H Q L L P D K L Q L R N I D
1261 GCA ACT TCT GGA CCA AAT AAA TCT ATC CCT TTG ACT AAC GAA ATC CCA ATG GGA GAT TTA
A T S G P N K S I P L T N E I P M G D L
1321 ATC GGA GGC AAA GGA GAG GAG AAT TTA TCA AGA ATC GGA TTT ACT AAG CAG ATG GTT TCA
I G G K G E E N L S R I G F T K Q M V S
1381 ATG GGG CAT CAA GCA TGT GGA GCT CTT GAG CTT TGG AAT TAT CCA ATA TGG ATG AGG GAT
M G H Q A C G A L E L W N Y P I W M R D

1441 CTT ATT GCT CAA GAT GTT GAT GGA ACA GAC AGG CCA CAT CAT GTT GAC CTT GCA GCA CTC
L I A Q D V D G T D R P H H V D L A A L
1501 GAA ATT TAT AGA GAC AGA GAA AGA AGT GTT GCT AGG TAC AAT GAA TTT CGA AGG AGA ATG
E I Y R D R E R S V A R Y N E F R R R M
1561 TTG CAA ATT CCC ATC ACT AAA TGG GAA GAT TTG ACG GAT GAT ATG GAA GTT ATT AAA ACA
L Q I P I T K W E D L T D D M E V I K T
1621 CTT CAT GAA GTT TAT GGT GAT GAT GTA GAA CAA TTG GAT CTG TTA GTT GGA ATG TCC GCG
L H E V Y G D D V E Q L D L L V G M S A
1681 GAG AAA AAA ATT AAA GGA TTT GCC ATC TCT GAG ACT GCA TTT TTC ATA TTC CTT CTC ATG
E K K I K G F A I S E T A F F I F L L M
1741 GCA TCA AGG AGG TTA GAG GCA GAT AGA TTT TTC ACA AGC AAT TAC AAC GAG GAG ACA TAC
A S R R L E A D R F F T S N Y N E E T Y
1801 ACA AAG AAA GGA TTA GAA TGG GTG AAT ACT ACT GAG AGT TTG AAA GAT GTG TTA GAT CGT
T K K G L E W V N T T E S L K D V L D R
1861 CAT TAT CCA GAA ATG ACT GAT AAA TGG ATG AAT TCA AAC AGT GCC TTC TCT GTT TGG GAT
H Y P E M T D K W M N S N S A F S V W D
1921 TCT TCT CCA CAA CCT CAC AAT CCT GTT CCA CTC TAC TTT CGT GTT CCT AAA CAT TAA ACT
S S P Q P H N P V P L Y F R V P K H *
1981 AAT AAT ACC TAA TTT ATT TAC TTA TCG AAA ATA AAA AAA AAA AAA AAA AAT TGT GTG GAA
2041 AAA AAA AAA AAT TTC CGT TTT TAC GTA ATT GTT TCA GTT TGT TAA ATT AAT GAT GTG GAT
2101 CCA TCC ATC TTT AAT TAA AGG TTC CTG G

Table 4-3 Results of *LE α -DOX2* BLASTN search against the EST database (February, 2005).

BLAST result*	GenBank Accession Number	Bit score	E value
cDNA library			
Tomato root	BE450706	1118	0
Tomato root	BE450352	967	0
Tomato root	BE450707	876	0
Potato <i>in vitro</i> root	CO502639	848	0
Tomato root	AW224574	848	0
Tomato root	AW224575	735	0
Potato mixed tissues of untreated and	BQ515397	678	0
<i>P. infestans</i> -treated			
JWS20 tomato salt-stressed roots	AW062238	456	e-124

*ESTs presented in this table are those with an E value equal to 0, with the exception of JWS20.

Table 4-4 Results of *LE α -DOX2* tBLASTX search against the non-redundant database (February, 2005).

TBLASTN result*	Gen Bank Accession Number	Bit score	E value
cDNA library			
<i>N. attenuata</i> pathogen-inducible α -DOX	AF229926	1534	0
<i>C. annuum</i> cyclooxygenase-like protein	AY040869	1481	0
<i>N. tabacum</i> mRNA for oxygenase	AJ007630	1407	0
<i>P. balsamifera</i> DNA	AC149545	184	6e-43
<i>C. arietinum</i> partial mRNA for α -DOX	AJ487467	137	1e-28
<i>M. truncatula</i> clone mth2-9b23	AC139526	96	4e-16
<i>O. sativa</i> α -DOX	AF229813	84	2e-12
<i>A. thaliana</i> α -DOX1	AY064666	74	2e-09
<i>L. esculentum</i> α -DOX 3	AJ850958	70	2e-08
<i>L. esculentum</i> fruit mRNA	BT013520	70	2e-08

*cDNAs presented in this table are those with E value equal to or less than 2e-08.

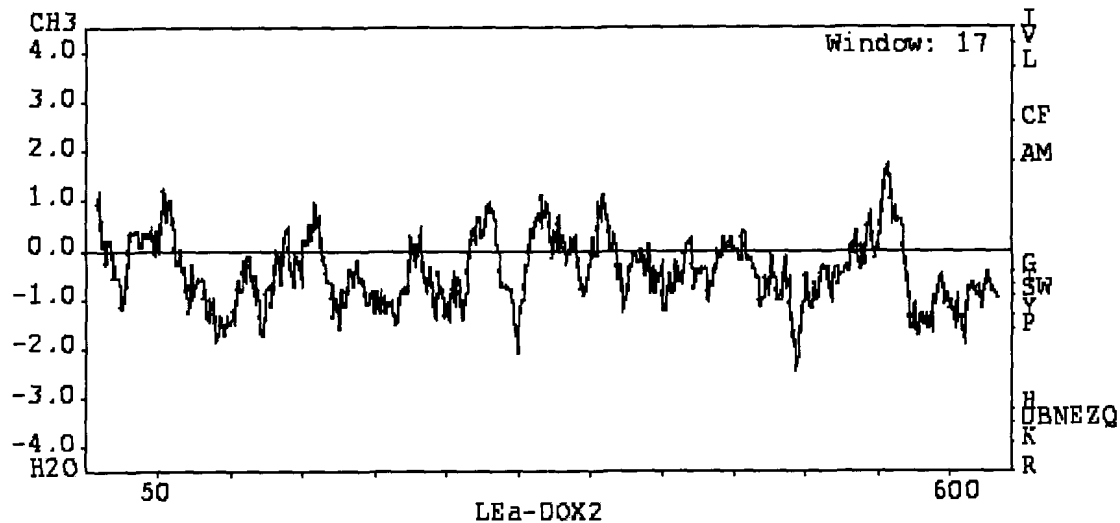


Figure 4-5 The hydropathy plot of *LEα-DOX2*.

The hydropathy profile of the *LEα-DOX2* deduced protein was calculated using the Kyte and Doolittle (1982) method with a window length of 17 amino acid residues. The x axis shows the amino acid residue number and the y axis shows the hydropathy value. Positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions. A hydropathy index greater than 1.6 is considered to be a significant transmembrane domain.

suggests that the the protein encoded by *LE α -DOX2* has one transmembrane helice and that its N-terminus is located outside the cell, and its C-terminal is located within the cytosol (Figure 4-6). Signal IP prediction showed that *LE α -DOX2*-encoded protein contains no signal peptide (Bendtsen et al., 2004).

The complete sequence of the cTOD20F16 clone contains an insert of 2128 nucleotides with an ORF that encodes a polypeptide of 632 amino acids (Figure 4-7) that has a predicted molecular weight of 85 kDa. In addition, it contains 49 nucleotides in the 5' UTR and 61 nucleotides in the 3' UTR including the stop codon. The cTOD20F16 clone hereafter will be renamed *LE α -DOX3* (BK001477). BLASTN searches against the EST database shows that the nucleotide sequence of *LE α -DOX3* shares high similarity to the nucleotide sequences of tomato and potato-derived ESTs (Table 4-5). Against the non-redundant database, the nucleotide sequence of *LE α -DOX3* shares high sequence similarity to α -DOX from various plants (Table 4-6). A Kyte and Doolittle hydropathy profile (Kyte and Doolittle, 1982) revealed that the protein encoded by the third α -DOX gene is mostly hydrophilic, except for regions that correspond to amino acids 11-30 and 534-554 (Figure 4-8). The TM topology (Hoffmann and Stoffel, 1993) prediction suggests that the the protein encoded by *LE α -DOX3* has two transmembrane helices, and that both its N- and C-terminus is located outside the cell (Figure 4-9). Signal IP prediction showed that the protein encoded by *LE α -DOX3* contains no signal peptide (Bendtsen et al., 2004).

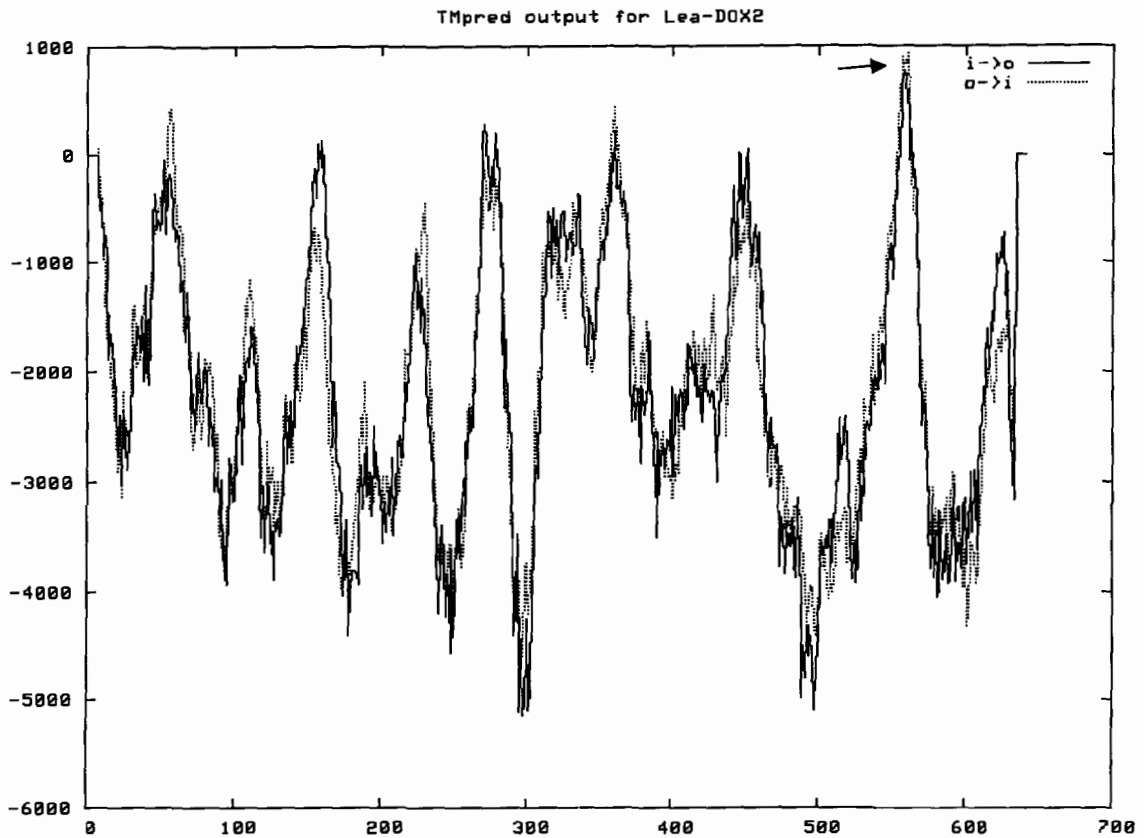


Figure 4-6 Transmembrane prediction of *LE α -DOX2*-encoded protein

The x axis indicates the amino acid residue number, and the y axis indicates the predicted TM value (a value larger than 500 is considered significant for a transmembrane region) . The *LE α -DOX2*-encoded protein contains one potential transmembrane helice, amino acid residue 550-571, with a score of 943 (Hoffmann and Stoffel, 1993).

Figure 4-7 The nucleotide and deduced amino acid sequences of *LE α -DOX3*.

The deduced single-letter amino acid sequence is given below the center nucleotide of each codon. A putative Kozak sequence (***RCCATGR***, in which R is a purine (A or G), Kozak, M., 1986) is both italicized and bolded. The initiating ATG and stop codon, are bolded and underlined.

1 G CTT

5 GAA TCC TTT TGA CTT ATT TGA CAG GTC AAC TGT TGA GAA ACT CTC

51 **ATG** GCA TTC TCA ATT TCT CTT CCA GAC TTT GTT CAT CCT CAA CTC
M A F S I S L P D F V H P Q L

96 CGG CAT GTT GTT GCA AAG ATG TCT TTC TTT GAC ACA ATT TTG TTC
R H V V A K M S F F D T I L F

141 TAT GTT GTA CAT CTT GTG GAC AAG TTT GAC TTA TGG CAT AGA TTG
Y V V H L V D K F D L W H R L

186 CCG GTG TTA CTG GGG GCA GCT TAC CTA GGA ATA AGA AGA CAC TTG
P V L L G A A Y L G I R R H L

231 CAC CAG CGA TAC AAT CTT TTA CAT GTA GGG AAA GTT AAT GGC AAG
H Q R Y N L L H V G K V N G K

276 AAA TAT GAC ACA GAA GAG TTT ACC TAT CGG ACT GCT GAT GGT TCA
K Y D T E E F T Y R T A D G S

321 TGC AAT CAT CCT GTG GAT CAT TTA GTG GGC AGT CAA GGA ACC TTC
C N H P V D H L V G S Q G T F

366 TTT GGC CGC AAC ATG CTA CCA TCC ACT TCA AGT TAT GCG CTG CTG
F G R N M L P S T S S Y A L L

411 GAA CCT CAC CCT GTA ACA GTG GCC TCC AAG CTC TTG GAA AGA AGA
E P H P V T V A S K L L E R R

456 AAA TAC ACA GAT TGT GGG GGC CAA TTC AAC ATG ATA GCG TGC GCG
K Y T D C G G Q F N M I A C A

501 TGG TTA CAA TTT ATG ATC CAT GAT TGG AAT GAC CAT ATG GAC GAC
W L Q F M I H D W N D H M E D

546 ACT GAA CAG GTG GAG CTT AGA GCT CCT CAA GAC GTT GCA GCA GGG
T E Q V E L R A P Q D V A A G

591 TGT CCG TTG AAG TCA TTT AAG TTC CTT AAA ACC AAA AAC TTT CCC
C P L K S F K F L K T K N F P

636 ACA GGT TCA CCT GAT CTG AAG TTT GGG CAT TTG AAT TCA AGG ACC
T G S P D L K F G H L N S R T

681 CCA TGG TGG GAT GGG AGT GTA ATA TAT GGA AAC AAC GAA GAG GGA
P W W D G S V I Y G N N E E G

726 ATG ATA AGG GTG AGA AGA TTT AAA GAT GGA AAG CTC AGG CTC TCA
M I R V R R F K D G K L R L S

771 GGT GAT GGA CTT CTG GAA CAT GAT GAT AAA GGC ATT CCA ATA TCT
G D G L L E H D D K G I P I S

816 GGA GAT GTC CGA AAC TAT TGG GCA GGC TAC TCT CTC TTG CAG GCC
G D V R N Y W A G Y S L L Q A

861 TTG TTT GTG AAG GAA CAT AAT GCC ATA TGT GAT ATG CTT AAG GAA
L F V K E H N A I C D M L K E

906 CAT TAC CCT GAA TTT GAT GAT GAA AAG GTT TAC CGA CAT GCA AGA
H Y P E F D D E K V Y R H A R

951 CTG ATA ACT TCA GCG GTC ATT GCA AAA ATC CAT ACT ATT GAT TGG
L I T S A V I A K I H T I D W

996 ACG CTT GAA ATT GTT AAG ACT GAT ACT CTT ATG GCA GGA ATG AGA
T L E I V K T D T L M A G M R

1041 ATC AAC TGG TAT GGC TTG TTG GGG AAG AGA GTC AAG GAT TTG TTA
I N W Y G L L G K R V K D L L

1086 GGA CCC AAA TTT GGA CCA GTA TTG AGT GGC TTA GTT GGT CTT AAA
G P K F G P V L S G L V G L K

1131 AAG CCT AGA GAT CAC GGA ACT CCC TAC TCA TTG ACT GAA GAG TTC
K P R D H G T P Y S L T E E F

1176 GTT AGC GTC TAC AGA ATG CAC TCA CTT TTA CCT GAC ACG ATT GTT
V S V Y R M H S L L P D T I V

1221 CTG AGG GAC CTG AAG TCG ACT ACA TCA GAA GAC AAA TCC TTG CCT
L R D L K S T T S E D K S L P
1266 ATT CAA GAT GAG ATT CCT ATG AGG GAA ATG ATA GGG AAA GAA GGA
I Q D E I P M R E M I G K E G
1311 GAA AAG AAC TTG TCG AAA ATC GGT ATG GAG CAG ATG CTA GTA TCG
E K N L S K I G M E Q M L V S
1356 ATG GGT CAC CAG TCA TCT GGA GCT GGT ACA TTG TGG AAT TTT CCA
M G H Q S S G A G T L W N F P
1401 TCA TGG ATG AGG AAC CTT GTT CCT CAT GAT ATC GAT GGA GAT GAT
S W M R N L V P H D I D G D D
1446 AGA CCT GAT TCA ATT GAC ATG GCC GCC TTG GAC ATT TAT AGA GAC
R P D S I D M A A L D I Y R D
1491 AGA GAG AGG GGA GTT CCG CGG TAC AAT GAG TTC AGA AGG AAT TTG
R E R G V P R Y N E F R R N L
1536 CTG ATG GTA CCA ATT AGC AAG TGG GAG GAT TTA ACT AAC GAC GAA
L M V P I S K W E D L T N D E
1581 GAA GTA ATT GAG GCT TTA CAA GAA GTA TAT GGC GAT GAT ATT GAG
E V I E A L Q E V Y G D D I E
1626 AAG CTA GAT CTC CAA ATT GGT TTG CAC GCG GAG AAG AAA ATT AAA
K L D L Q I G L H A E K K I K
1671 GGC TTT GCC ATT AGT GAG ACT GCC TTT TTT ATA TTT CTG CTC ATT
G F A I S E T A F F I F L L I
1716 GCT TCA AGG AGG TTA GAG GCT GAT CGA TTC TTC ACA ACT GAT TTC
A S R R L E A D R F F T T D F
1761 AAT TCG CGA ACC TAC ACA GAG AAA GGC TTT GAA TGG GTA AAC AAG
N S R T Y T E K G F E W V N K
1806 ACA GAG ACA TTG AAA GAT GTC ATC GAT CGA TAC TTC CCT GAA ATG
T E T L K D V I D R Y F P E M
1851 ACA GAG AAA TAC ATG AGA TGC ACA AGT GCA TTC TCA GTG TGG AGT
T E K Y M R C T S A F S V W S
1896 TCA GAT CCA GAT CCT AAA CAC TAC TTA CCT CTT TAT CTG AGA CCA
S D P D P K H Y L P L Y L R P
1941 GCA ACC TAA TGG TAG TAC TAT ATT TGG TGA TCA ACT TTG TAT GGG
A T *
1996 GTG ATT ATG TTA AAA CAA TGG T

Table 4-5 Results of *LE α -DOX3* BLASTN search against the EST database (February, 2005).

BLAST result*	GenBank Accession Number	Bit score	E value
cDNA library			
Potato abiotic stress (leaves and roots)	CK267946	1411	0
Tomato flower	BI934764	1289	0
Tomato ovary	AI489322	1283	0
Tomato ovary	AI489252	1241	0
Tomato flower	BI934766	1189	0
Potato roots	BM112529	1174	0
Tomato flower buds	BE354006	1094	0
Potato swollen stolon	CV302863	1065	0
Potato sprouting eyes	BI175977	1037	0
Tomato <i>Pseudomonas</i> Susceptible leaves	CN385174	991	0
Tomato roots	CN385174	971	0
Potato abiotic stress (leaves and roots)	CK267947	963	0
Tomato shoot/meristem	BG124577	926	0
Potato sprouting eyes	BG593366	823	0
Potato sprouting eyes	BG598877	785	0
Potato roots	BM404158	656	0

*ESTs presented in this table are those with an E value equal to 0.

Table 4-6 Results of *LE α -DOX3* tBLASTX search against the NR database (February 2005).

BLASTN result*	GenBank Accession Number	Bit score	E value
cDNA library			
<i>L. esculentum</i> α -DOX 3	AJ850958	3901	0
<i>L. esculentum</i> fruit mRNA	BT013520	2349	0
<i>L. esculentum</i> feebly	U35643	2068	0
<i>L. esculentum</i> feebly	U35644	432	e-117
<i>L. esculentum</i> feebly	AY345858	151	8e-33
<i>A. thaliana</i> feebly like protein	AY128743	100	3e-17
<i>M. truncatula</i> clone mth2-9b23	AC139526	74	1e-09
<i>P. sativum</i> mRNA for α -DOX1	AJ784963	62	6e-06
<i>N. tabacum</i> mRNA for oxygenase	AJ007630	62	6e-06
<i>P. balsamifera</i> DNA	AC149545	60	2e-05

*cDNAs presented in this table are those with an E value equal to or less than 2e-05.

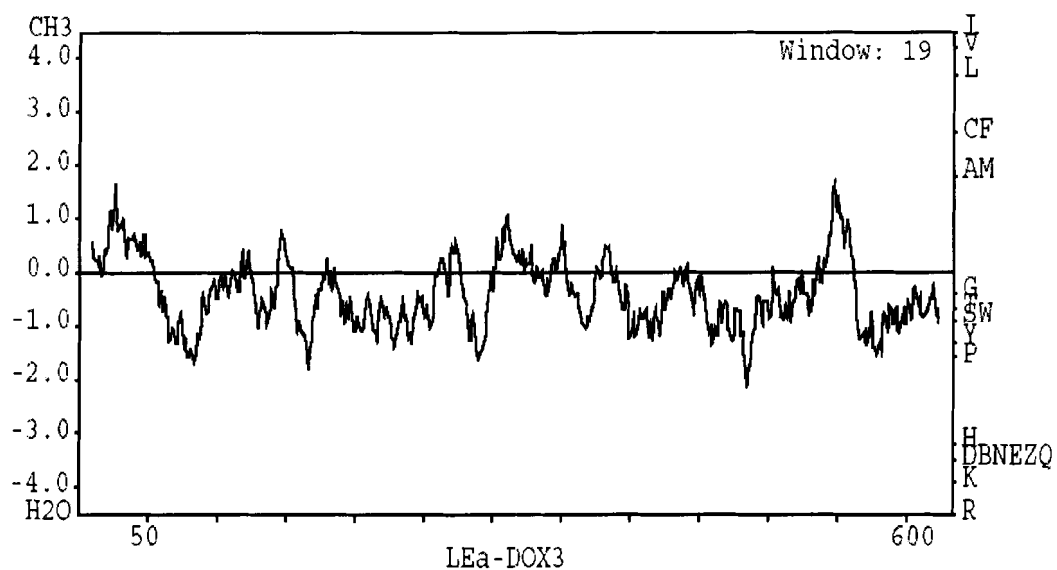


Figure 4-8 The hydropathy plot of *LEα-DOX3*.

The hydropathy profile of the *LEα-DOX3* deduced protein was calculated using the Kyte and Doolittle (1982) method with a window length of 19 amino acid residues. The x axis shows the amino acid residue number and the y axis shows the hydropathy value. Positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions. A hydropathy index greater than 1.6 is considered to be a significant transmembrane domain.

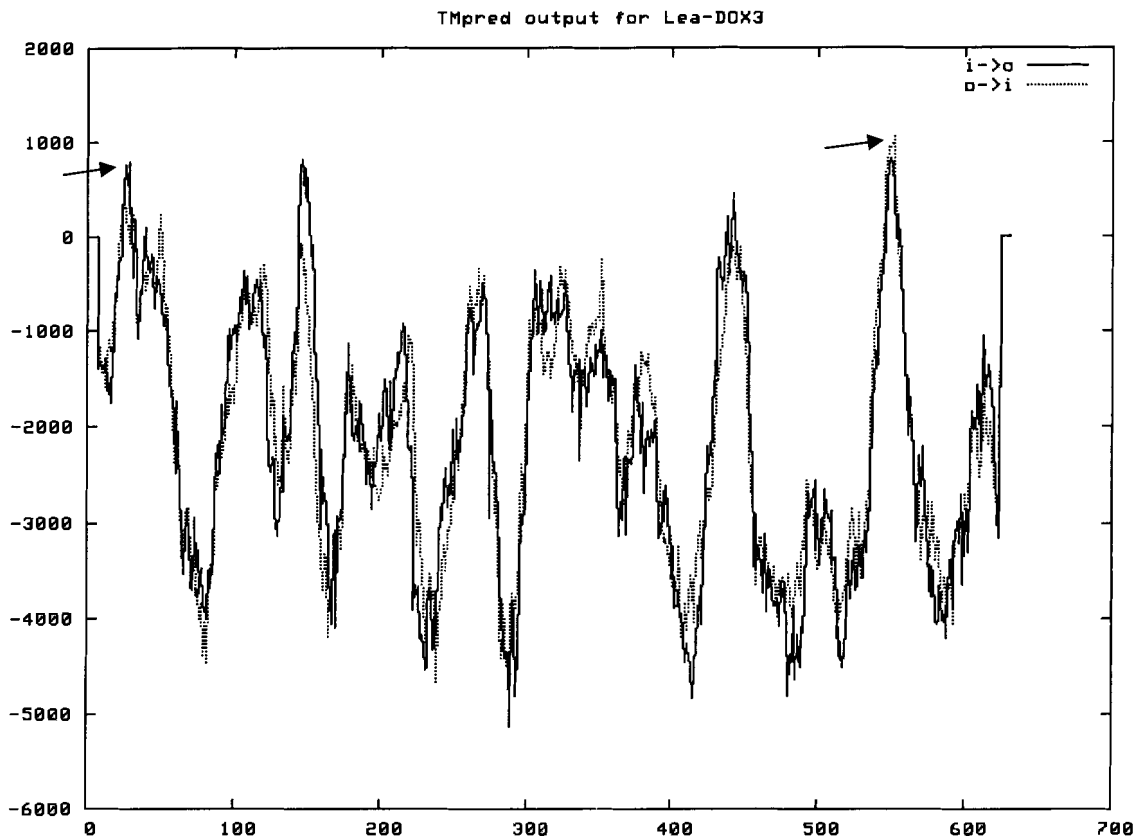


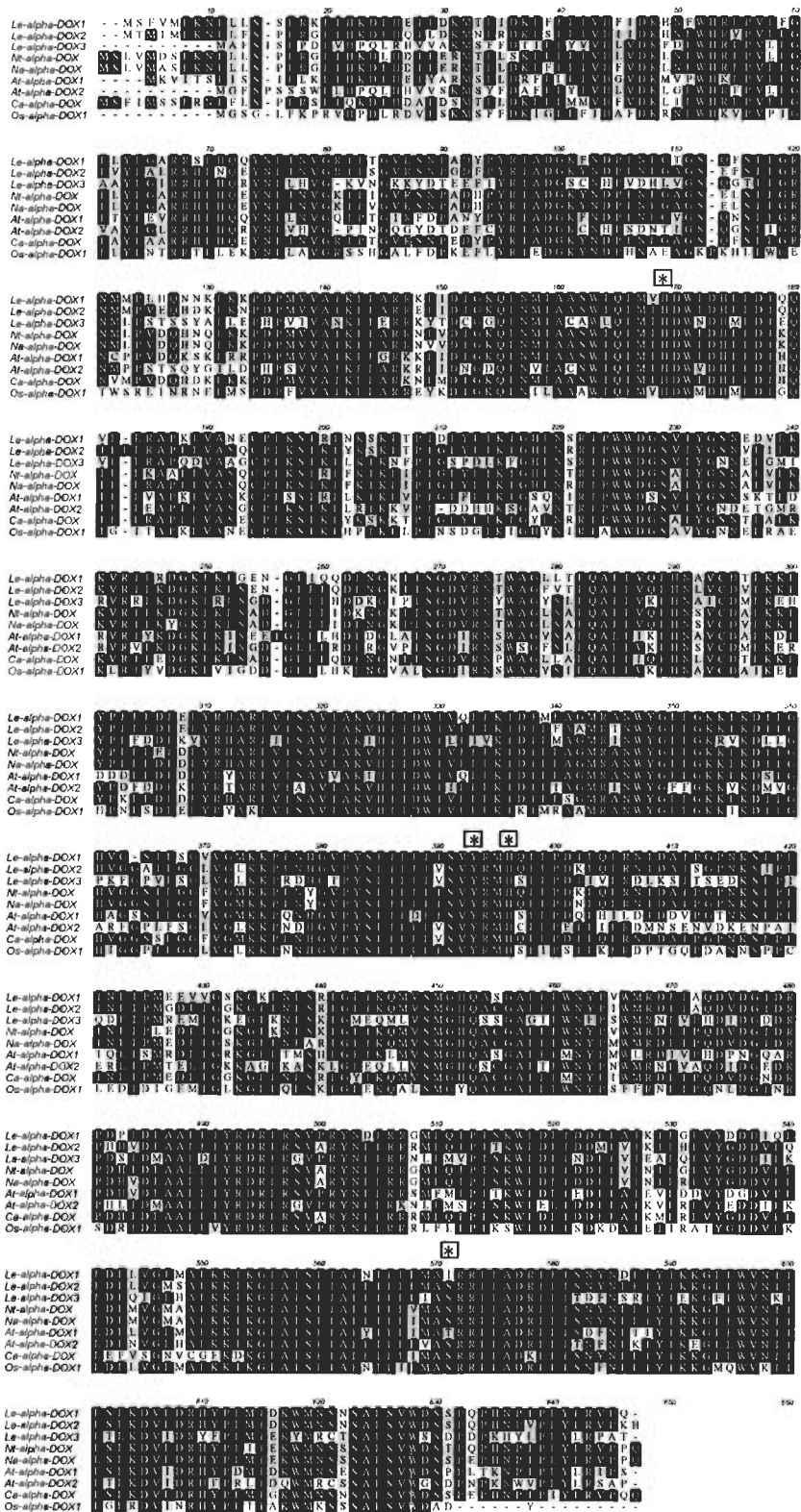
Figure 4-9 Transmembrane prediction of *LE α -DOX3*-encoded protein

The x axis indicates the amino acid residue number, and the y axis indicates the predicted TM value (a value larger than 500 is considered significant for a transmembrane region) . The *LE α -DOX3*-encoded protein contains two potential transmembrane helices, amino acid residue 18-37 and 541-562, with a score of 796 and 1076, respectively (Hoffmann and Stoffel, 1993).

The nucleotide sequences of *LE α -DOX1* and *LE α -DOX2* were very similar to each other (85% identity) whereas *LE α -DOX3* was less similar (63% and 66% identity to *LE α -DOX1* and *LE α -DOX2*, respectively). BLASTN searches against the TIGR Tomato Gene Index database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato) shows that *LE α -DOX1* corresponds to TC126269, *LE α -DOX2* to TC127979, TC122317, and the singleton BF098372, and *LE α -DOX3* corresponds to TC119265, TC119649, and BG124577. BLASTN searches against TIGR tomato gene index database also shows that a potential fourth α -DOX gene exists, and is represented by BE432966. The potential *LE α -DOX4* gene shares 85% and 82% nucleotide sequence identity to *LE α -DOX1* and *LE α -DOX2*, respectively. Sequence similarity BLASTP searches with the nucleotide sequence of *LE α -DOX1* against the non-redundant databases revealed significant similarity to plant α -DOX sequences (Figure 4-10). The polypeptide encoded by *LE α -DOX1* shares high similarity with α -DOX from *Nicotiana attenuata* (85% identity), *N. tabacum* (84% identity), *Arabidopsis thaliana* (α -DOX1; 73% identity) and *Oryza sativa* (64% identity), a cyclooxygenase-like protein from *Capsicum annuum* (82% identity) and the feebly-like protein from *A. thaliana* (α -DOX2; 62% identity). *LE α -DOX3* corresponds to the *FEEBLY* gene isolated as a result of insertional mutagenesis of tomato (van der Biezen *et al.*, 1996; Meissner *et al.*, 2000), and it is more similar to the Arabidopsis feebly-like

Figure 4-10 Deduced amino acid sequence alignment of α -DOX1, -2 and -3 of *L. esculentum* and α -DOX from *N. tabacum*, *N. attenuata*, *A. thaliana*, *C. annuum* and *O. Sativa* .

Lycopersicon esculentum (Le-alpha-DOX1, Le-alpha-DOX2, Le-alpha-DOX3), α -DOX from *Nicotiana tabacum* (Nt-alpha-DOX GenBank accession: AJ007630), α -DOX from *N. attenuata* (Na-alpha-DOX: AF229926) α -DOX1 and -2 from *Arabidopsis thaliana* (At-alpha-DOX1: AF334402, At-alpha-DOX2: AAG52078), α -DOX from *Capsicum annuum* (Ca-alpha-DOX: AY040869), and α -DOX from *Oryza sativa* (Os-alpha-DOX: AAF64042). Identical amino acid residues are indicated by white letters on a black background, and conserved residues as black letters on a gray background. Amino acids involved in the α -DOX reaction mechanism are indicated by an asterisk.



(α -DOX2) protein than to *LE α -DOX1* (71% identity versus 63% identity, respectively).

Alpha-dioxygenases are heme enzymes that incorporate dioxygen into fatty acids and share structural similarity with mammalian prostaglandin-H synthases (PGHS). Amino acid residues involved in heme binding (His-165 and His-389 of *LE α -DOX1*), and initiating the oxygenation reaction (Tyr-386) are conserved in plant α -DOX presented in Figure 4-10, whereas a Ser residue involved in substrate binding (Ser-564) is not conserved in the *LE α -DOX1* or *Arabidopsis* α -DOX1 polypeptides (Figure 4-10).

4.3.2 Cross Hybridization of *LE α -DOX* Isoforms

In tomato, α -DOX belongs to a small gene family consist of at least three members: *LE α -DOX1*, *LE α -DOX2* and *LE α -DOX3*. There was high nucleotide sequence identity between *LE α -DOX1* and *LE α -DOX2* (85%) and less similarity between *LE α -DOX1* and *LE α -DOX3* (63%). To determine the extent of cross hybridization between the less similar *LE α -DOX1* and *LE α -DOX3* sequences, DNA dot blot analyses were performed. *LE α -DOX1* and *LE α -DOX3* cDNAs were blotted and hybridized against *LE α -DOX1*, and there was approximately 12% cross hybridization between the *LE α -DOX1* and *LE α -DOX3* cDNAs (Figure 4-11).

Due to the high degree of nucleotide sequence identity between *LE α DOX1* and *LE α -DOX2*, a gene specific probe for each isoform was

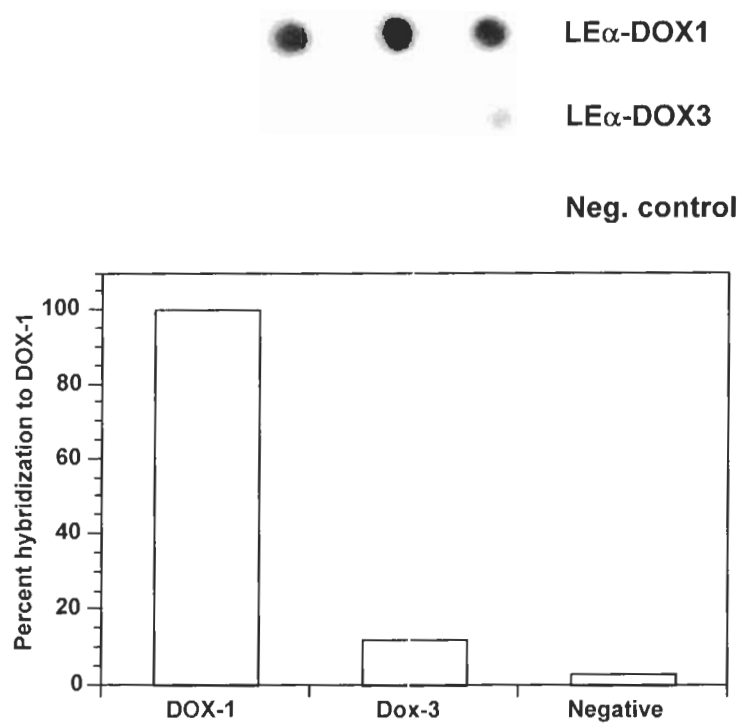


Figure 4-11 Cross-hybridization between $LE\alpha$ -DOX1 and $LE\alpha$ -DOX3.

$LE\alpha$ -DOX1 and $LE\alpha$ -DOX3 DNA (2 ng) was blotted in triplicate onto a nylon membrane together with a negative control (clone cLEG11M5 encoding a protein kinase-like protein, from CUGI). The plot shows percent hybridization obtained following hybridization with $LE\alpha$ -DOX1.

prepared from the 5'-end of the cDNA. Northern analyses using either the *LE α -DOX1* full-length or *LE α -DOX15'* probes resulted in a similar pattern of transcript accumulation. The expression pattern obtained with either the *LE α -DOX1* full-length or *LE α -DOX1 5'*-probes was distinctive from that obtained when using the *LE α -DOX2 5'*-probe or the *LE α -DOX3* full-length probe (Figures 4-13 and 4-16).

4.3.3 Genomic Southern Hybridization Analyses

Genomic southern analyses were performed in order to confirm the number of α -*DOX* genes in the tomato genome. Genomic DNA was digested with *EcoRI* (E), *XhoI* (X), *HindIII* (H), or *BamHI* (B). Southern blot analyses were performed using full-length *LE α -DOX1*, *cLEW26H11* and *LE α -DOX3* as probes (Figure 4-12).

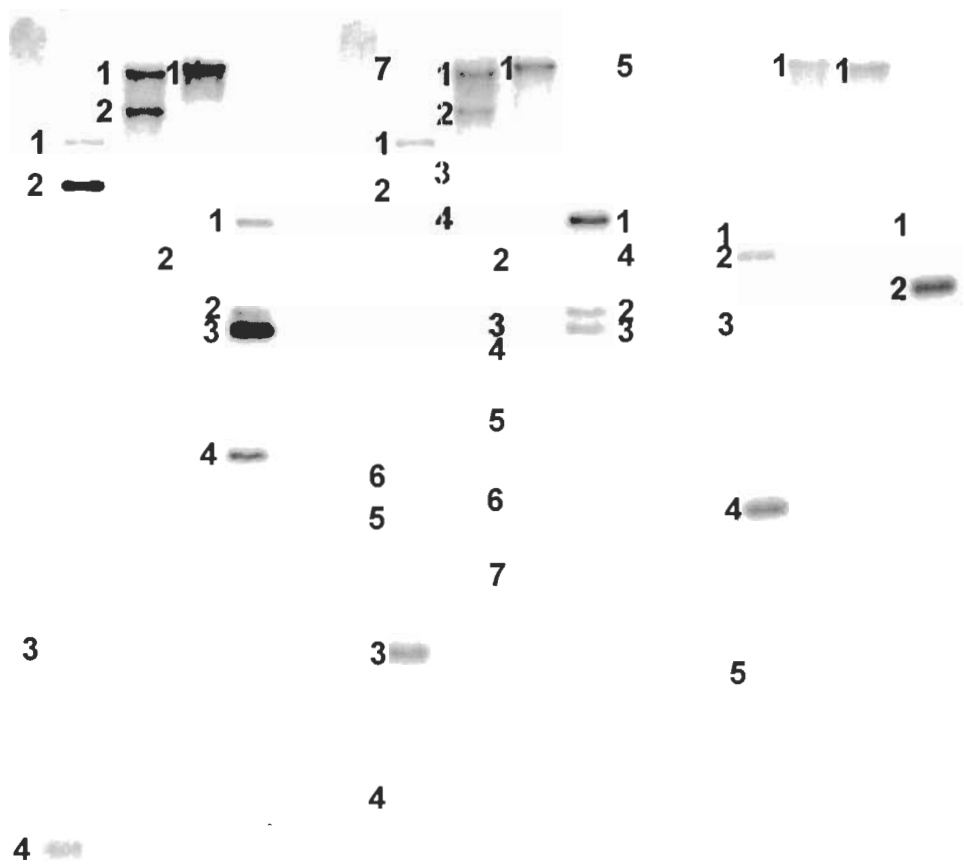
In genomic DNA digested with *EcoRI*, the *LE α -DOX1* probe detected four bands, of which one (band number two) gave the strongest signal (Figure 4-12A). Two bands were detected in *XhoI* and *BamHI* digested genomic DNA. The two *XhoI* bands gave similar signal intensities whereas in *BamHI* digested DNA, band number one gave a strong signal relative to band number two (Figure 4-12A). Four bands were detected in *HindIII* digested genomic DNA, of which band number three gave the strongest signal.

The *cLEW26H11* probe detected seven, four, seven and five bands in *EcoRI*, *XhoI*, *BamHI* or *HindIII* digested genomic DNA, respectively (Figure 4-12B). In *EcoRI*-, *BamHI*- or *HindIII*-digested genomic DNAs, band number three, one, and one gave the strongest signals, respectively, whereas in *XhoI*-digested genomic DNAs, both bands gave equal signal intensities (Figure 4-12B). Due to

Figure 4-12 Southern genomic analyses of tomato α -DOX isoforms.

Tomato genomic DNA was digested with *EcoRI* (E), *XhoI* (X), *BamHI* (B), and *HindIII* (H), size separated on a 0.7% agarose gel in 0.5X TBE running buffer and probed with *LE α -DOX1* (4-12A), *cLEW26H11* (4-12B) and *LE α -DOX3* (Fig. 4-12C). Undigested (U) represents genomic DNA that was not subjected to restriction enzyme digestion. Numbers indicate bands present following hybridization. For blots hybridized with *LE α -DOX1* (4-12A) and *cLEW26H11* (4-12B), identical numbers were assigned to the same bands.

U E X B H U E X B H U E X B H



A. LE α -DOX-1

B. cLEW26H11

C. LE α -DOX-3

the high sequence similarity shared by *LE α -DOX1* and *LE α -DOX2*, the common DNA fragment detected in the digested genomic DNA, may have arisen due to cross hybridization between the *LE α -DOX1* and *LE α -DOX2* probes (Figures 4-12 A,B). Since at least another α -DOX isoform may be present in the tomato genome, it may account for the additional bands detected by southern blot analyses, such as the extra bands present in *Bam*HI-digested genomic DNAs (Figure 4-12B).

The full-length *LE α -DOX3* probe detected a more distinct pattern of hybridizing DNA fragments. Five bands in *Eco*RI, one band in *Xho*I and *Bam*HI and two bands in *Hind*III digested genomic DNA, respectively, hybridized to *LE α -DOX3* (Figure 4-12C). In general the Southern analyses confirm the presence of α -DOX gene family.

4.3.4 Effect of a Salt Treatment on α -DOX Expression in Roots

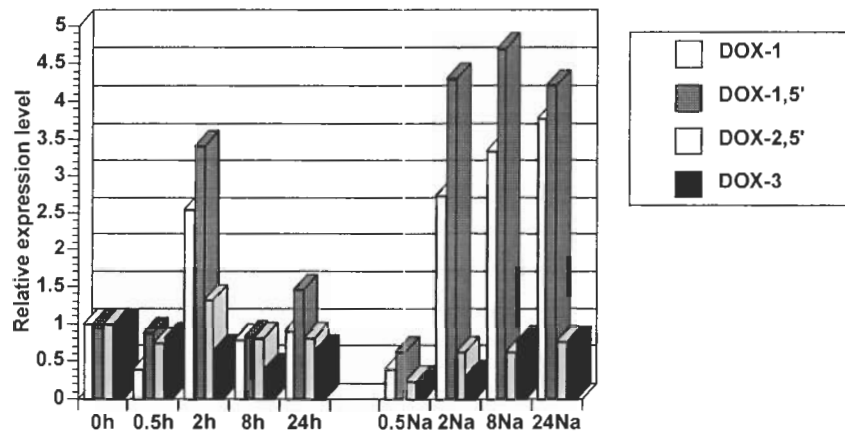
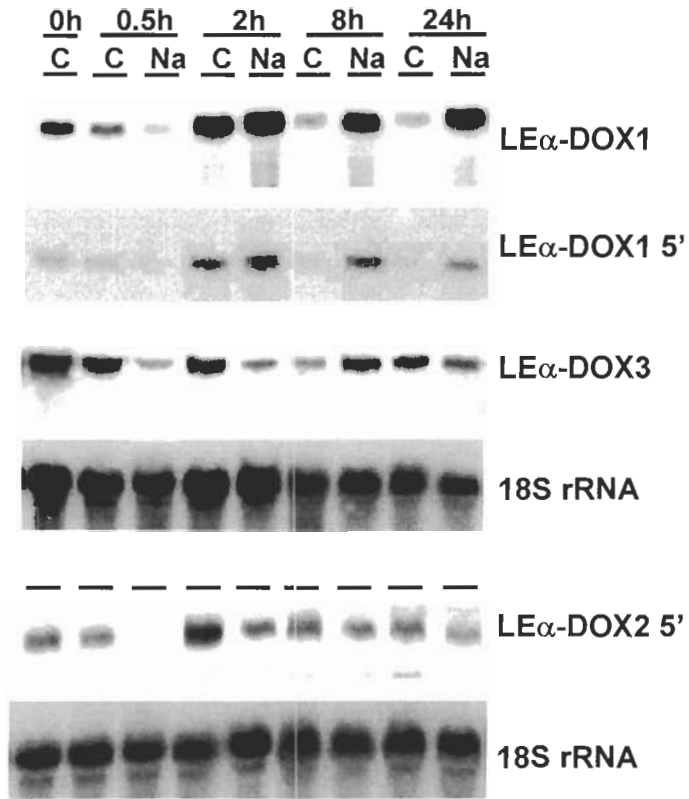
In tomato α -DOX was initially identified as a salt responsive partial cDNA (JWS20, Chapter II, Figure 2-1D), and gene(s) corresponding to JWS20 were demonstrated to be up regulated following the application of a salt treatment (Chapter II, Figure 2-12). The nucleotide sequence of JWS20 corresponds to the 3' end of the *LE α -DOX2* isoform; an area in which high nucleotide similarity among the three α -DOX isoforms exists (Figure 4-10). Consequently, when using JWS20 as a probe, there is a high possibility that it will hybridize to multiple α -DOX isoforms. It is therefore necessary to determine whether salt treatment has an effect on *LE α -DOX1*, *LE α -DOX2*, and *LE α -DOX3*, respectively. *LE α -DOX1* expression in roots was up-regulated by a salt treatment at 8h, and 24h

(Figure 4-13). The expression of *LE α -DOX2* was not affected by salt at these times, whereas *LE α -DOX3* expression was slightly up-regulated at 8h but not at 24h in response to a salt treatment. At 0.5h time point, salt treatment down regulated all *LE α -DOX* isoforms, whereas at 2h time point, a salt treatment resulted in a slight up regulation of *LE α -DOX1* expression and down regulation of both *LE α -DOX2* and *LE α -DOX3* expression (Figure 4-13).

The expression of *LE α -DOX3* in control roots remained constant throughout all the time points, with the lowest level obtained at the 8h time point (Figure 4-13). In control roots, the expression of both *LE α -DOX1* and *LE α -DOX2* noticeably peaked at two hours and returned to lower levels by 24h. To ascertain whether this was due to circadian regulation of gene expression, non-treated root tissue was harvested at various intervals during a 26h period. Northern analysis using the full-length *LE α -DOX1* probe showed that its expression peaked after two hours, and another smaller peak was observed 12 hours later (Figure 4-14). The highest peak coincided with the two hour sampling time for the salt and control time course experiments, thus suggesting that this may be due to the movement of plants at the beginning of the experiment. This result was subsequently confirmed when a similar experiment was conducted, but at a different time of day. *LE α -DOX1* expression peaked at 2h after the experiment started (Kwok and Plant, unpublished data). Therefore, a handling effect may be responsible for the expression of *LE α -DOX1* detected in control roots 2h after transfer to fresh media.

Figure 4-13 α -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 *LE α -DOX1*, a partial probe derived from the 5' end of *LE α -DOX1* (*LE α -DOX1* 5') or *LE α -DOX-2* (*LE α -DOX2* 5'), *LE α -DOX-3*, and with an 18S rRNA probe that served as a loading control to obtain normalized expression values. The plot shows the expression level obtained relative to the 0 h control sample.



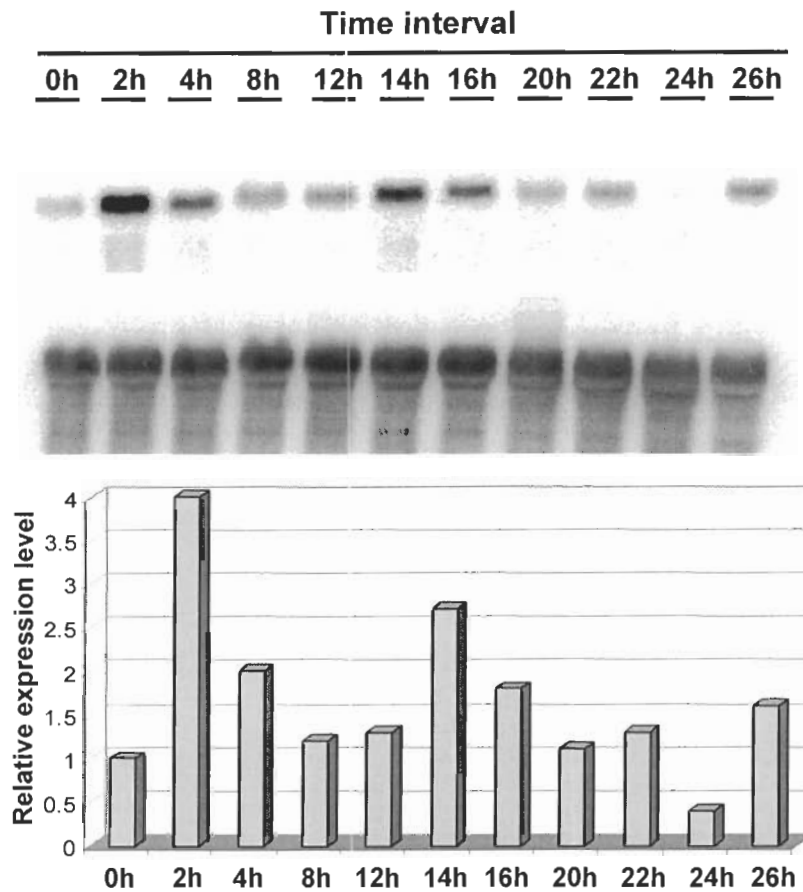


Figure 4-14 *LEα-DOX1* expression in non-treated root tissues at various time intervals. RNA was isolated from roots at 0, 2, 4, 8, 12, 14, 16, 20, 22, 24, and 26 h after transfer to MS media. The blot was hybridized with the *LEα-DOX1* probe and then with an 18S rRNA probe that served as a loading control to obtain normalized expression values. The plot shows the expression level obtained relative to the 0 h sample.

To determine whether the salt enhanced *LE α -DOX1* expression at 24h was a consequence of the applied salt treatment, salt-treated plants were transferred to nutrient medium containing no salt for a period of 48h. The expression level of *LE α -DOX1* present at the end of the 48h recovery period was lower than that obtained following a 24h salt treatment, and is similar to that in the non-treated roots (Figure 4-15).

4.3.5 Effect of Wounding and Pathogen Treatments on α -DOX Expression in Roots

In the leaves of tobacco (Sanz et al., 1998; Hermsmeier et al., 2001), hot pepper (Kim et al., 2002) and Arabidopsis (de Leon Ponce et al., 2002), α -DOX expression is responsive to biotic stress imposed by pathogen infection or caterpillar feeding. To assess whether α -DOX will also respond to comparable cues in tomatoes, two treatments were performed. The first one involved mechanical wounding in which roots were wounded by pinching with forceps, and the second involved exposing roots to biotic stress imposed by treatment with *Pythium aphanidermatum*. Wounding treatment elicited strong up-regulation of *LE α -DOX1* at 8h and 24h (Figure 4-16). The expression of *LE α -DOX2* was also wound responsive at 8h and 24h whereas *LE α -DOX3* was not wound responsive. Following *P. aphanidermatum* exposure for 24h, the *LE α -DOX1* expression was upregulated (Figure 4-16). Since *LE α -DOX1* is both salt- and wound-responsive, this isoform was chosen to be the focus for further study.

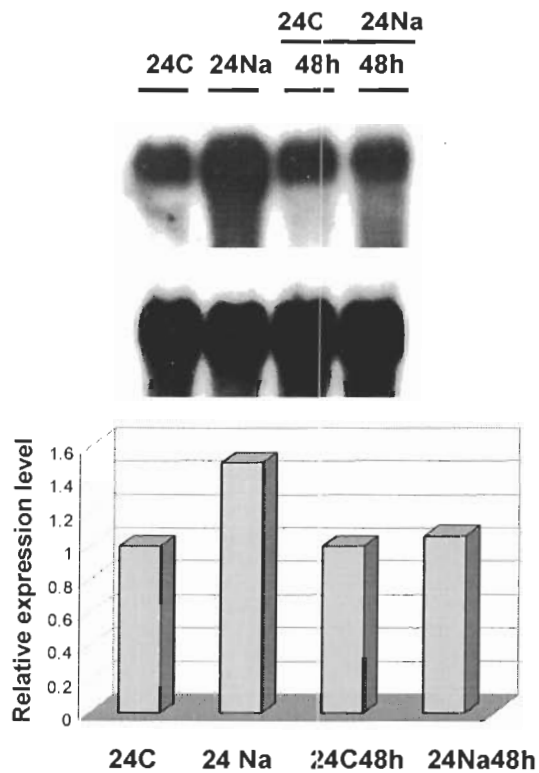
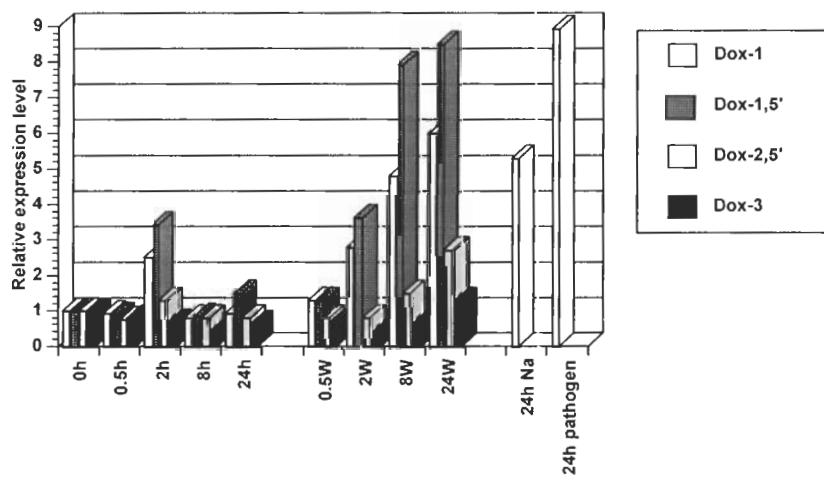
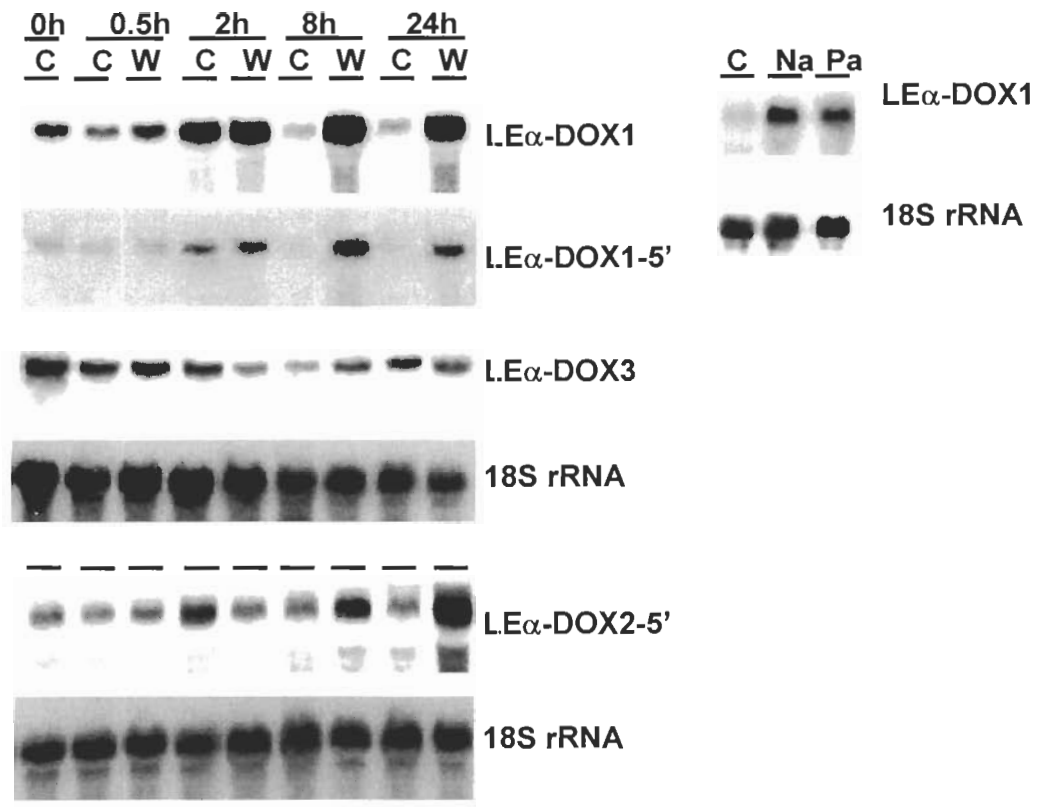


Figure 4-15 *LEα-DOX1* expression in roots exposed to a salt treatment followed by a 48h recovery in MS media with no salt.

RNA was isolated from roots 24 h after transfer to MS media containing salt or no salt with no recovery: 24Na, 24 C; or with a 48h period of recovery: 24Na48h, 24C48h. Blot was hybridized with *LEα-DOX1* and with an 18S rRNA probe that served as a loading control to obtain normalized expression values. The plot shows the expression level obtained relative to the 24 h control sample.

Figure 4-16 α -DOX expression in wounded and pathogen-treated roots.

RNA was isolated from roots 0, 0.5, 2, 8, and 24 h after wounding (W) or transfer to MS media (C). RNA was also isolated from tissue harvested 24h after treatment with *P. aphanidermatum* (Pa) or salt (Na). Blots were hybridized with *LE α -DOX1*, a partial probe derived from the 5' end of *LE α -DOX1* (*LE α -DOX1* 5') or *LE α -DOX-2* (*LE α -DOX2* 5'), *LE α -DOX-3*, and finally with an 18S rRNA probe. The plot shows the relative expression level relative to the 0 h sample.



4.3.6 Spatial Expression Analyses of *LE α -DOX1* Expression

To determine whether *LE α -DOX1* is expressed in organs other than the roots, RNA was extracted from young, mature and senescent leaves, roots, open and closed flowers, green and red fruit and the seeds extracted from green and red fruit of green house grown plants. *LE α -DOX1* expression was detected in the roots and not in any other organs (Figure 4-17).

4.3.7 Effect of ABA on *LE α -DOX1* Expression in Roots

It was shown previously that *LE α -DOX* expression is ABA responsive in roots (Chapter II, Figure 2-12). Application of ABA up regulated *LE α -DOX1* expression (Figure 4-18) in roots, confirming our previous result. In this experiment, various ABA (+/- mixed isomers) concentrations ranging from 7.5 to 120 μ M were applied for a period of 24h. At lower ABA concentrations (7.5 and 15 μ M), *LE α -DOX1* expression was slightly upregulated; the expression level increased further as the ABA concentration increased to 30 μ M. However, the *LE α -DOX1* expression level did not increase further when the ABA level was increased to 120 μ M.

The role of ABA in regulating *LE α -DOX1* expression was further investigated using *Flacca (flc)*. In *flc*, a salt treatment up regulated the expression of *LE α -DOX1* at 24h (Figure 4-19), and the degree of salt-induced up regulation in *flc* at this time, was comparable to that obtained in wild-type plants. No salt induction was observed in *flc* at the 8h time point due to high levels of expression in control roots; the high expression level in control roots of *flc* was also observed in earlier studies (Chapter II, Figure 2-14).

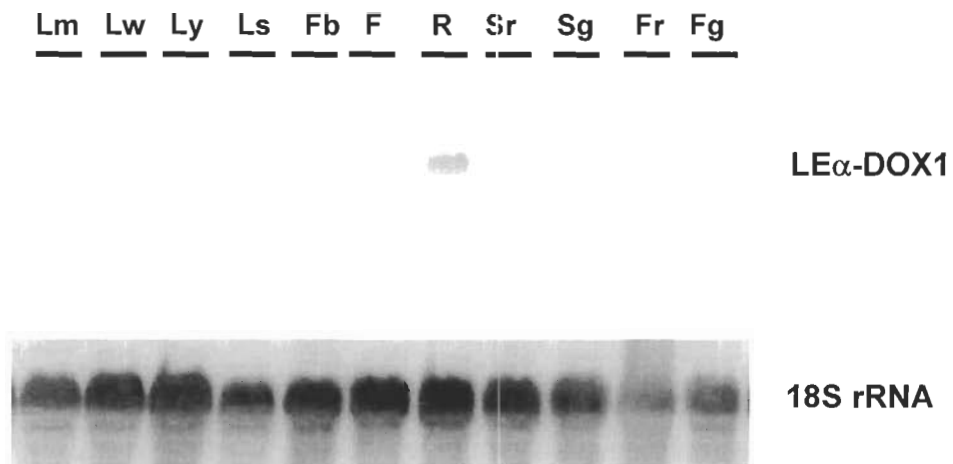


Figure 4-17 Spatial expression of *LE α -DOX1*.

RNA was isolated from mature leaves (L.m), wounded leaves (Lw, 24 h), young leaves (Ly), senescent leaves (Ls), flower buds (Fb), open flowers (F), roots (R), seeds extracted from red (Sr) and green (Sg) fruit, and pericarp tissue from red (Fr) and green (Fg) fruit. Blots were hybridized sequentially with *LE α -DOX1*, and then the 18S rRNA probe. RNA isolation was conducted by Agnes Tsui, blot and hybridization were performed by Ananchanok Tirajoh.

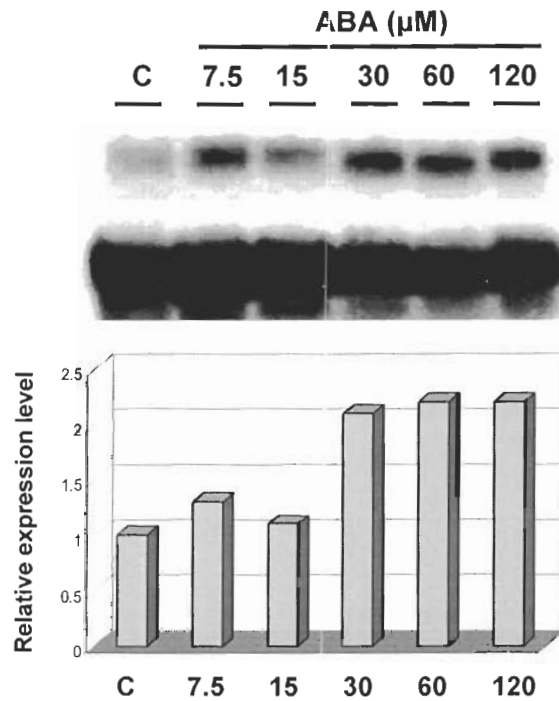


Figure 4-18 Influence of ABA on *LE α -DOX1* expression.

RNA was isolated from roots following exposure to 7.5, 15, 30, 60 or 120 μM ABA (+/- mixed isomers) or to MS media (C) for 24 h. Blots were hybridized sequentially with the *LE α -DOX1* 5' probe and then the 18S rRNA probe. The plot shows normalized expression levels relative to the control values.

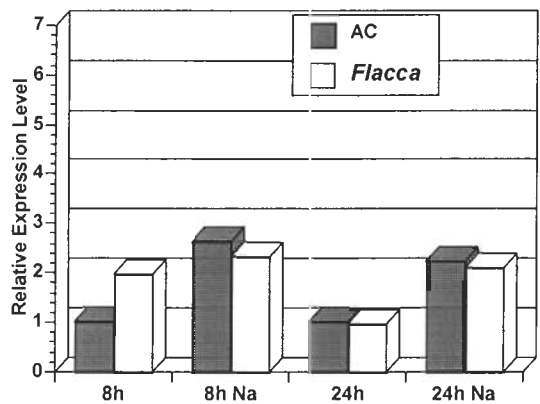
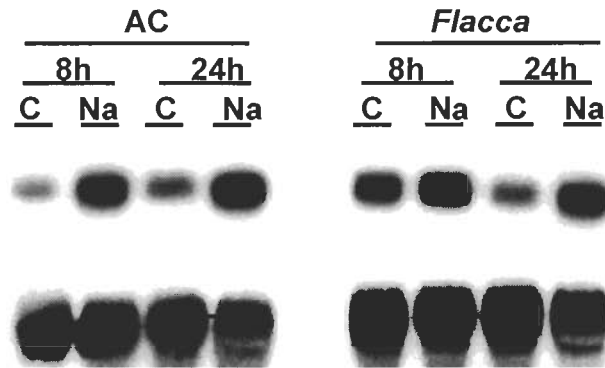


Figure 4-19 Influence of ABA on $LE\alpha$ -DOX1 expression.

RNA was isolated from roots following exposure to MS media (C) or salt (Na) for 8 h or 24 h for AC and *flc*. Blots were hybridized sequentially with the $LE\alpha$ -DOX1 5' probe and then the 18S rRNA probe. The plot shows normalized expression levels relative to the control values. For comparison between AC and *flc* the AC control values were set to one.

Due to the ability of *flacca* to accumulate some ABA following salt stress (Chen and Plant, 1999), FLU was applied as a pre-treatment. *LE α -DOX1* expression was marginally higher in the roots of FLU-pretreated plants than in non-treated roots (Figure 4-20). A subsequent salt treatment resulted in an increased expression of *LE α -DOX1* that was higher than that obtained in FLU pre-treated control roots. Interestingly, the *LE α -DOX1* transcript level in salt-treated roots following FLU pre-treatment was higher than that observed in salt-treated roots with no FLU pre-treatment. A similar result was also obtained with a FLU pretreatment followed by a pathogen challenge (Figure 4-21).

4.3.8 Effect of Ethylene on *LE α -DOX1* Expression in Roots

Spollen et al., 2000 demonstrated that one of ABAs functions in osmotically stressed seedling roots is to prevent excess ethylene production. It has been previously demonstrated that in FLU-pretreated seedlings the root ABA levels are reduced substantially (Chen and Plant, 1999). Therefore, it is possible that the enhanced expression of *LE α -DOX1* in roots of FLU-pretreated plants is due to the increased ethylene evolution that results from the low level of ABA. To address this possibility, it was necessary to determine whether ethylene plays a role in regulating *LE α -DOX1* expression. Plants were exposed to both the ethylene generating agent ethephon, and the ethylene biosynthetic precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). The expression of *LE α -DOX1* in ACC and ethephon-treated roots was distinctly enhanced (Figure 4-22).

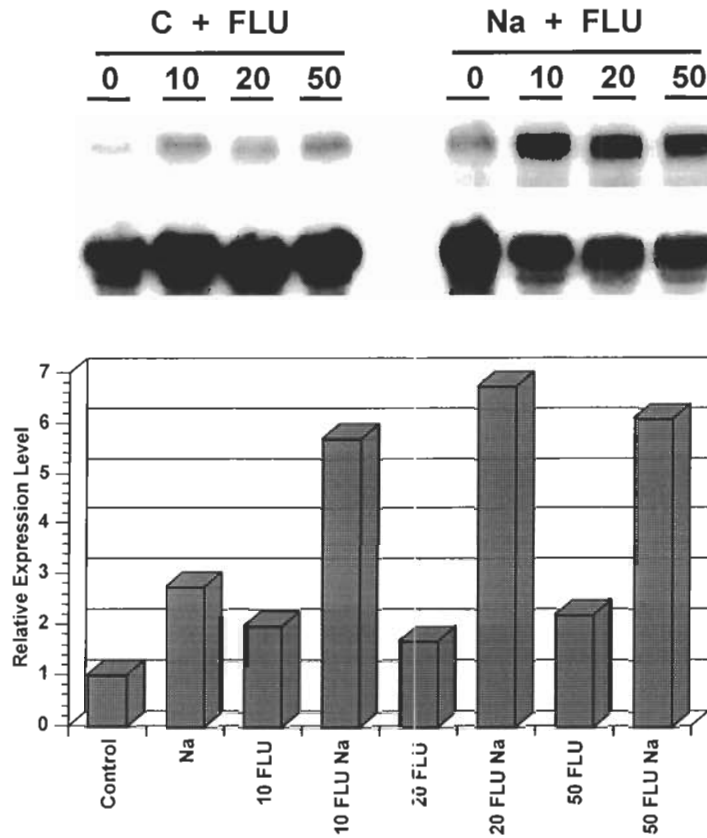


Figure 4-20 Influence of ABA on *LE α -DOX1* expression.

RNA was isolated from roots following a 24h exposure to MS media (C) or salt (Na) following a pre-treatment (24h) with no FLU (0) or with 10, 20 or 50 μ M FLU. Blots were hybridized with the *LE α -DOX1* 5' probe and then the 18S rRNA probe. The plot shows normalized expression levels relative to the control (C) value.

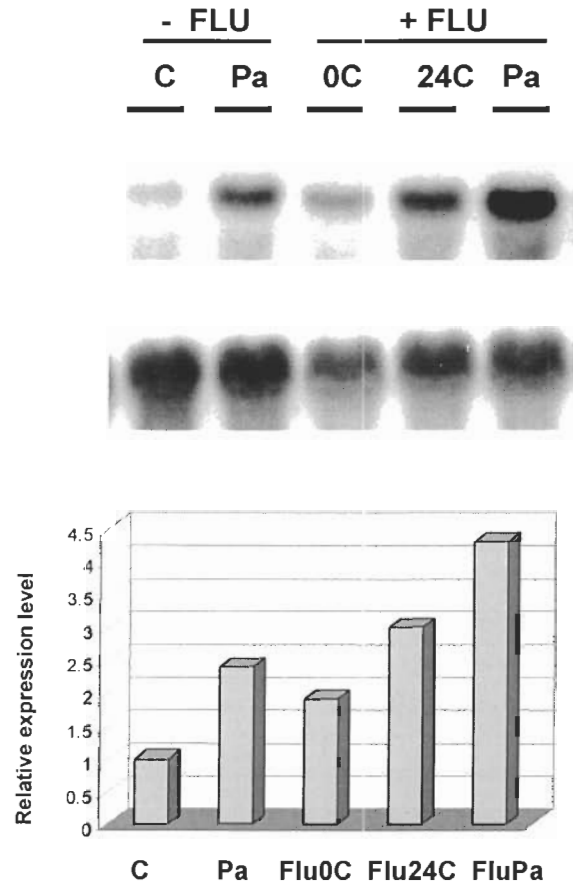


Figure 4-21 Influence of pathogen challenge on *LEα-DOX1* expression.

RNA was isolated from roots following exposure to MS media (C) or *P. aphanidermatum* (Pa) or to a 50 μ M FLU pre-treatment without pathogen for 0h (FLU0C), 24h (FLU24h) and with pathogen for 24h (FLUPa). The blot was hybridized with the *LEα-DOX1* probe and then the 18S rRNA probe. The plot shows normalized expression levels relative to the control (C) value.

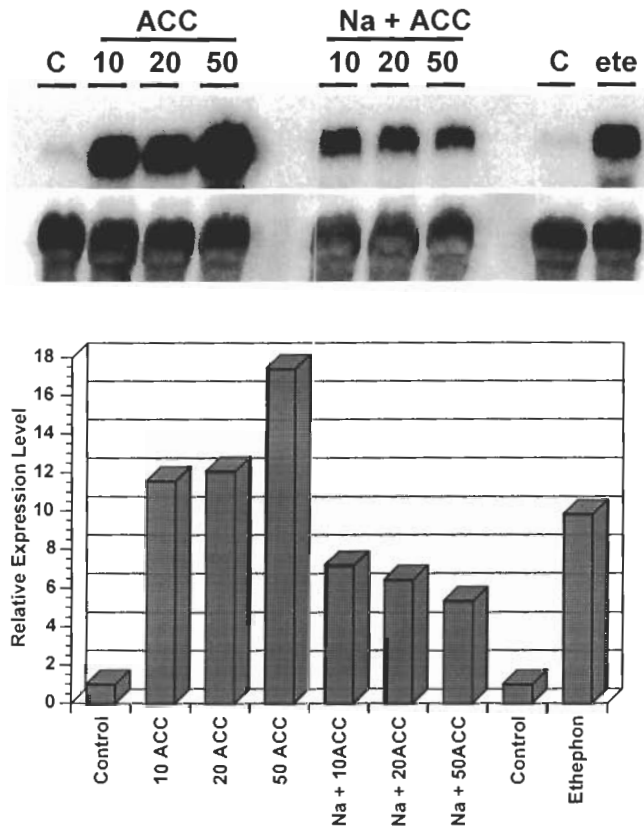


Figure 4-22 Influence of ethylene on *LEα-DOX1* expression.

RNA was isolated from roots of plants transferred to MS media (C), or exposed to 10, 20, or 50 μ M ACC in the absence (ACC) or presence of salt (Na + ACC) or to 500 μ M ethephon (ete) for 24h. Blot was hybridized sequentially with the *LEα-DOX1* 5' probe and then the 18S rRNA probe. Plot shows the expression level relative to the control samples.

To further investigate the role of ethylene in regulating *LE α -DOX1* expression, an inhibitor of ethylene biosynthesis 1- aminoethoxyvinylglucine (AVG) was used. In salt-treated roots, AVG application did not have a major effect on the expression of *LE α -DOX1* (Figure 4-23), although, at higher AVG concentrations, a slightly increased expression level of *LE α -DOX1* was observed.

To gain a better understanding of the relationship between ethylene and salt stress, silver thiosulfate (STS), an inhibitor of ethylene action was used at concentrations ranging from 50 to 500 μ M. At all concentrations tested, STS treatment alone slightly increased *LE α -DOX1* expression in roots (Figure 4-24). However, STS in combination with a salt treatment caused a dramatic reduction of *LE α -DOX1* expression at all concentrations tested (Figure 4-24).

4.3.9 Effect of ABA and Ethylene in Regulating *LE α -DOX1* Expression in Roots

Expression of *LE α -DOX1* is both ABA and ethylene responsive. However, ethylene resulted in a stronger induction of *LE α -DOX1* expression than ABA. Because 1) ABA functions in osmotically stressed roots to suppress ethylene effects (Spollen et al., 2000), and 2) ABA levels increase in the roots of tomato following salt stress (Chen and Plant, 1999), it is possible that ABA and ethylene interact to regulate *LE α -DOX1* expression in roots. When salt and the ethylene precursor ACC were applied together, the expression of *LE α -DOX1* was reduced to a level closer to that caused by salt treatment alone (Figure 4-25). To further test for an interaction between ABA and ethylene, a combination of ABA and ACC were applied to plants. In accordance with the *LE α -DOX1* expression level

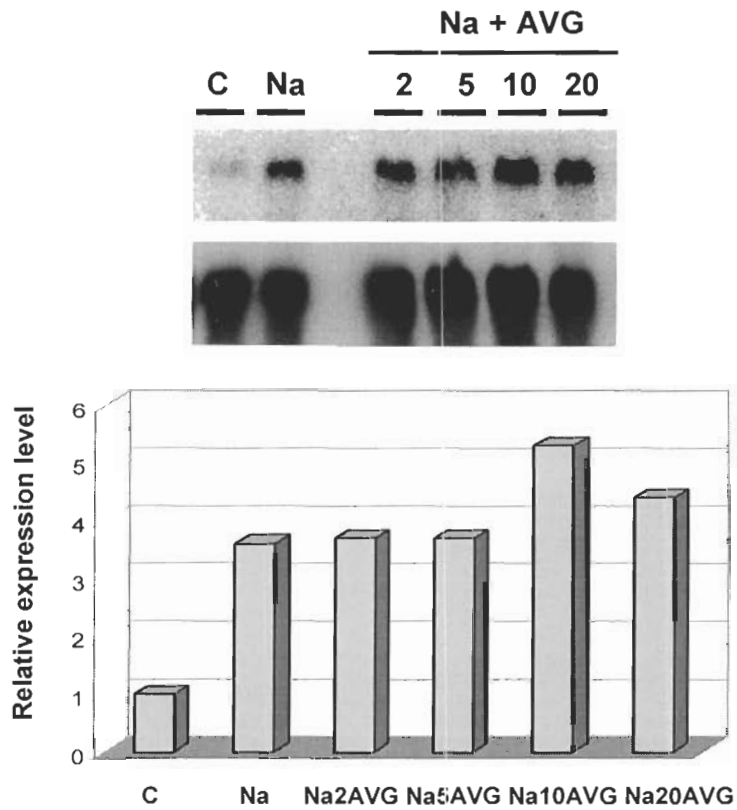


Figure 4-23 Influence of ethylene on *LE α -DOX1* expression.

RNA was isolated from roots of plants transferred to MS media (C), or exposed to salt (Na) or salt plus 2, 5, 10 or 20 μ M AVG (Na + AVG) for 24h. The blot was hybridized sequentially with the *LE α -DOX1* 5' probe and then the 18S rRNA probe. Plot shows the expression level relative to the control samples.

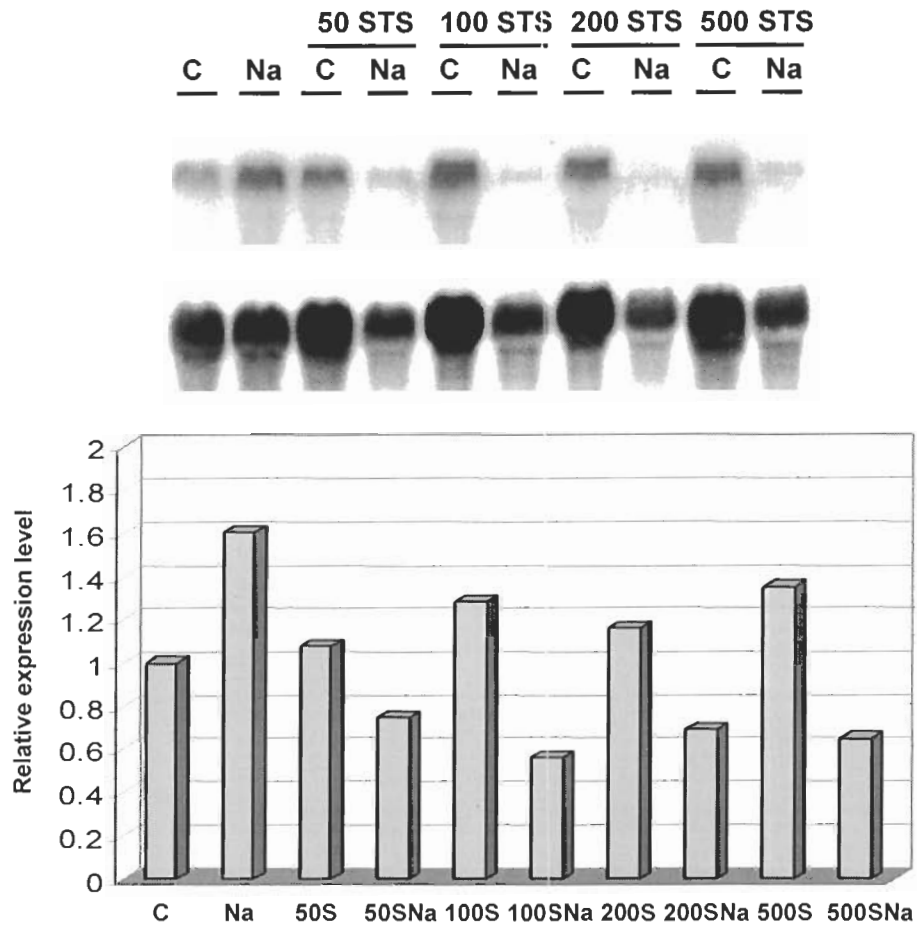


Figure 4-24 Influence of ethylene on *LEα-DOX1* expression.

RNA was isolated from roots of plants transferred to MS media alone (C) or with salt (Na) or exposed to MS media with 50, 100, 200 or 500 μ M STS in the absence (STS-C) or presence of salt (STS-Na) for 24h. The blot was hybridized sequentially with the *LEα-DOX1* full-length probe and then the 18S rRNA probe. Plot shows the expression level relative to the control samples (C).

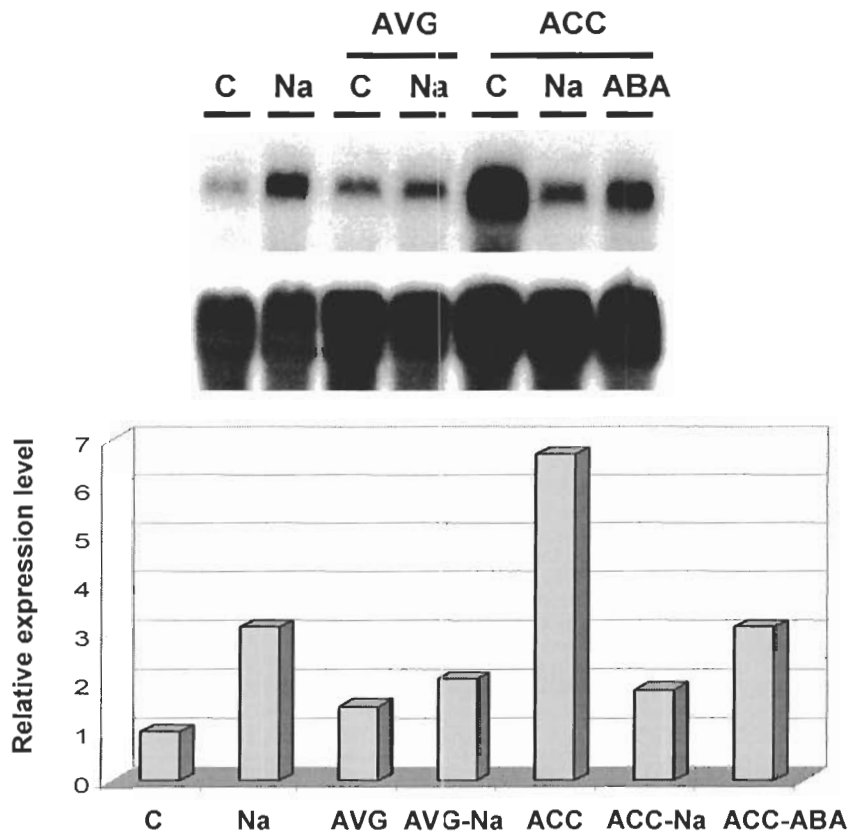


Figure 4-25 Influence of ethylene and ABA on *L.Eα-DOX1* expression.

RNA was isolated from roots of plants transferred to MS media (C), or salt (Na), AVG (10 μ M) or ACC (10 μ M) in the absence or presence of salt; or to ACC together with ABA (60 μ M) for 24h. Blots were hybridized sequentially with the *L.Eα-DOX1* 5' probe and then the 18S rRNA probe. Plot shows the expression level relative to the control samples.

detected when ACC and Na were applied together (Figures 4-22, 4-25), the expression of *LE α -DOX1* in roots exposed to ACC together with ABA was lower than that in ACC-treated roots (Figure 4-25).

4.4 Discussion

It was recently discovered that in plants in addition to lipoxygenase, there exists another group of dioxygenases that add O₂ to fatty acid substrates. *ALPHA DIOXYGENASE* was initially identified in tobacco as a pathogen induced oxygenase, and it shares similarity to mammalian prostaglandin endoperoxide synthases while being structurally unrelated to lipoxygenases (Sanz et al., 1998; Hamberg et al., 1999). In addition to tobacco, *α -DOX* has been identified from several other plants including *Arabidopsis*, cucumber, pea, and rice (Sanz et al., 1998; de Leon Ponce et al., 2002; Borge et al., 1999; Saffert et al., 2000; Keokuda, 2002).

Studies using recombinant *α -DOX* from tobacco demonstrated that *α -DOX* catalyzes the introduction of oxygen at the C-2 (α) position of saturated and unsaturated fatty acids to form unstable 2-hydroperoxy fatty acid intermediates that represent a new class of oxylipins (Hamberg et al., 1999; Hamberg et al., 2002). These unstable 2-hydroperoxides are further converted by non-enzymatic reactions to yield a one carbon shortened fatty aldehyde (83%), a 2-hydroxy fatty acid (15%) and the next lower fatty acid homolog (2%) (Hamberg et al., 2002). In contrast to these *in vitro*-derived data, a more recent study showed that the 2-hydroxy fatty acids were the major product to accumulate in tobacco leaves *in vivo* (90-95%) following bacterial infection. In addition, one carbon shortened

fatty acids were detected (5-10%) but there were no linolenic acid derived-fatty aldehydes (Hamberg et al., 2003). The mechanism of the reduction of the 2-hydroperoxy fatty acids to yield 2-hydroxy fatty acids is unknown, but it was suggested to involve peroxidase activity (Hamberg et al., 2003). In this context, the α -DOX enzyme complex purified from pea seeds possesses peroxidase activity (Saffert et al., 2000). Recombinant tobacco α -DOX1 does not possess peroxidase activity (Hamberg et al., 1999); however, it can not be ruled out that such activity is absent in the native tobacco α -DOX1 enzyme.

Evidence thus far indicates that oxylipins play important roles during defense reactions that take place in plants following bacterial and fungal infection, insects, and other pathogens attack (Blee, 2002). Furthermore, other oxylipins particularly those belonging to the jasmonate family, can function as signaling molecules leading to the activation of specific defense genes (Weber 2002). Currently, alpha-oxidation degradation of fatty acids is a pathway of unknown physiological significance in plants. de Leon Ponce et al (2002) demonstrated that *Arabidopsis* with reduced α -DOX1 were more susceptible to pathogen challenge as indicated by the larger size of the necrotic lesions, conversely, increased level of α -DOX1 resulted in a slower manifestation of necrotic lesions. Therefore, he suggested that α -DOX may play a role in generating lipid-derived molecules, which are important in protecting plants from oxidative stress. It was also demonstrated that bacterial infection increases the level of α -DOX (Sanz et al., 1998; de Leon Ponce et al., 2002), and the levels are higher and occur earlier when the infection results in a hypersensitive

reaction (de Leon Ponce et al., 2002). In addition, incompatible pathogen interactions in transgenic tobacco and Arabidopsis plants that overexpress α -DOX result in a massive accumulation of 2-hydroxy fatty acids (de Leon Ponce et al., 2002; Hamberg et al., 2003). Furthermore, application of the α -DOX-derived 2-hydroxy fatty acids together with the bacterial inoculum reduced the extent of cell death (Hamberg et al., 2003). Therefore, it has been proposed that one possible function of α -DOX in plants is to generate product(s) that can protect plants from undergoing excessive cell death, and the α -DOX-derived 2-hydroxy fatty acids may be the prime candidate.

In tomato, α -DOX-like gene was initially identified as a salt responsive partial cDNA using mRNA differential display (Chapter II, Wei et al., 2000). To my knowledge, this is the first report that describes the salt stress-responsive nature of α -DOX, and hence the first that suggests oxylipins may be involved in mediating the response of roots to salt stress. α -DOX-like genes in tomato are represented by a small gene family comprised of at least three members: *LE α -DOX1*, *LE α -DOX2*, and *LE α -DOX3*. At the nucleotide sequence level, *LE α -DOX1* and *LE α -DOX2* are similar to each other, whereas *LE α -DOX3* is less similar (Figure 4-7). Arabidopsis possesses two α -DOX genes: *At α -DOX1* and *At α -DOX2*. *At α -DOX1* is more similar to *LE α -DOX1* and -2, whereas *At α -DOX2* is more similar to *LE α -DOX3* (Figure 4-10). The *LE α -DOX3* gene has been disrupted by insertional mutagenesis which results in a distinct phenotype called *feebly* (van der Biezen et al., 1996; Meissner et al., 2000). *Feebly* contains high anthocyanin levels at the seedling development stage. Mature *feebly* plants are

small with pale green leaves and they produce deformed fruit. Therefore, it is speculated that FEEBLY is involved in a metabolic pathway and that its absence results in physiologically disturbed plants (van der Biezen et al., 1996).

Hydropathy analyses and TM prediction showed that the proteins-encoded by *LE α -DOX1*, *LE α -DOX2*, and *LE α -DOX3*, respectively are anchored to the membrane. This result is in accordance to the proposed role of α -DOX to generate lipid-derived molecules.

Genome analyses of stress-responsive expression showed that α -DOX expression is up regulated in drought-stressed roots of barley and Arabidopsis (Ozturk et al., 2002; Seki et al., 2002a). GENEVESTIGATOR-generated digital northern data reveal that the expression of both *At α -DOX1* and *At α -DOX2* is enhanced following salt, drought, wounding and oxidative stresses. Moreover, *At α -DOX1* is expressed predominantly in roots whereas *At α -DOX2* is expressed predominantly in shoots. Interestingly, neither *At α -DOX1* nor *At α -DOX2* is ethylene responsive and only *At α -DOX1* is ABA responsive. Of the three members of the tomato α -DOX family, only *LE α -DOX1* was up regulated by salt (Figure 4-13). *LE α -DOX2* was down regulated by salt, which was in accordance to the initial pattern detected for JWS20 by DD-PCR.

Spatial analyses of *LE α -DOX1* showed that in tomato *LE α -DOX1* expression is mostly detected in the root (Figure 4-17), which is in accordance with the BLASTN result against the EST database, although expression in callus tissue is noted (Table 4-1). BLASTN result against the EST database for *LE α -DOX2* indicates that its expression is restricted to roots (Table 4-3). Wounding of roots

up-regulated expression of *LE α -DOX2*, whereas both wounding and *P. aphanidermatum* treatments up regulated *LE α -DOX1* expression (Figures 4-16, 4-21). This result suggests a general role for α -DOX in protecting roots against a variety of stresses. *LE α -DOX3* expression is detected in roots, but its expression was not responsive to either salt or wounding treatment (Figures 4-13, 4-16). BLASTN result for *LE α -DOX3* showed that its expression is not limited to roots (Table 4-5), thus, it is possible that *LE α -DOX3* may be wound responsive in other organs such as leaves.

α -DOX expression is responsive to chemicals that elicit the generation of ROS (Sanz et al., 1998; Hermsmeier et al., 2001; de Leon Ponce et al., 2002; Weber et al., 2004). Salt stress-induced oxidative stress and PCD in roots have been reported (Katsuhara, 1997; Katsuhara and Shibasaka, 2000; Huh et al., 2002; Shalata et al., 2001). In plants, ROS are continuously produced as byproducts of various metabolic pathways that occur in different cellular compartments including mitochondria and chloroplasts (Asada, 1994). Under physiologically steady state conditions, ROS are scavenged by antioxidative defense systems that are often confined to particular compartments (Foyer and Harbinson, 1994). Oxidative stress occurs when the antioxidative systems fail to detoxify ROS, and this is commonly observed under various stress conditions including salinity. Salinity stress has been demonstrated to upregulate the antioxidative systems in both roots and shoots, resulting in an alleviation of salt-induced oxidative stress (Mittova et al., 2003; Mittova et al., 2004). Programmed cell death is essential for maintaining cellular homeostasis, regulation of

development, and control of ageing in multicellular organisms (Jones, 2000). One type of PCD present in plants involves lysosomal degeneration, which results in the release of hydrolases that eliminate organelles and other cellular components. In plants, PCD has been demonstrated to facilitate cellular differentiation and homeostasis, and defense against cellular insult. Tracheary element and phloem differentiation, root cap and aerenchyma formation, aleurone and endosperm cell death, leaf senescence, and the hypersensitive response are some examples of processes in plants that involve PCD (Beers and McDowell., 2001; Drew et al., 2000; Fukuda, 2000; Kawasaki et al., 1999; Vaux and Kormeyer, 1999). The most characterized PCD in plants is the hypersensitive response elicited by an incompatible interaction between a host and pathogen (Lam et al., 1999).

In both barley and *Arabidopsis* primary roots, salt ions can trigger PCD (Katsuhara and Shibasaka, 2000; Huh et al., 2002). It is postulated that salt-induced PCD is one form of adaptation that allows the formation of new roots that not only can maintain water and mineral uptake but are also believed to have a higher tolerance to the saline environment (Huh et al., 2002). It is therefore speculated that salt-induced PCD may be an important aspect of salt tolerance in roots. In this regard, it is tempting to speculate that α -DOX-generated oxylipins may be involved in protecting roots from oxidative damage and cell death associated with salinity stress.

One of my research objectives was to investigate the role of ABA in regulating changes of gene expression in salt-treated roots of tomato. *LE α -DOX1*

expression is up regulated by ABA (Figure 4-18); however its expression was also detected in salt-treated *flc* roots (Figure 4-19). *Flacca* has been demonstrated to have the ability to accumulate some ABA in roots following salt stress (Chen and Plant, 1999), as well as having an impaired ABA transport system from root to shoot (Sagi et al., 1999). Therefore, it is possible that the expression of *LE α -DOX1* in salt-treated *flc* roots is due to their ability to accumulate some ABA. When FLU was used to reduce ABA levels, *LE α -DOX1* expression was higher in salt-treated roots following a FLU pre-treatment than in those without a FLU pretreatment (Figure 4-20). A similar response was also obtained following *P. aphanidermatum* treatment of FLU pre-treated roots (Figure 4-21). Since ABA has been demonstrated to restrict ethylene production in roots of osmotically-stressed plants (Spollen et al., 2000), this result points to the possibility that ethylene may play a role in regulating *LE α -DOX1* expression. The increased *LE α -DOX1* expression following ACC or ethephon treatment supports a role for ethylene in regulating *LE α -DOX1* (Fig. 4-22).

Ethylene mediates responses to both pathogenic (Diaz et al., 2002), non-pathogenic organisms (Knoester et al., 1999), to soil compaction (Hussain et al., 1999), and to flood-induced anoxia which results in aeranchyma formation via PCD (Drew et al., 2000). In cultured tomato cells, both hypoosmotic and hyperosmotic stresses have been shown to induce ACC synthase, the key enzyme regulating ethylene biosynthesis (Felix et al., 2000). However, whether osmotic stress induces ethylene evolution in intact plants remains unclear (Morgan et al., 1990; Narayana et al., 1991). To further investigate the role of

ethylene in regulating *LE α -DOX1* expression during salt stress, an inhibitor of ethylene biosynthesis, AVG, and an inhibitor of ethylene action, STS, were used. During salt stress, AVG had a minimal effect on *LE α -DOX1* expression (Figure 4-23); suggesting that the expression of *LE α -DOX1* during salt stress may not be dependent on newly accumulated ethylene. Since salt stress elevates ABA levels in roots, it is possible that salt-responsive *LE α -DOX1* expression is mediated by ABA.

Relatively low levels of STS (ranging from 50 to 500 μ M) were used to avoid any negative effect due to its toxicity. The appearance of STS-treated plants was similar to that of the control plants. In the absence of salt stress, STS treatments marginally increased *LE-DOX1* expression. However, STS together with salt caused a dramatic reduction of *LE α -DOX1* expression at all concentrations tested (Figure 4-24). These results indicate that in non-stressed roots the expression of *LE α -DOX1* is not ethylene mediated; however the dramatic reduction of *LE α -DOX1* expression in STS/salt-treated plants indicates a potential role for ethylene signalling in regulating *LE α -DOX1* expression during salt stress.

Accumulating evidence indicates that the ability of plants to respond to ethylene coincides with a rise in ethylene production. However, responses to ethylene with unchanged ethylene production have been reported (Mayak et al., 2004). Thus, sensitivity to ethylene plays a role in some stress responses (Lehman et al., 1996; Visser et al., 1996). Recently, Zhao and Schaller (2004) demonstrated that exposure of *Arabidopsis* plants to salt stress results in

reduced expression of the ethylene receptor *ETR1*. Since ethylene receptors serve as negative regulators of the ethylene signal transduction pathway in the absence of ethylene (Hua and Meyerowitz, 1998; Tieman et al., 2000), a decrease in receptor level is predicted to increase the plants sensitivity to ethylene. Ethylene binding is mediated by a copper cofactor and silver is believed to replace the copper cofactor (Rodriguez et al., 1999). Ethylene receptors that contain silver have the ability to bind ethylene; however, the binding site is perturbed and therefore unable to inactivate CTR1, a negative regulator of the ethylene response pathway (Kieber et al., 1993). It is therefore tempting to speculate that, although newly synthesized ethylene may not be necessary for the expression of *LE α -DOX1* during salt stress, a functional ethylene signaling pathway may be required.

The ability of ABA and salt (which triggers ABA accumulation) to reduce ACC-responsive *LE α -DOX1* expression (Figure 4-25) provides evidence for an interaction between ABA and ethylene in regulating *LE α -DOX1* expression in tomato roots. Interactions between ABA and ethylene occur at many levels including at the level of synthesis or effect (Spollen et al., 2000; Sharp et al., 2000; Lenoble et al., 2004) and between their signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Gazzarrini and Mc Court, 2001). The interaction between ABA and ethylene was first proposed by Wright (1980). ABA has long been associated as an inhibitor of shoot growth (Trewavas and Jones, 1991; Davies, 1995) based on the fact that ABA accumulates at high concentration in plants that are experiencing water deficit stress, which

corresponds to growth inhibition. In addition, ABA application generally results in inhibition of growth. Ironically, ABA deficient mutants have poor shoot growth as shown by their reduced height and smaller leaves, and this phenotype can be rescued by exogenous ABA treatment (Imbar and Tal, 1970; Bradford, 1983). However, since wiltiness is a trademark of ABA deficient mutants, their reduced shoot growth have always been associated with an impaired water balance (Bradford, 1983; Neil et al., 1986; Nagel et al., 1994). It has been documented that ethylene production is higher in ABA deficient mutants of tomato (Tal et al. 1979) and Arabidopsis (Rakitina et al., 1994). In addition, morphological symptoms, which are associated with excess ethylene such as leaf epinasty and adventitious rooting are also present in tomato ABA deficient mutants (Tal, 1966; Nagel et al., 1994). Spollen et al (2000) have demonstrated that during water deficit stress, the rate of ethylene production in ABA deficient seedlings of maize was higher than in wild type, and this effect can be completely prevented by increasing the ABA level. They further showed that during water deficit stresses, root elongation of ABA deficient seedlings was restored when ethylene production was reduced to a normal level using inhibitors of ethylene synthesis. This finding suggests that ethylene suppression by ABA is an important factor in maintaining root growth during water deficit stress. In more recent work, Le Noble and colleagues (Le Noble et al., 2004) demonstrated that normal levels of endogenous ABA are necessary to maintain shoot development in well-watered Arabidopsis plants, a finding that support a similar data obtained by Sharp et al (2000) using tomato plants. Taken together, these results suggest that normal

levels of ABA are required to maintain shoot and root growth in both water-limiting and well-watered conditions.

Interactions between ABA and ethylene at the level of signaling pathways was revealed based on observations that several hormone response mutants also have altered sensitivities to other hormones. The ethylene insensitive mutant (*ein2*) of *Arabidopsis* has been independently isolated from various genetic screens including screens for mutants that have increased sensitivity to ABA (Beaudoin et al., 2000; Ghassemian et al., 2000). The *Era3* (Enhanced response to ABA3) mutant was initially identified by screening for seeds unable to germinate in the presence of low levels of exogenous ABA (Cutler et al., 1996). Concurrent with the identification of *era3*, in an effort to screen for mutations with either enhanced or suppressed the *Arabidopsis abi* phenotype, another new allele of *EIN2* was discovered (Beaudoin et al., 2000). These data suggest that *EIN2* is positioned at the crossroads between the ethylene and ABA response pathways.

A hypothetical model for the role of ABA, ethylene and ethylene signalling in regulating the expression of *LE α -DOX1* in roots is presented in Figure 4-26. This study showed that the expression of *LE α -DOX1* is upregulated following salt treatment. ABA treatment alone up regulates *LE α -DOX1* expression, and salt stress is known to cause an increased level of ABA in roots (Chen and Plant, 1999), therefore the ability of salt stress to up regulate *LE α -DOX1* expression may be mediated in part by the increased ABA level. Since increased ABA is one of the many changes that occur during salinity stresses, it can not be ruled out

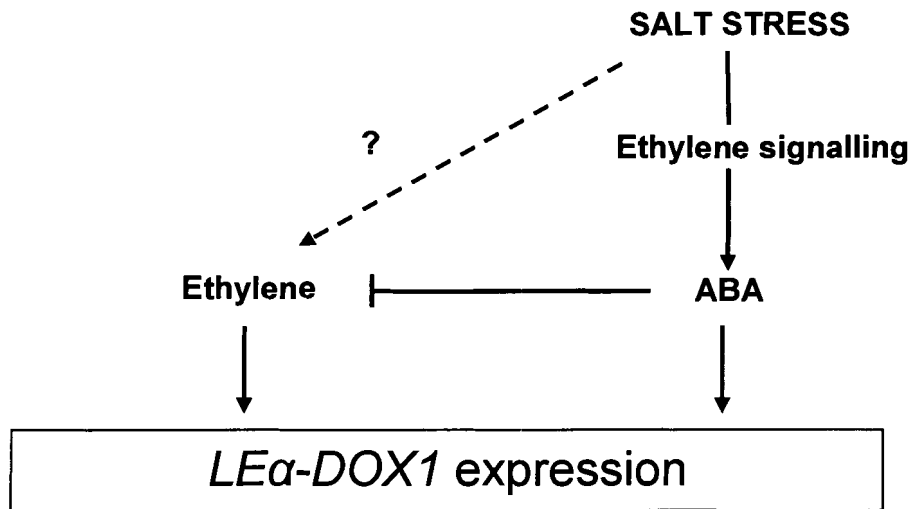


Figure 4-26 A hypothetical model for the role of ABA and/or ethylene, and ethylene signalling in regulating *LEα-DOX1* expression in tomato roots.

LEα-DOX1 was up-regulated by both salt and ABA treatment. *LEα-DOX1* expression was also up-regulated by ethylene. This study indicated that a functional ethylene signaling pathway may be required for *LEα-DOX1* expression during salt stress, and the ethylene-induced *LEα-DOX1* expression can be antagonized by ABA.

that other factors may play a role in regulating *LE α -DOX1* expression in roots. The expression of *LE α -DOX1* is up-regulated following wounding, and wounding is known to increase ethylene level (Kende, 1993; O'Donnell et al., 1996), thus the ability of wounding to up regulate *LE α -DOX1* expression may be mediated, in part, by increased ethylene levels. *LE α -DOX1* expression was strongly up-regulated by ethylene, however, during salt stress, blocking ethylene synthesis with AVG had minimal effect on *LE α -DOX1* expression. Thus, an increased ethylene level may not be necessary for *LE α -DOX1* expression in salt-stressed roots. However, a functional ethylene signaling pathway may be required since the *LE α -DOX1* expression was strongly affected when a combination of STS and salt treatments were applied together. The reduced expression obtained when ACC was applied together with either salt or ABA suggests that ABA and ethylene interact to regulating *LE α -DOX1* expression.

5 SUMMARY AND FUTURE PROSPECTS

5.1. Summary

The objectives of my thesis were to isolate and characterize novel salt responsive genes from the roots of tomato (*Lycopersicon esculentum* L Mill), and to assess the role of ABA in regulating their expression following salinity stress.

Examination of tomato root mRNA profiles by DD-PCR demonstrated that a salt treatment induced, up-regulated or down-regulated the expression of several genes. RNA from ABA-treated roots gave rise to DD-PCR profiles that differed from those caused by a salt treatment. Nine partial cDNAs were identified, six were isolated, and three were eventually chosen for further analyses - JWS19, JWS20 and JWS27. The JWS19, JWS20 and JWS27 partial cDNA inserts share high nucleotide sequence similarity with an auxin-regulated protein of tomato, a hydrogen peroxide-induced tobacco cDNA fragment, and alpha-dioxygenase enzymes, respectively. Full-length cDNAs were subsequently isolated for JWS19 (JWL19), JWS20 (*LE α -DOX*), and JWS27 (JWL27).

The nucleotide sequence of JWL19 shares high similarity to a tomato auxin-regulated protein, and also contains a domain of unknown function (DUF 966), which is present in a family of plant proteins with unknown function. The nucleotide sequence of JWL27 contains two introns indicating it may be derived either from an unprocessed transcript(s) or a pseudogene. Exogenous ABA treatments had no major effect on the expression of genes corresponding to both

JWL19 and JWS27, suggesting that ABA does not play a role in regulating their expression during salt stress. This conclusion was confirmed when salt-treated *flc* or fluridone-treated plants were examined for salt- responsive *JWL19* and *JWS27* expression in which gene expression was similar to that detected in salt-treated wild-type roots.

JWS20 corresponds to an *ALPHA-DIOXYGENASE*-like gene. In tomato α -*DOX* is represented by a small gene family with at least three members: *LE α -DOX1*, *LE α -DOX2*, and *LE α -DOX3*, and cDNA clones for all three α -*DOX* genes were obtained from the Clemson University Genomics Institute (CUGI). The nucleotide sequences of *LE α -DOX1* and *LE α -DOX2* were very similar (85% identity), whereas *LE α -DOX3* was less similar (63% and 66% identity to *LE α -DOX1* and *LE α -DOX2*, respectively). Among the three α -*DOX* isoforms, only *LE α -DOX1* was salt responsive. In addition, *LE α -DOX1* expression was responsive to pathogen challenge and mechanical wounding. Spatial expression analyses showed that *LE α -DOX1* is expressed primarily in roots. The JWS20 partial cDNA corresponding to *LE α -DOX2* was identified as a salt down-regulated gene by DD-PCR. This is consistent with the result obtained when the expression of salt-treated AC roots were examined, in which the expression of *LE α -DOX2* was not up regulated by the applied salt treatment.

The expression of *LE α -DOX1* was ABA responsive; however, its expression increased when endogenous ABA levels were reduced during salt stress or following a pathogen treatment. This may result from the fact that one function of ABA in osmotically stressed roots is to suppress ethylene synthesis

and/or action (Sharp et al., 2000). Both ethephon and ACC treatments distinctly increased *LE α -DOX1* expression, and this effect was countered by ABA, suggesting that ABA and ethylene interact in regulating the expression of *LE α -DOX1*. Blocking ethylene biosynthesis during salinity stress with AVG did not have a drastic effect on the expression of *LE α -DOX1*, indicating that ABA and not ethylene may be responsible for the enhanced expression of *LE α -DOX1* following salinity stress. However, blocking ethylene signaling with STS during salt stress markedly reduced the expression of *LE α -DOX1*. Together, these results suggest that, although newly accumulated ethylene may not be required, a functional ethylene signaling pathway may be essential for the expression of *LE α -DOX1* during salinity stress.

5.1 Future Prospects

In this study, salt was applied as a salt shock treatment, and this condition is not what plants experience in their natural habitats. Thus, it is of interest to know whether genes identified in this study are expressed in a similar manner if a more natural salt stress approach is employed. In a lab setting experiment, this can be achieved by gradually increasing the NaCl concentration to the level that was used in my study (170 mM NaCl) over a period of time.

The expression of *LE α -DOX1* was both ABA and ethylene responsive. In tomato roots, salinity stress causes an increased endogenous ABA level (Chen and Plant, 1999), however, to my knowledge there has not been any report with regard to the involvement of ethylene during salt stress in roots. Therefore measuring the endogenous ethylene level and/or determining whether enzyme(s)

corresponding to the limited step of ethylene biosynthesis pathway is up regulated by a salt stress are some of the essential future experiments that can be conducted.

Recent work by Zhao and Schaller (2004), showed that in *Arabidopsis* salt stress causes a reduction in the expression of the ethylene receptor, *ETR1*. Since ethylene receptors serve as a negative regulators of the ethylene signal transduction pathway, decreased receptor levels is predicted to increase sensitivity to ethylene; whether salt stress causes a similar effect in tomato roots. that remains to be discovered. This study also demonstrated that ABA and ethylene can interact in regulating the expression of *LE α -DOX1*. Further experiments using ABA response and the ethylene signaling mutants should help us to gain a better understanding of how and at what level (is it at the level of synthesis?, signaling? or both?) these two hormones interact in regulating the *LE α -DOX1* expression.

Finally, this is the first study to my knowledge that demonstrates the salt responsive nature of α -DOX. It is therefore of great interest to know the contribution of α -DOX to plant responses during salt stress. This can be achieved by; 1). Suppressing the *LE α -DOX1* expression using RNA interference, and 2). Determining the enzymatic activity of *LE α -DOX1* in plants in which the *LE α -DOX1* has been suppressed as well as in wild type plants under both non-stressed and salt-stressed conditions.

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APPENDIX

Appendix-1. List of ESTs clones that were used to generate TC162290.

#	EST Id	GB#	Clone	Library name
1	TIGR EST310456	AW979397	cLEW1H10	tomato nutrient deficient roots
2	TIGR EST357552	AW931709	cLEF46E13	tomato mature green fruit
3	TIGR EST275366	AW031912	cLEC21L15	tomato callus tissue
4	TIGR EST354530	BE463307	cTOC12F9	tomato preanthesis flower buds
5	TIGR EST400839	BE449952	cLEY11B17	tomato root at pre-anthesis
6	TIGR EST468703	BG135811	cTOE23N23	tomato crown gall
7	TIGR EST555147	BI935258	cTOD22G4	tomato flower, anthesis
8	TIGR EST555070	BI935181	cTOD22G3	tomato flower, anthesis
9	TIGR EST242631	AI483154	cLEB8D19	tomato shoot
10	TIGR EST328040	AW649586	cLEI8D10	tomato germinating seed
11	TIGR EST584175	BM409848	cLEG50I18	tomato breaker fruit
12	I00B JWS-27	AW062242	JWC37-11	Salt-stressed tomato roots
13	TIGR EST585531	BM411204	cLEG56A19	tomato breaker fruit
14	TIGR EST412712	BE461293	cLEG38K7	tomato breaker fruit
15	TIGR EST309721	AW399221	cLPT6L7	<i>L. pennellii</i> trichome
16	TIGR EST414232	BE458940	cLEM5E15	tomato immature green fruit
17	TIGR EST532432	BI421766	cLEC67J16	tomato callus tissue
18	TIGR EST532475	BI421809	cLEC68A17	tomato callus tissue
19	TIGR EST413461	BE461946	cLEG40P10	tomato breaker fruit
20	TIGR EST532483	BI421817	cLEC68C17	tomato callus tissue
21	TIGR EST281859	AW035705	cLEC36A16	tomato callus tissue

Appendix-1 Continued.

#	EST Id	GB#	Clone	Library name
22	TIGR EST264629	AI895186	cLEC7C17	tomato callus tissue
23	TIGR EST282815	AW035956	cLEC37N12	tomato callus tissue
24	TIGR EST413638	BE458346	cLEM1F22	tomato immature green fruit
25	I018 LECAD02B21	CK714948	LECAD02B21	seedlings treated with CdCl2
26	TIGR EST252373	AI771177	cLED29C5	tomato ovary
27	TIGR EST320416	AW618430	cLPT13C23	<i>L. pennellii</i> trichome
28	TIGR EST552212	BI932323	cTOC22D20	tomato flower buds
29	TIGR EST361184	BE449746	cLEY14C13	tomato root at pre-anthesis
30	TIGR EST361206	BE449768	cLEY14G19	tomato root at pre-anthesis
31	TIGR EST326197	AW647743	cLEI2I11	tomato germinating seed
32	TIGR EST326246	AW647792	cLEI2E14	tomato germinating seed
33	TIGR EST299571	AW222760	cLEN9E8	tomato red ripe fruit
34	TIGR EST338388	AW929600	cTOC9F21	tomato flower buds
35	TIGR EST401934	BE451047	cLEY16E8	tomato root at pre-anthesis
36	TIGR EST243631	AI485327	cLED5E23	tomato ovary
37	TIGR EST245931	AI487609	cLED9B6	tomato ovary
38	TIGR EST247263	AI488924	cLED18F21	tomato ovary
39	TIGR EST247491	AI489152	cLED15G3	tomato ovary
40	TIGR EST252445	AI771345	cLED29E14	tomato ovary
41	TIGR EST244833	AI486512	cLED6K8	tomato ovary
42	TIGR EST267663	AI898220	cLED32I2	tomato ovary
43	TIGR EST465600	BG132708	cTOE8N23	tomato crown gall
44	TIGR EST360293	BF096341	cLEW11A21	tomato nutrient deficient roots
45a	TIGR EST301229	AW218749	cLEX1C23	tomato root at fruit set
45b	TIGR EST301230	AW218750	cLEX1C23	tomato root at fruit set
46	TIGRcC-esflcLEL7N03a1BG631434		cLEL7N3	tomato flower

Appendix-2. Nucleotide sequence alignment of JWL27 and TC 162290. Numbers on the alignment correspond to the nucleotide numbers of JWL27 and TC162290, respectively.

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162290: 5    ctctttcaatattctgtgagagacgccgtaacctattctagggtttccagaaattttg 64
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 8    ctctttcaatattctgtgagagacgccgtaacctattctagggtttccagaaattttg 67

162290: 65    attctgcgattcaatcacaagtaatcgtttgatcgcgccaaatcgatctgaaatctgaa 124
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  :68    attctgcgattcaatcacaagtaatcgtttgatcgcgccaaatcgatctgaaatctgaa 127

162290: 125   ccatagtgttgatTTTTTtgcgatttTTTTtgttttttggtaaagaaagggaggggaagt 184
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 128   ccatagtgttgatTTTTTtgcgatttTTTTtgttttttggtaaagaaagggaggggaagt 187

162290: 185   ctcagatctgaaatcgaaaagggatttgtctgtgtattgaggatgtctcggtgcttcca 244
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 188   ctcagatctgaaatcgaaaagggatttgtctgtgtattgaggatgtctcggtgcttcca 247

162290: 245   taccaccacctggctattcgctgaatagatccagcaatgaagccttgatcgaatcgatt 304
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 248   taccaccacctggctattcgctgaatagatccagcaatgaagccttgatcgaatcgatt 307

162290: 305   aaggtttgttgaaacaaagagcacttgaatttatgtgtttacgctcttgttgctgatat 364
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 308   aaggtttgttgaaacaaagagcacttgaatttatgtgtttacgctcttgttgctgatat 367

162290: 365   aatctagcagggatctatactctatgtaaatctgtaaggaaaatttgggttttcgtggg 424
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 368   aatctagcagggatctatactctatgtaaatctgtaaggaaaatttgggttttcgtggg 427

162290: 425   ttgtgcattggttgtcaacttggatttagggttttaggatttgattgctgctgggataa 484
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 428   ttgtgcattggttgtcaacttggatttagggttttaggatttgattgctgctgggataa 487

162290: 485   attgacgtttttgtaagccttttgtgtggatttattaattatacctggttagaaatgagg 544
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 488   attgacgtttttgtaagccttttgtgtggatttattaattatacctggttagaaatgagg 547

162290: 545   attagatataactTTTTtgtgctcaccttggatttaacagtatTTaatgaattctacg 604
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 548   attagatataactTTTTtgtgctcaccttggatttaacagtatTTaatgaattctacg 607

162290: 605   agaataggttatgcgaaatctgagcaatttgatatacttaacctttggtaaaggagag 664
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 608   agaataggttatgcgaaatctgagcaatttgatatacttaacctttggtaaaggagag 667

162290: 665   gtgataagtttaggcaacttttaggctttcaatttgtgttggaggaaatcgtaatg 724
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 668   gtgataagtttaggcaacttttaggctttcaatttgtgttggaggaaatcgtaatg 727

162290: 725   agaagttagaagtgatgcaaacattgaggtgtaattgtattgTTTgaggaagaagtcca 784
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 728   agaagttagaagtgatgcaaacattgaggtgtaattgtattgTTTgaggaagaagtcca 787

162290: 785   gcagaaattgtaggcaaaaacctgtgcttgaatagattttctaacttgaatatacttct 844
           | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
JWL27  : 788   gcagaaattgtaggcaaaaacctgtgcttgaatagattttctaacttgaatatacttct 847

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162290:1805 acatgagcctgctgtgtgtttcacagaattccagctgctcatccgacagcaccagaacag 1864
|||||
JWL27 :1808 acatgagcctgctgtgtgtttcacagaattccagctgctcatccgacagcaccagaacag 1867

162290:1865 taataaaaggaagaggcctacttcaccttcaccttcacgaggtggcatacaggcgcgatgg 1924
|||||
JWL27 :1868 taataaaaggaagaggcctacttcaccttcaccttcacgaggtggcatacaggcgcgatgg 1927

162290:1925 tagcattatcagaattagattgtctaaaaaggggtgtgcaaggtgaaatatcagtatccaa 1984
|||||
JWL27 :1928 tagcattatcagaattagattgtctaaaaaggggtgtgcaaggtgaaatatcagtatccaa 1987

162290:1985 agagaagcacttgccaaaacctgctcaacaagttgcggaagtactgtcaggacatctgc 2044
|||||
JWL27 :1988 agagaagcacttgccaaaacctgctcaacaagttgcggaagtactgtcaggacatctgc 2047

162290:2045 cgaaagagccaaccctttgttgaaaactacaacaacgaagttgtcctcctcctgtagc 2104
|||||
JWL27 :2048 cgaaagagccaaccctttgttgaaaactacaacaacgaagttgtcctcctcctgtagc 2107

162290:2105 agtttcagagccaagtagcatccaattgtgggtgggttgaccgtgtggctgaagacaacgc 2164
|||||
JWL27 :2108 agtttcagagccaagtagcatccaattgtgggtgggttgaccgtgtggctgaagacaacgc 2167

162290:2165 aactccatcttgttccaaagtgcacgagaacagcatagagtttcagtacaagaatttgat 2224
|||||
JWL27 :2168 aactccatcttgttccaaagtgcacgagaacagcatagagtttcagtacaagaatttgat 2227

162290:2225 tgagaactggcttccaccaagtctgccaagtgataatctagatttgaggatgatcaatc 2284
|||||
JWL27 :2228 tgagaactggcttccaccaagtctgccaagtgataatctagatttgaggatgatcaatc 2287

162290:2285 atggctgtttcaaagaaaacctaagcaagctcgagttgaggaaaaaaacttagggcgcg 2344
|||||
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JWL27 :2588 tgccagttgctatcttcagaaatccattgttctacatcaatgaaatgatcaatatagta 2647

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