FUNCTIONAL ANALYSIS OF RCD-SRO (RADICAL-INDUCED CELL DEATH-SIMILAR TO RCD-ONE) RELATED GENES FROM TOMATO (Solanum lycopersicum L.) IN SALT STRESSED ROOTS

by

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ABSTRACT

JWL-26 is a novel salt-regulated gene from *Solanum lycopersicum* L. that shares similarity with several *Arabidopsis* proteins including RCD1 (Radical-induced Cell Death 1) and SRO (Similar to RCD-One). These proteins regulate hormone signalling cascades involved in programmed cell death. JWL-26 shares the highest amino acid sequence similarity with SRO5, which is expressed in a salt-responsive manner in Arabidopsis and is proposed to protect cells from reactive oxygen species (ROS). In tomato JWL-26 was expressed exclusively in salt-stressed roots. To determine whether JWL-26 is involved in protecting salt-stressed roots against ROS, JWL-26 together with SRO5 were analyzed in transgenic plants. Transgenic lines were generated that over-expressed SRO5 or JWL-26 in wild-type Arabidopsis and in an sro5 knock-out line. Over-expression of SRO5 or JWL-26 improved survival during salt stress and reduced levels of ROS. Thus, JWL-26 may be involved in ROS detoxification pathways in roots and this may confer salt tolerance.
To Mom and Dad

With love
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CHAPTER 1: INTRODUCTION

Salt is a major factor that limits agricultural production throughout the world. Soil salinization occurs when high concentrations of dissolved salts accumulate within the soil. Although a number of different salts including sodium chloride (NaCl), sodium sulphate (Na₂SO₄), sodium nitrate (NaNO₃) magnesium sulphate (MgSO₄) and magnesium chloride (MgCl₂) are present in saline soils, sodium chloride (NaCl), which is the most abundant and toxic salt causes the most serious problems for higher plants in nature. Soil salinity can occur naturally, referred to as primary salinization or can be induced by human agricultural activities, referred to as secondary salinization (Szalbocs, 1994).

The source of primary salinization is mostly seawater, which is carried inland by winds and deposited by rainfall (Chapman, 1974). In addition, submergence of coastal areas under seawater does also result in primary salinization. In some areas low rainfall, which prevents the leaching of salts from soil, leads to salt accumulation. Secondary salinization generally occurs within arid agricultural settings. In these areas, irrigation is a common cause of salt accumulation at the soil-root interface because dissolved salts in irrigation water remain in the soil after water is lost by evaporation and transpiration (Bernstein, 1974). As a consequence, salts from irrigation water will build up in the soil if continued irrigation is used with improper drainage.
The effects of salt stress on agriculture can be dramatic. High salinity imposes two primary effects on plants the first is osmotic stress, which results in loss of cellular turgor and the second is ionic toxicity leading to inhibition of cellular function due to an increase in the intercellular concentration of Na\(^+\). These primary effects of salinity stress can impose negative effects on various physiological processes including growth, photosynthesis, stomatal closure and cytosolic metabolism. In addition to these stresses, salt also induces the production of Reactive Oxygen Species (ROS) (see section 1.6). As a result of ionic toxicity, osmotic stress and ROS, in extreme cases plants may die under salinity stress.

1.1 Glycophytes and Halophytes

Plants are classified as glycophytes or halophytes according to their ability to grow on high saline medium (Flowers et al., 1977). Most crop plants are glycophytes and can not tolerate salinity stress; the majority of glycophytes are killed on media containing 100 mM NaCl, whereas halophytes (plants adapted to saline habitats) can grow and survive in medium containing 500 mM NaCl (Smith and McComb, 1981, Hasegawa et al., 2000). Halophytes can continue to grow in a saline medium 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 and Zhu, 2000). However, the presence of a special mechanism in halophytes whereby they cope with severe salt stress is still not clear. It has been suggested that halophytes are more successful in using
mechanisms that exist in glycophytes (Zhu, 2001). The use of Arabidopsis (a glycophyte) and Arabidopsis-related halophytic salt cress (*Thellungiella halophila*) (Taji et al., 2004) in a comparative genomics approach resulted in a better understanding of the mechanisms involved in salt tolerance in halophytes and glycophytes. Arabidopsis contains most or nearly all of the salt tolerance genes that exist in halophytes (Zhu, 2000; Shinozaki et al., 2003). The absence of morphological adaptations in salt cress such as salt glands, which usually exist in halophytes led to the hypothesis that tolerance mechanisms in salt cress are similar to those in glycophytes (Bressan et al., 2001; Zhu, 2001). The ability of salt cress to tolerate salt stress is likely because of pre-existing mechanisms that are present prior to stress, these include its ability to express various abiotic or biotic stress-inducible genes under non-stressed conditions.

### 1.2 Na⁺ toxicity

Growth inhibition imposed by Na⁺ toxicity is one of the primary effects of salt stress in plants. The sodium ion (Na⁺) is very harmful when its concentration exceeds the optimal level in the cytosol (1-10 mM). Potassium (K⁺), on the other hand, is a crucial and abundant cation in the cytosol and its concentration needs to be maintained between 100-200 mM for efficient metabolic functioning (Taiz & Zeiger, 2002). Potassium ions play a role as co-factors in the cytosol to activate more than 50 enzymes, which are susceptible to high Na⁺ concentration in the cytosol (Munns, James & Lauchli 2006). Under salt stress, Na⁺ ions compete with K⁺ ions for uptake by plant cells. High concentrations of cytosolic Na⁺ cause metabolic toxicity by a competition between Na⁺ and K⁺ for enzyme binding sites.
Ion cytotoxicity is caused by replacement of K\(^+\) with Na\(^+\) in biochemical reactions, which results in conformational changes and loss of protein function as Na\(^+\) and Cl\(^-\) ions penetrate the hydration shells and interfere with noncovalent interactions between amino acids (Zhu 2002). In addition, elevated levels of Na\(^+\) can change the permeability of the plasma membrane by displacing Ca\(^{+2}\) from the plasma membrane and inducing K\(^+\) leakage from the cell. Therefore, maintenance of intercellular K\(^+\) levels and avoidance of Na\(^+\) influx into the cytosol is vital for a variety of cellular functions such as enzyme activities, maintenance of membrane potential and an appropriate osmotic-dependant regulation of the cell volume. In serious cases, high salinity disrupts water potential homeostasis and, together with ion toxicity at the cellular and whole plant levels, can lead to molecular damage, growth arrest and even death (Zhu 2001 and Zhu et al., 2003).

1.3 Osmotic stress component

Salt-induced osmotic stress reduces water availability for plants and since water-generated turgor pressure is a driving force for cell expansion this can reduce growth (Munns, 2002). Low water availability is also a major limiting factor for photosynthesis and therefore, plant productivity. One of the responses to osmotic stress arising from salinity is abscisic acid (ABA) redistribution and accumulation, which triggers stomatal closure. However, there has been a continuous debate with regards to whether the negative effect of salt stress on photosynthesis is caused as a result of stomatal closure or metabolic impairment. It was recently elucidated that salinity stress mainly limits CO\(_2\) uptake into the
leaves by inducing stomatal closure and not the biochemical capacity to assimilate CO₂ (Flexas et al., 2004).

It is believed that reduced growth due to stress is an adaptive feature to save energy resources to cope with stress conditions and later recovery (Zhu, 2001). During salt stress, the inhibition of shoot growth is greater than that of root growth (Saab et al., 1990). It was proposed that less growth reduction in roots during salt stress would allow plants to explore more soil volume in order to absorb water and minerals. It also adjusts the balance between shoot system and root system sizes with regards to ability of roots to provide water to shoot.

1.4 Sensors of salt stress

The sensing of salt stress and subsequent signalling events to turn on adaptive responses are critical steps for plants exposed to salinity stress. Presumably, plants are able to sense salt stress through both hyperosmolarity and ion-specific signals. However, little is known about how ionic and osmotic stress signals are sensed under salinity, although several osmosensors including histidine kinases are reported to be involved in osmotic stress signal perception (Zhu 2003, Tamura et. al. 2003 Raitt and Saito, 2003). In theory, Na⁺ ions can be sensed either when they enter the cell, or before entering the cell, or both. Membrane receptors are hypothesized to act as sensors of extracellular Na⁺, whereas membrane proteins or cytoplasmic Na⁺-sensitive enzymes are suggested as sensors of intracellular Na⁺. Zhu et al (2002) introduced the plasma-membrane Na⁺/H⁺ antiporter SOS1 (SALT OVERLY SENSITIVE1) as a possible Na⁺ sensor.
SOS1 contains 10-12 transmembrane domains and a long 700 amino acid tail that is predicted to be located in the cytoplasm. Evidence suggests that membrane transporters with long cytoplasmic tails such as bacterial glucose transporter function as a sensor of the solute that they transfer (Chen et al., 1997; Ozcan et al., 1998). The presence of the long cytoplasmic tail in SOS1 suggests that this protein can act as both an Na\(^+\) transporter and sensor (Zhu, 2000). However, how plants sense Na\(^+\) in the cells and whether they sense toxic ions inside the cells or outside need to be elucidated in detail.

1.5 Mechanisms of salt entry into roots

1.5.1 Apoplastic Na\(^+\) influx

Endodermal cells control the radial transport of ions from soil solution to the root xylem since the Casperian strip is an impermeable barrier to apoplastic movement of solutes. Nevertheless, an apoplastic bypass flow is a potential pathway for Na\(^+\) entry into the plant cells. The exact mechanism of the apoplast pathway has not yet been well defined. However, this bypass flow has been suggested to be located at points where the root branches. The apoplastic bypass flow appears to be the main pathway for Na\(^+\) entry in rice (Yeo and Flowers, 1985; Yeo et al., 1987). The importance of this pathway to uptake Na\(^+\) was also supported by some data from salt-tolerant plants. Halophytes have developed several anatomical adaptations such as the formation of a second endodermis to minimize Na\(^+\) entry through the apoplastic pathway (Stelzer and Lauchli, 1977). In cotton (Gossypium hirsutum) seedlings, salt stress induces suberization and the formation of an exodermis (Reinhardt and Rost, 1995).
1.5.2 Transmembrane Na\(^+\) influx

The movement of Na\(^+\) into the roots follows a passive pathway since the concentration of Na\(^+\) in saline soil is generally much higher than that in the cytosol of a root cell (Cheeseman, 1982). Although no Na\(^+\)-selective channels have been identified to date, different uptake mechanisms for Na\(^+\) including the involvement of various cation channels were proposed. The cation channels could be voltage-dependent or voltage-independent. The non-selective voltage-dependent cation channels (NSCCs) have been suggested to be the dominant pathway for Na\(^+\) entry into the plant roots (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, which have a high selectivity for cations over anions. To date there are many candidate genes that encode NSCC's; however, the molecular identity of these NSCCs is still unclear (Demidchik et al., 2002). The two proposed candidates for NSCC's are the cyclic nucleotide-gated channels (CNGCs) and the putative glutamate-activated channels (GLRs) (Maathuis and Sanders, 2001; Leng et al., 2002). It has been shown that CNGCs are expressed in roots and they affect cation uptake (Sunkar et al., 2000; White et al., 2000). The involvement of the glutamate-activated channels (GLRs) in Na\(^+\) influx needs further confirmation.

The involvement of voltage-independent channels in Na\(^+\) transport is better understood than the voltage-dependent channels (Xiong and Zhu, 2002). High-affinity potassium transporters (HKTs) are considered to be the major route for Na\(^+\)-influx into the plant cells (Amtmann and Sanders, 1999; Schachtman and
Liu, 1999; Tyerman and Skerrett, 1999; White, 1999). Given the similarities between Na\(^+\) and K\(^+\), potassium influx transporters have long been suggested to be involved in sodium influx (Epstein et al., 1963). In wheat (Triticum spp.), screening of a cDNA library of K\(^+\)-starved roots resulted in isolation of HKT1, which belongs to the high affinity K\(^+\) uptake system. The HKT transporter was isolated from various plant species including Arabidopsis (Uozumi et al., 2000), eucalyptus (Eucalyptus globules) (Fairbairn et al., 2000; Liu et al., 2001), common ice plant (Mesembryanthemum crystallinum) and rice (Oryza sativa) (Golldack et al., 2002; Horie et al., 2001). In rice, eight functional HKT1 genes (Os-HKT1-8) were isolated (Uozumi et al., 2000, Horie et al., 2001, Golldack et al 2002 and Garciadeblas et al., 2003). In Arabidopsis, only one HKT1, AtHKT1, was identified whereas in eucalyptus two genes with homology to HKT1 were identified (Uozumi et al., 2000; Horie et al., 2001; Garciadeblas et al., 2003).

Expression analysis of AtHKT1 in Saccharomyces cerevisiae and Xenopus oocytes showed that at low external Na\(^+\) concentrations, HKT1 functions as an active K\(^+\) transporter whereas at high external Na\(^+\) concentrations, it can function as a low affinity Na\(^+\) transporter. Rubio et al., 1995). AtHKT1 acts as a Na\(^+\)-transporter and plays an important role in controlling cytosolic Na\(^+\) detoxification (Sunarpi et al., 2005). Sunarpi et al., (2005) suggested that AtHKT1 functions by unloading Na\(^+\) from xylem vessels to xylem parenchyma cells and thus is involved in salt tolerance by protecting the plant leaves from salt stress.
1.5.3 Prevention or alleviation of salt-induced damage

Plants cope with salinity stress either by avoidance or tolerance mechanisms. Avoidance to salt stress involves the restriction of salt uptake. On the whole plant level, this can be achieved by inhibition of salt uptake by roots. However, such a mechanism is not always efficient. Therefore, various strategies have evolved to limit the transportation of salt into the sensitive organs or tissues. In general, glycophytes are known to exclude salt whereas halophytes accumulate salt to maintain a low cellular osmotic potential. Exclusion of salt by glycophytes includes either precluding Na$^+$ entry into the plant at the root surface or Na$^+$ entry into the xylem in the roots. Halophytes sequester Na$^+$ within leaf vacuole or accumulate extracellularly salt in glands or salt bladders. Salt bladders are hair-like structures on leaf surfaces and consist of stalk cells, which support a balloon-shaped cell. The stalk cells transport the salt ions into the vacuole of the bladder cell. Salt glands have a simple structure and directly transport salt ions out of the plants. Another mechanism developed in halophytes to avoid salt is succulence. Succulence is a modification in which high water content is maintained per leaf surface area (Longstreth and Nobel, 1979). This adaptation minimizes the effect of excessive salt accumulation within the leaf tissue. In addition, most halophytes distribute Na$^+$ to older mature leaves rather than to the developing tissues. This adaptation mechanism, however, is not limited to the halophytes since some glycophytes can also compartmentalize salt in older tissues (Flowers and Yeo, 1986, Gorham, 1990).
Tolerance mechanisms involved in adaptation to salt stress can be classified as those that mediate osmolyte biosynthesis, ion homeostasis, and toxic radical scavenging (Bohnert et al., 1995). Plants respond to osmotic stress via osmotic adjustment mediated by the accumulation of organic solutes that do not inhibit metabolic processes, called compatible solutes. Compatible solutes include simple sugars (fructose and glucose), sugar alcohols (glycerol and methylated inositoles), complex sugars (trehalose, raffinose and fructans) (Bohnert et al., 1996), ions (K⁺), charged metabolites (glycine betaine), and amino acids such as proline. (Nuccio et al., 1999). These components are capable of preserving the activity of enzymes in saline solutions. Many osmolytes are considered to function as osmoprotectants, because their concentrations are not sufficient to adjust cell osmotic potential sufficiently for osmotic adjustment. For example, glycine betaine maintains the integrity of the plasma membrane and thylakoid during salt stress (Rhodes et al., 1993). Na⁺ is an effective osmolyte for osmotic adjustment when compartmentalized into the vacuole. The accumulation of sodium ions inside the vacuole makes the vacuolar osmotic potential more negative, which results in a more negative water potential inside the vacuole than in the cytosol. In the meantime, the accumulation of compatible osmolytes in the cytosol lowers the water potential equivalent to the water potential inside the vacuole. These events result in a more negative water potential inside the cell than the surrounding media, which favors water uptake by the cell under salinity (He et al., 2005).
Plants accumulate LEA proteins (late embryogenesis abundant proteins) and chaperons in response to salt stress (Ingram and Bartels from thesis 1996). These molecules are involved in the protection of enzymes, lipids and mRNA (Yamaguchi-Shinozaki et al., 2002). LEA proteins are abundantly produced in embryo tissues during seed desiccation. A variety of LEA proteins have been identified in vegetative tissues of water-stressed plants. LEA gene expression and or protein accumulation is correlated with dehydration tolerance and stress tolerance in a number of plant species. For example, the levels of LEA proteins in the roots of rice are higher in salt-tolerant varieties compared with sensitive varieties (Moons et al., 1995). Xu et al., (1996) showed that transgenic rice over-expressing a barley LEA gene (HVA1) had significantly enhanced tolerance to water and salt stress and delayed development of damage symptoms during stress conditions (Xu et al., 1996).

Transmembrane transport proteins are involved in ion homeostasis. The molecular identity of many of these proteins that mediate Na\(^+\), Cl\(^-\), K\(^+\) and Ca\(^{2+}\) homeostasis has been determined. These proteins include H\(^+\) translocating ATPases and pyrophosphatases, Ca\(^{2+}\)-ATPases, secondary active transporters, and channels mediate ion fluxes (Sze et al., 1999). The SOS–stress signalling cascade regulates Na\(^+\) and K\(^+\) homeostasis and salt tolerance (Zhu 2000). According to the suggested model for the SOS-signal transduction pathway, accumulation of Na\(^+\) initiates changes in cytosolic free Ca\(^{2+}\). SOS3, a myristoylated calcium (Ca\(^{2+}\))-binding protein, senses the cytosolic Ca\(^{2+}\) changes elicited by salt stress and interacts with SOS2, a serine/threonine protein kinase,
to form the SOS3-SOS2 complex. This complex in turn increases the activity of the plasma membrane Na\(^+\)/H\(^+\) exchanger encoded by the SOS1 gene (Zhu 2000 and Liu et al., 2000). SOS1 removes Na\(^+\) from the cytosol into the apoplast through secondary active transport, which is energized by a H\(^+\)-ATPase. Over-expression of SOS1 enhances salt tolerance in Arabidopsis (Zhu 2002 and Shi et al., 2003). SOS1 restricts the loading of Na\(^+\) into the root xylem and therefore limits the accumulation of Na\(^+\) into the shoot.

Vacuolar compartmentalization is another strategy for plants to cope with salt stress (Tester and Davenport 2003). Compartmentalization into the vacuole eliminates the toxicity of Na\(^+\) in the cytosol. This is an advantage for growth and osmotic adjustment since the vacuole occupies almost 90% of mature cells (Zhu 2003). The protein involved in Na\(^+\) transport into the vacuole is the tonoplast Na\(^+\)/H\(^+\)-antiporter. The Na\(^+\)/H\(^+\)-antiporter is energized by the vacuolar H\(^+\)-ATPase. Vacuolar H\(^+\)-ATPase plays an important role in salt tolerance in Saccharomyces cerevisiae (Hamilton, Taylor and Good, 2002) and in many plant species (Vera-Estrella et al., 2005). Increasing the activity of the tonoplast Na\(^+\)/H\(^+\)-antiporter or the activity of the vacuolar H\(^+\)-ATPase can enhance ion accumulation in the vacuole and reduce the potential of Na\(^+\) toxicity in the cytoplasm (Gaxiola et al. 2002). Over-expression of AtNHX1, which encodes a vacuolar Na\(^+\)/H\(^+\)-antiporter resulted in salt tolerance in Arabidopsis (Apse et al., 1999). In a similar approach over-expression of the rice gene OsNHX1, which encode a vacuolar Na\(^+\)/H\(^+\)-antiporter improved salt tolerance in rice (Fukuda et al., 2004). Gaxiola et al. (2001) showed that over-expression of the Arabidopsis
gene AVP1 that encodes a vacuolar H+-pyrophosphatase can increase drought and salt tolerance.

1.6 Salt and oxidative stress

A consequence of salt-induced and ionic stresses is an oxidative stress (Chinnusamy et al., 2005). Reactive oxygen species (ROS) such as superoxide (O2⁻), hydrogen peroxide (H₂O₂), hydroxyl radicals (·OH) and singlet oxygen are unavoidably generated in mitochondria, chloroplasts and peroxisomes as by-products of aerobic metabolic processes such as respiration and photosynthesis. The main sites of ROS production during salt stress are the apoplast, chloroplast and mitochondria. ROS are strongly reactive and generally inactivate enzymes and damage cellular components. In the absence of a protective system, ROS can cause oxidative damage to proteins, DNA and lipids (Gechev & Hilde 5005 and Apel & Hirt 2004). However, increasing evidence indicates that the ROS generated during stress also act as signalling molecules in the stress-response signal transduction pathway. ROS regulates signal transduction pathways through modulating the activity of ion channels (Neill et al., 2002).

1.6.1 ROS scavenging mechanisms

The cytotoxic properties of ROS explain the development of sophisticated ROS-scavenging mechanisms in plants. ROS detoxifying systems are classified as nonenzymatic and enzymatic antioxidants. The main nonezymatic antioxidants include ascorbate (APX) and glutathione (GSH), which directly or
through the ascorbate-glutathione cycle (Halliwell-Asada cycle) scavenge ROS from the cell. Enzymatic ROS scavenging systems include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SODs are considered to be the first line of defense against ROS. SODs reduce superoxide to form H$_2$O$_2$ and O$_2$. Catalases (CAT) in turn convert H$_2$O$_2$ to water and oxygen. Three classes of SODs have been identified according to the metal co-factor they utilize: iron SOD (Fe SOD), manganese SOD (Mn SOD), and copper-zinc SOD (Cu-Zn SOD). Typically, Fe SODs are in the chloroplast, Mn SODs are in the mitochondrion and the peroxisome, and Cu-Zn SODs are located in the chloroplast and the cytosol (Alscher et al., 2002 and Apel 2004).

APX, GPX and CAT are the major H$_2$O$_2$ metabolising systems. In contrast to CAT, detoxification of ROS by APX necessitates the presence of ascorbate and the GSH regeneration system, the ascorbate-glutathione cycle. Components of the ascorbate-glutathione cycle are extensive in the cellular compartments where ROS detoxification is necessary, such as the chloroplasts, mitochondria and cytosol. This indicates that the cycle functions as a key factor to keep ROS generation under control (Yabuta et al., 2004).

1.6.2 ROS production mechanisms

1.6.2.1 Salt-induced ROS in the chloroplast

In salt stressed plants, ROS contribute to cellular damage. ROS in the chloroplast arise due to decreases in intercellular CO$_2$ concentrations as a result of stomatal closure. This decreases the efficiency of the Calvin cycle and results in insufficient regeneration of NADP$^+$ (Miyake et al., 2005). This initiates the
reduction of O$_2$, which results in the formation of ROS. Hernandez et al. (1995) showed that salinity can decrease chlorophyll content and PSII activity and at the same time increase the concentration of H$_2$O$_2$.

Antioxidant systems of the chloroplast keep the levels of ROS under control (Gonzalez et al., 2002). The ascorbate/glutathione cycle is the major ROS scavenging system in the chloroplast (Møller 2001). The activity of the chloroplastic antioxidants is changed under salinity. Hernandez et al. (2001) observed an increase in the activity of cytosolic and chloroplastic CuZn-SODII in the pea plants exposed to salt. The expression of Fe-SOD increased in the chloroplast of salt-treated Lycopersicon pennellii (Lpa) plants. Salinity also induced the activity of ascorbate peroxidase and GST (glutathione-S-transferase) in the chloroplast of salt-treated Lpa plants (Hernández 2001 and Mittova 2004).

1.6.2.2 Salt-induced ROS in the mitochondria

In addition to the chloroplasts, ROS can be generated in the mitochondria. The electron transport chain (ETC) is the major site of ROS production. The ETC contains complexes I-IV, an alternative oxidase (AOX) and four NAD(P)H dehydrogenases, which are a potential site of ROS generation (Møller 2001). Damage to mitochondria is an early event under high saline conditions. Hamilton and Heckathorn (2001) showed that salt stress causes damage to mitochondrial electron transport in Zea mays (Hamilton and Heckathorn 2001). Salinity affects the mitochondrial electron transport chain by damaging Complex I (NADH dehydrogenase) and II (succinate dehydrogenase). Oxygen, as an electron acceptor, directly interacts with complex I to oxidize NAD(P)H dehydrogenases...
on the inner surface of the inner mitochondrial membrane (IMM) (Møller 2001). The leakage of electrons from complex I under salt stress leads to the formation of ROS (Mittova et al., 2004). Accumulation of H$_2$O$_2$ in mitochondria coincides with disintegration of the mitochondrial matrix and with the appearance of the first symptoms of leaf damage (Pellinen et al., 1999). Under salt stress, plants protect the mitochondrial electron transport chain with osmoprotectants (proline, betaine, and sucrose), antioxidants and small heat shock proteins (sHsps). Antioxidants and sHsps protect complex I whereas, complex II is protected by osmoprotectants. The osmoprotectants protect complex II either through balancing osmotic potential or direct stabilization of membranes and/or proteins (Hamilton 2001).

The protective role of the root mitochondrial antioxidant system against oxidative stress has hardly been studied (Mittova 2004). The adaptation of salt tolerant LPa roots to oxidative stress resulted from salt-induction of the mitochondrial SOD and APX. Elevated levels of leaf mitochondrial H$_2$O$_2$ under salt stress was reported for pea and tomato plants (Dixit et al., 2002). Similar salt-induced activities of SOD and APX were also observed in leaf mitochondria (Boveris 1984). Furthermore, the levels of ASC and GSH increased in the mitochondria of Lpa plants under salinity, which indicate that non-enzymatic mechanisms of H$_2$O$_2$ detoxification also contribute to decrease the salt-induced H$_2$O$_2$. 

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1.6.3 Salt-induced ROS in the apoplast

Little is known about the generation of apoplastic ROS under salinity. Likely, the main enzyme generator of apoplastic ROS in plants is NADPH oxidase enzymes similar to those present in mammalian phagocytes (Apel et al., 2004). Cross et al., (1999) proposed that NADPH oxidase is activated by cations (Cross et al., 1999 and Kawano et al., 2001). Furthermore, application of diphenylene iodonium (DPI), an inhibitor of NAD(P)H oxidase significantly reduced the accumulation of superoxide and H$_2$O$_2$ under salt stress (Dixit et al., 2001).

Salt stress can induce necrotic lesions on plant leaves. Salt-induced necrotic lesions (SINLs) are localized initially on the minor veins and are observed during the first stages of injury. SINLs are reminiscent of the leaf “microbursts” observed in response to pathogens where H$_2$O$_2$ is accumulated. The development of necrotic lesions in the vicinity of veins, cell death and ROS accumulation have been observed in ozone exposed plants. Nitroblue tetrasolium (NBT) staining of SINLs of minor veins proved the accumulation of both O$_2^-$ and H$_2$O$_2$ in the necrotic lesions. Thus, SINLs are a result of apoplastic ROS (Hernández et al., 2002). The presence of SOD, APX and catalase activities have been reported in the apoplast of both barley (Hordeum vulgare) and oat (Avena sativa) leaves. Further studies need to be performed to determine the mode of action of NADPH oxidase and ROS scavenging mechanisms in the apoplast under salt stress (Cross et al., 1999 and Hernández et al., 2002).
1.7 Research background

Application of differential display-polymerase chain reaction (DD-PCR) to isolate novel salt-responsive genes from tomato roots resulted in the identification of JWS-26, a partial cDNA derived from a gene that was clearly up-regulated in salt-treated tomato roots (Wei et al., 2000). The expression of the gene corresponding to JWS-26 was up-regulated transiently, peaking at 2h after salt treatment (Wei, et al. 2000). A full length cDNA clone (JWL-26) was isolated from a cDNA library constructed with mRNA isolated from tomato roots exposed to salt for 2 hr. The cDNA corresponding to JWS-26, hereafter called JWL-26, encodes a polypeptide with amino acid sequence similarity with several Arabidopsis proteins that include RCD1 (Radical-induced Cell Death 1) and the SRO (Similar to RCD-One) family members (Effendy 2002). Thus, JWL-26 may be related to RCD1-SRO gene family.

Lesions in RCD1 confer an ozone sensitive phenotype and the rcd1 mutant was isolated as such by Overmyer et al., (2000) and independently in a screen for UV-B resistant mutants by Fujibe et al., (2004) (Overmyer et al., 2000 and Fujibe et al., 2004). RCD1 mediates hormone signalling pathways involved in the regulation of ROS-dependant programmed cell death (PCD) in Arabidopsis (Overmyer et al. 2000). PCD shares similar features with apoptosis in humans, such as the degradation of nuclear DNA into internucleosomal fragments, shrinking of the nucleolus and condensation of the chromatins. The plant hormone ethylene (ET) is a regulator of PCD in plant-pathogen interactions and senescence (Overmyer et al. 2005). A key role for ethylene as a regulator of cell
death in ozone responses has also been suggested (Overmyer et al. 2000). The 
rcd1 mutant was described as a novel class of ROS-responsive lesion-mimic 
mutants (LMM). Ozone exposure of rcd1 showed HR (hypersensitive response)-
like symptoms similar to that induced by incompatible pathogens. Analysis of 
rcd1 revealed the involvement of three separate processes during lesion 
formation including initiation, propagation and containment. The rcd1 mutant 
accumulates SA and ET upon ozone exposure. Salicylic acid (SA), a natural 
inducer of plant defense against pathogens, has a central role in generating the 
oxidative burst and development of HR. Ozone exposure induces SA 
biosynthesis, which acts in concert with ROS to initiate lesion formation through 
the same mechanisms that regulate lesion formation during HR (Overmyer et al. 
2000 and Ahlfors et al. 2004). SA enhances H2O2 levels through inactivating 
enzymes involved in H2O2 degradation, such as catalase and ascorbate 
peroxidase and by increasing activities of Cu,Zn-SOD. During the propagation 
phase of lesion formation signals are generated, which amplify cell death to the 
neighboring cells. H2O2 and superoxide serve as signaling molecules in lesion 
amplification processes. Ethylene biosynthesis and signaling are required for 
lesion propagation. It has also been determined that ethylene acts upstream of 
superoxide accumulation. Analysis of an rcd1 ein2 (ethylene insensitive) double 
mutant indicated that lesion initiation is ethylene independent whereas lesion-
propagation is an ethylene-dependent phenomena. Jasmonic acid (JA) and its 
methyl ester, methyl jasmonate (MeJA) are suggested to be responsible for the 
containment of lesion spread. In ozone responses, ethylene and JA oppose
each other and JA signaling prevents the spread of cell death. Application of MeJA to rcd1 after O3 exposure inhibits lesion propagation (Rao et al. 2000 and Ahlfors et al. 2004).

The RCD1 protein interacts with several transcription factors, including DREB2A (drought-responsive element binding 2A), which binds to DRE/CRT (dehydration-responsive element/C-repeat) cis-acting elements to regulate the expression of genes involved in the response to drought and low-temperature (Ahlfors et al., 2004). Furthermore, work by Katiyar et al. (2006) has shown that SOS1 interacts through its C-terminal cytosolic tail with RCD1 to regulate the accumulation of ROS during salt stress. Such an interaction may either regulate the transport of ROS and/or reductant across the cell membrane or affect the expression of ROS-scavenging genes, which are regulated by RCD1 and SOS1 (Katiyar et al. 2006). The increased expression of SOD genes in the rcd1 and sos1 mutants may explain the increased tolerance of these mutants to herbicide methyl viologen (MV), which generates superoxide in the chloroplast, and the higher sensitivity to H2O2.

The RCD1-SRO gene family contains, in addition to RCD1, five genes (SRO1-SRO5), which encode proteins of unknown function that are very similar to RCD1. RCD1 and SRO1 possess nuclear localization (NLS) motifs and a WWE domain involved in protein-protein interactions in ubiquitin and ADP-ribose conjugation systems (Aravind et al., 2001 and Ahlfors et al., 2004). These conserved domains are not present in SRO2-SRO5. However, RCD1 and all SROs carry a poly(ADP-ribose) polymerase (PARP) signature, which is located
upstream of the C-terminal protein interaction domain. PARP catalyses the poly(ADP-ribosyl)ation of a limited number of proteins involved in DNA repair and chromatin architecture (De Murcia, and de Murcia 1994, Ahlfors et al., 2004 and Ame et al., 2004). PARP also might transfer an ADP-ribose moiety from its substrate nicotinamide adenine dinucleotide (NAD), to a target molecule (Corda and Di Girolamo, 2003). Together, some PARPs may play a role in genome protection, whereas others may function in telomere replication and cellular transport (De Murcia G., and de Murcia J. M. 1994 and Ame et al., 2004).

JWL-26 lacks the NLS motifs and WWE domain. However, JWL-26 contains a PARP domain and has sequence identity with the C-terminal protein interaction domain. Thus, JWL-26 could interact with other proteins for poly(ADP-ribosyl)ation purposes. Since starting my research it has been shown, in Arabidopsis, that SRO5 is expressed in salt stressed seedlings (Borsani et al., 2005).

1.8 Research objectives

Reverse and forward genetic approaches together with molecular and physiological analyses were carried out to characterize the function of JWL26 and putative tomato orthologs for RCD1 during salt stress. I hypothesize that:

1. JWL-26 and the putative tomato orthologs for RCD1 are involved in salt tolerance.

2. JWL-26 and the putative tomato orthologs for RCD1 are involved in oxidative stress detoxification pathways in roots during salt stress.
To test these hypotheses I undertook the following approaches:

1. To isolate RCD1-like cDNAs from tomato.

2. To determine the nucleotide and predicted amino acid sequences of RCD1-like cDNAs in order to gain information about their encoded polypeptides.

3. To analyse the expression of *JWL-26* and *RCD1-like* genes in parallel with *At-RCD1* and *At-SRO5* in salt treated plants.

4. To generate and analyse transgenic lines over-expressing: *Le-RCD1-Like*, *JWL-26* and *SRO5* and to characterize the role of these genes in salt stressed plants.

5. To assess the extent of ROS production and scavenging in salt-stressed wild-type and transgenic lines with altered *SRO5* and *JWL-26* expression.
CHAPTER 2: ISOLATION AND EXPRESSION OF LE-RCD1-LIKE GENES

2.1 Introduction

Application of differential display-polymerase chain reaction (DD-PCR) to isolate novel salt-responsive genes from tomato roots resulted in the identification of JWS-26, a partial cDNA derived from a gene that was clearly up-regulated in salt-treated tomato roots (Wei et al., 2000). The expression of the gene corresponding to JWS-26 was up-regulated transiently, peaking at 2h after salt treatment (Wei et al., 2000). A full length cDNA clone (JWL-26) was isolated from a tomato cDNA library constructed with mRNA isolated from tomato roots exposed to salt for 2 hr. The nucleotide sequence of the cDNA corresponding to JWS-26, hereafter called JWL-26, is 1459 bp long and contains a 1035-nucleotide ORF that encodes a predicted 344-amino acid protein (Effendy 2002).

BLAST-based homology searches revealed similarity between JWL-26 and several Arabidopsis proteins that include RCD1 (Radical-induced Cell Death 1) and the SRO (Similar to RCD-One) family members (Effendy 2002). Lesions in RCD1 confer an ozone sensitive phenotype and the rcd1 mutant was isolated as such by Overmyer et al., (2000) and independently in a screen for UV-B resistant mutants by Fujibe et al., (2004). RCD1 mediates hormone signalling pathways involved in the regulation of ROS-dependent programmed cell death in Arabidopsis (Overmyer et al., 2000). In addition to RCD1, the RCD1-SRO gene
family contains five genes (SRO1-SRO5) of unknown function. RCD1 and SRO1 possess nuclear localization signal (NLS) motifs and a WWE domain involved in protein-protein interactions in ubiquitin and ADP-ribose conjugation systems (Aravind et al., 2001 and Ahlfors et al., 2004). These conserved domains are not present in SRO2-SRO5. However, RCD1 and all SROs carry a poly(ADP-ribose) polymerase (PARP) signature, which is located upstream of the C-terminal protein interaction domain. PARP catalyses the poly(ADP-ribosylation) of a limited number of proteins involved in DNA repair and chromatin architecture (De Murcia and de Murcia 1994, Ahlfors et al., 2004 and Ame et al., 2004). PARP also might transfer an ADP-ribose moiety from its substrate nicotinamide adenine dinucleotide (NAD), to a target molecule (Corda and Di Girolamo, 2003). Together, some PARPs may play a role in genome protection, whereas others may function in telomere replication and cellular transport (De Murcia G., and de Murcia J. M. 1994 and Ame et al., 2004). The JWL-26 amino acid sequence is most closely related to SRO5 (At5g62520.1) protein (Effendy 2002). The NLS motifs and WWE domain are not present in JWL-26. However, like SRO5, JWL-26 contains a PARP signature and has sequence identity with the C-terminal protein interaction domain.

2.2 Research objectives

The objectives of the work in this chapter were to:

1. Isolate RCD1-like cDNAs from tomato.
2. Determine the nucleotide and predicted amino acid sequences of the isolated cDNAs in order to gain information about their encoded polypeptides.

3. Analyse the expression of RCD1-like genes in parallel with At-RCD1 and At-SRO5 in salt treated plants.

2.3 Materials and methods

2.3.1 Materials

Control and salt treated tomato and Arabidopsis root and shoot tissues were kindly provided to me by Agnes Kwok and Theingi Aung, respectively.

The original JWL-26 cDNA clone was provided by Jollanda Effendy. The tomato cTOD6A15 and cLEN4A3 cDNA clones were obtained from CUGI (Clemson University Genomics Institute, http://www.genome.clemson.edu). The Arabidopsis SRO5 cDNA clone was obtained from TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org) and the RCD1 cDNA clone was kindly provided by Dr. Jaakko Kangasjärvi at the University of Helsinki, Finland.

2.3.2 Isolation of Le-RCD1-Like cDNA clones

The nucleotide sequence of the cDNA insert of cLEN4A3 was determined using universal T3 (5’-AATTAACCCTCACTAAAGGG-3’) and T7 (5’-TAATACGACTCACTATAGGG-3’) primers and gene specific primer (5’-GATGCTGATGAAAA TGGCG-3’).
Total RNA extracted from 6-week-old tomato roots exposed to 170 mM salt for 8 h was used for RT-PCR (Reverse transcriptase polymerase chain reaction) to isolate the full-length cDNA clone corresponding to cTOD6A15. RNA (2.5 µg) was reverse-transcribed by M-MuLV reverse transcriptase (Fermentas) from an anchored oligo-dT primer using standard methods in a reaction volume of 20 µL. Each reverse transcription (RT) reaction contained 2.5 µg RNA, 20 U RNase inhibitor (Fermentas), 20 U reverse transcriptase (Fermentas), 10 mM reaction buffer, 10 µM oligo-dT and 20 µM each dNTP and was incubated at 70°C for 5 min followed by 42°C for 90 min.

The open reading frame (ORF) of cLEN4A3 and cTOD6A15 cDNAs were directly amplified by polymerase chain reaction (PCR) with the following pair of primers: 5'-CACCGCATTTTCTTCAATATGATGTAGC-3' (Le-RCD1-likeA forward), 5'-CAACCATCTAAACGATCTCAC-3' (Le-RCD1-likeA reverse), 5'-CACCTGACAGACCTGTGCTATACTAG-3' (Le-RCD1-likeB forward), 5'-AGAAACATAATAATGCGAAAAC-3' (Le-RCD1-likeB reverse). Each PCR reaction contained 0.2 µM of each primer, 10 µM dNTP mixture, 1.25 mM MgCl₂, 2U Taq DNA polymerase, supplied with its own buffer (Fermentas) and 20 ng template cDNA. Thermal cycling was carried out in a Thermo Hybaid MBS 0.2G thermal cycler programmed to complete one cycle of 95°C for 2 min, then 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 min followed by one cycle of 72°C for 5 min. After PCR the products were separated in a 1% agarose gel in 1X TAE (Tris-Acetate-EDTA, pH 8.0) and bands of the expected size were excised and purified using QIAquick PCR purification kit (QIAGEN)
according to the manufacture's instructions. Re-amplified cDNA products were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen) following the manufacturer's instructions. The plasmid DNA was then transformed into *Escherichia coli* TOP10 (Invitrogen) by the heat-shock method whereby the reaction was incubated on ice for 30 min and subsequently heat-shocked in a water bath for 30 seconds at 42°C without shaking. The transformed cells were immediately transferred to ice, followed by addition of 250 μL LB medium. The cultures were then incubated at 37°C for 2 h with shaking (250 rpm). Aliquots of bacterial culture (50-100 μL) were spread on pre-warmed agar plates containing 50 μg/ml kanamycin, and incubated at 37°C overnight. Plasmid DNA was isolated using the Qiagen plasmid purification kit following the manufacturer's instructions (Qiagen, Missisauga, Ontario, Canada). Positive clones were confirmed by PCR using the same primers used to amplify the original cDNAs. PCR products were separated in a 1% agarose gel in 1X TAE. The nucleotide sequence of positive cDNA clones was determined using the M13 reverse (5'-CAGGAAACAGCTATGACC-3') and M13 forward (5'-GTAAAACGACGGCCAGT-3') universal primers and gene specific (Le-RCD1-likeA-F-Sequencing: 5'-GATGCTGATGAAAATGGCG-3' and Le-RCD1-likeB-F-Sequencing: 5'-TGGCAGAAAATCAACAGG-3') primers. Sequencing was carried out by an ABI 311 automatic sequencing machine (Perkin Elmer, Foster City, CA, USA) at the NAPS unit (Biotechnology Laboratory, University of British Columbia, BC, Canada). Gene specific primers were designed using OLIGOTM Version 5.0
software (National Biosciences Plymouth, MN, USA), and were tested with PCR before use in sequencing.

2.3.3 Nucleotide and deduced amino acid sequence analyses

Similarity searches were performed by submitting nucleotide sequences to the National Center for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). The analysis was conducted using BLASTX against the nr (non-redundant protein sequences) database. Full-length nucleotide sequences were translated using DNASTAR software. Nuclear localization predictions (NLS) were performed using the PredictNLS server (http://cubic.bioc.columbia.edu/predictNLS). Conserved domains were analysed using the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and InterProScan sequence search (http://www.ebi.ac.uk/InterProScan). Amino acid sequences were aligned using multiple sequence alignment software, CLUSTALW (MacVector 7.2). In the alignment, similarity among amino acids was determined using default settings. Analyses of Arabidopsis microarray data were conducted using the GENEVESTIGATOR (https://www.genevestigator.ethz.ch) toolbox.

2.3.4 RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen). Frozen tissues (300 mg) were ground to a fine powder in liquid nitrogen. One millilitre of Trizol was added per 100 mg (FW) of sample, and ground until an ice-cream-like consistency was obtained. The ground tissue was transferred to a 15 ml tube.
and mixed thoroughly by vortexing. The mixture was then incubated at 65 °C for 5 minutes followed by centrifugation at 5000 rpm in a SS34 SORVALL rotor for 40 min at 4°C. Following centrifugation, the top aqueous layer was removed and transferred to a new 15 ml tube. Chloroform (0.2 ml CHCl₃ per ml Trizol) was added to the supernatant, mixed thoroughly, and then centrifuged as before. Following the second centrifugation, the top layer was removed and transferred to a new 15 ml tube. A half volume of cold isopropanol and a half volume of 0.8 M Na₃-citrate was added, mixed thoroughly, followed by a ten minute incubation at room temperature. The sample was centrifuged as before for 40 min at 4°C. Following centrifugation, the supernatant was discarded, and 70% (v/v) ethanol was added to the pellet and the sample was centrifuged at the same speed as before for 30 min. The pellet was briefly air-dried and dissolved in 100 μl DEPC-ddH₂O and transferred to a 1.5 ml microfuge tube. The concentration of RNA was measured using a 1:100 dilution of the RNA in a spectrophotometer (BIO-RAD SmartSpec™ 3000, Mississauga, Ontario) at 260 nm and 280 nm in water.

2.3.5 Preparation of radiolabelled probes

Le-RCD1-like cDNA clones were used to generate probes for northern blot analyses. PCR was performed (as described in section 2.3.3) using the same primers that were used to amplify the ORF of original cDNAs. The cDNA insert of JWL-26 was amplified using forward (5’-CGGTCTGAGGCGGCAAGA-3’) and reverse (5’-GGTTGGAGGGTTGAAAG-3’) primers. The At-SRO5 cDNA insert was amplified using the uni51 forward (5’-CTGTGAGGCTCAAATCG-3’) and uni51 reverse (5’-TGGCTGGAACCTTAGGAGGCAC-3’) primers. The
cDNA insert of At-RCD1 was amplified using M13 universal primers. To assess RNA loading, tomato 18S rRNA and Arabidopsis ubiquitin were used as constitutive probes. The inserts of the 18S rRNA and ubiquitin clones were amplified using the T3 (5'-AATTAACCCTCACTAAAGGG-3'), T7 (5'-TAATACGAC TC ACTATAGGG-3'), and uni51 (ubi) universal primers, respectively. PCR conditions were similar to that described in section 2.3.2, with the exception of the annealing temperature, which was 55°C. PCR products were purified using the QIAquick gel extraction kit (QIAGEN) following the manufacturer's instructions. The concentration of the DNA probes was determined using a spectrophotometer at 260 nm. To verify the concentration, the probes were electrophoretically separated at 85V for 1.5h in a 1% agarose gel in 1X TAE buffer against a DNA Mass Ladder (Invitrogen). The detection of DNA fragments was performed under UV light using ethidium bromide staining.

Approximately 15 ng DNA was denatured by boiling for 3 min and immediately cooled in ice. Subsequently, 1X Tris-EDTA (TE Buffer, pH 8) was added to the denatured DNA to a total volume of 45μl. The denatured probe was then transferred to an oligolabelling reaction mix vial (Amersham Rediprime™ II, Random prime labelling system, Piscataway, USA). Fifty μCi α-32P dCTP (at 3000Ci/mmol) (NEN, PerkinElmer Life Sciences, Markham, Ontario, Canada) was added, carefully mixed and incubated at 37°C for 30 min. The labelled probe was separated from unincorporated nucleotides by applying the probe mixture to a microspin column (MicroSpin™ S-300 HR Columns, Amersham Pharmacia Biotech) and centrifuging at 3,000 rpm for 2 min. The flow-through
was collected, and 1 μl aliquot of the flow-through was added directly to a vial containing scintillation cocktail (BCS Biodegradable Counting Scintillant, Amersham, Oakville, Ontario, Canada) to measure the total cpm from which the specific activity of the probe was calculated. The probe was then denatured at 90°C for 3 min chilled on ice for 5 min and added to hybridization buffer.

2.3.6 Northern hybridization analyses

RNA aliquots (15 μg) were separated in a formaldehyde-denaturing agarose gel (1.2% w/v) 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) by electrophoresis. 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 containing 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 the sample was loaded. The gel was run in 1X MOPS at 60 V for 3h, after which it was rinsed in DEPC-treated ddH2O and then soaked in 20X SSC (3.0 M NaCl, 0.3 M sodium citrate pH 7.0) for 5 min. Prior to blotting a positively charged nylon membrane (Boehringer Mannheim, Laval, Quebec, Canada) was soaked briefly in DEPC-treated ddH2O, following by soaking in 20X SSC for 5 min. Blotting by capillary transfer was performed in 20X SSC overnight as described by Sambrook et al. (1989). Following blotting, the nylon membrane was soaked in 6X SSC for 5 min and air dried. The transferred denatured RNA was fixed onto the nylon membrane by exposure to UV light for 30 seconds using the auto crosslink setting a UV Stratalinker™ 2400.
The membrane was then baked at 80°C for 30 min. The membrane was prehybridized in 10 mL hybridization solution (100 mM NaH₂PO₄, 50 mM Na₂P₂O₇, 1 mM EDTA and 7% SDS) containing denatured salmon sperm DNA (100 µg/ml) for 2 h at 65°C in a rotary hybridization oven (Techne Hybridiser HB-2D, Mandel, Guelph, ON, Canada). Fifty microliters of the denatured radiolabelled probe was added, and the mixture was incubated overnight at 65°C. Following hybridization, the blot was washed three times in 10 ml wash solution I (2X SSC, 0.1% SDS) at room temperature for 5 min, then washed two times in wash solution II (1X SSC, 0.1%SDS) at 65°C and at 68°C, each for 45 min. The membrane was thereafter washed in wash solution III (0.5 XSSC, 0.1% SDS) at 65°C for 45 min and then briefly air-dried and sealed with saran wrap. Finally, the blot was exposed to either an X-ray autoradiography film (Kodak Scientific Imaging Film X-Omat Blue XB1, NEN™ Life Science, Boston, MA, USA) at -80°C with an intensifying screen for 24h, or to a phosphoscreen for a minimum of one hour. The phosphoscreen was detected with a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Inc., Sunnyvale, CA).

Band intensity from autoradiography films was quantified using the Scion Image Beta software version 1.62c (Scion Corporation, Frederick, MD, USA). The expression level was normalized by dividing the signal obtained for a salt responsive cDNA probe by that obtained for the 18S rRNA probe. Band intensity from phosphoscreen was quantified using Image Quant 5.2 (Molecular Dynamics, Piscataway, NJ) and subsequently normalized by dividing the signal.
obtained for a salt responsive cDNA probe by that obtained for the ubiquitin probe.

2.4 Results

2.4.1 Isolation and characterization of Le-RCD1-like cDNAs

In order to identify RCD1-like genes in tomato, At-RCD1 was used as a query to search against the *Lycopersicon esculentum* EST (expressed sequence tag) database at TIGR (http://www.tigr.org). Searching against TIGR database with the BLASTP program resulted in only two cDNA clones, cLEN4A3 and cTOD6A15 with significant sequence similarity to the 5' end of RCD1, which were obtained form Clemson University Genomics Institute (CUGI). TC 162509 and TC159230, which contain partial cDNA sequences of cTOD6A15 and cLEN4A3, respectively, were identified at the *Lycopersicum esculentum* EST database.

The entire cDNA insert of cLEN4A3 is 1953 nucleotides in length and contains a single open reading frame (ORF) encoding a polypeptide of 594 amino acids. Alignment of the nucleotide sequence of cLEN4A3 obtained using T3 and T7 universal primers against TC159230 showed no differences between these two sequences. CLEN4A3 contains a 138-bp-long 5'-untranslated region (5' UTR) and a 89-bp-long 3'-untranslated region (3' UTR). The ORF of cLEN4A3 was amplified using specific primers and cloned into the GATEWAY pENTR vector. The cLEN4A3 clone is renamed hereafter as Le-RCD1-likeA.

The nucleotide sequence of cTOD6A15 obtained using the universal T3 and T7 primers was aligned against TC 162509, which contains a partial cDNA
sequence of cTOD6A15 and a stop codon was found in the middle of the single potential ORF. Therefore, in order to isolate a cDNA corresponding to cTOD6A15, total RNA isolated from tomato roots was subjected to RT-PCR using primers designed to amplify the ORF from cTOD6A15. A cDNA of approximately 2.5 kb was amplified from root total RNA using these ORF specific primers and cloned into the GATEWAY pENTR vector. The nucleotide sequence was determined and found to be 2301 nucleotides in length with a single ORF capable of encoding a polypeptide of 597 amino acids. The isolated cDNA corresponding to cTOD6A15 contains a 77 bp 5' UTR and a 430 bp 3'UTR and is renamed hereafter as Le-RCD1-likeB. The complete nucleotide and deduced amino acid sequence of Le-RCD1-likeA and B are presented in Figure 2-1.
Figure 2-1: Le-RCD1-like A and B nucleotide and deduced amino acid sequences.

The deduced single-letter amino acid sequence is given below the center nucleotide of each codon. The initiating ATG is underlined, and the stop codon is designated with an asterisk (*).
Le-RCD1-likeA

10  20  30  40  50  60  70  80  90  100
GCATTTCCTTTCTTCAATATGATGTAGCTTCCCGGAAATTTATCACAGATGGTTTTTACTA'ITAGTATTTTAGCCIIIIIIIGGTACTTGTAAATAGATAG'ITAT
110 120 130 140 150 160 170 180 190 200
ATCTGTAGAT~CCAAAGGGGG'ITGGATTTTTAGATAGTGGCCGTAGGACTGTGG'ITGGAGAAAAGAGTAAAGTGG'ITACTCAGAATTTGGCA
M E K S K V V T Q N L A

210 220 230 240 250 260 270 280 290 300
CATCTGCTTAGAGCCAGCAGTGAGAAAATATCAA'ITCAATCCAACTGTGATAGAAAGCTTGAGAAGAGGAAAAGGATCG'ITCACTGCGAGTCTAATAGTC
H L L R ASS E K I S I Q S N CDR K L E K R K R I V H C E S N

310 320 330 340 350 360 370 380 390 400
AATCACATCTCAGGATATCTGAACACAAGAACTACCTGAACT'ITAAGAGAAGCAGACTGCCGCTACGTGTACTGTTTTATCAGAACGGTGAATGGACCGA
Q S H L R I S E H K N Y L N F K R S R L F L R V L F Y Q N G E W T D

410 420 430 440 450 460 470 480 490 500
CTTCCCTCAAGATA'ITA'ITCCTATAG'ITAAAGAAGATTTT=CGAAAAAGACTG'ITA'ITGAGGTGAAAGTTTGTGACT'ITCATATAA'ITCTTGATA'IT
F F Q D I I P I V KED F R A K K T V I E V K V C D F H I I L D I

510 520 530 540 550 560 570 580 590 600
CTACATATGG'ITCAAATAGATGTAATAAATGGTTTACAGAAACCTA'ITGCCTGGATAGATGAAGTAGGTAGGTGTTTCTTCCCTGAGTCTTATCTTATCA
L H M V Q I D V I N G L Q K P I A WID E V G R C F F F E S Y L I

610 620 630 640 650 660 670 680 690 700
GTAGTGAAATGCTTGGGAA'ITTTGAAACCCTGTCAAAGAGAACTGAAGAGTTTATGACAACTGAGCCAGATAGGATAACTGATATGAAG'ITGCAACTCGA
SSE M L G N F BTL S K R TEE F M T T E P D R lTD M K L Q L D

710 720 730 740 750 760 770 780 790 800
CACTGACTTAAATGGACTGGATAATCGCAATTTGGAAGAAGATGTGGAAGAGTCAAATA'ITGG'ITACAAGAGGAATAAAGTATGTCCTCTTAAAGACAGT
T D LNG L D N R N LEE D VEE S N I G Y K R N K V C F L K D S

810 820 830 840 850 860 870 880 890 900
CAAGAAG'ITGCTGA'ITATAAAAAATCAGATGCAAAAATTGCACAAG'ITGCAGAAAATAAACAGAACCAAGAAACACCGTCTCCTGACTTAGAAGCTAGCC
Q E V A D Y K K S D A K I A Q V A E N K Q N Q E T P S P D LEA S

910 920 930 940 950 960 970 980 990 1000
TGAAATTTGTGAATGCAGAATCTGTCAAAAATATGTTTA'ITATGGGAATGAATG'ITAACCCTAACAAGTGTGAGATCAAAA'ITAACAAATGCTCTAGTAA

910 920 930 940 950 960 970 980 990 1000
TGAAATTTGTGAATGCAGAATCTGTCAAAAATATGTTTA'ITATGGGAATGAATG'ITAACCCTAACAAGTGTGAGATCAAAA'ITAACAAATGCTCTAGTAA
L K F V NAB S V K N M F I M G M N V N P N K C ELK INK C S S N

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
ATTTTGACAACTCGCTTAGAACTCT'ITGAGAAGCAGGTTGAAATAACGCAAAAATATCGTGGAAACTCAAATG'ITCGATATGCATGGCTTGCCGCTTCT
Y L T T R L ELF Y L T T R L ELF Y L T T R L ELF Y L T T R L ELF

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
AAAGATTTGATATCTACAA'ITATGAAGTACGGCCTTGCACCTGGAGGATCTAAGTATAGGCCTAAA'ITTGGAG'ITGGTG'ITCACCTTAGTGCTCTCCA'IT

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
AGATGACATCGATCTCGAGAATCCTACTCA'ITATG'ITA'ITTGGAATATGAATTTGAAC
2.4.2 Analyses of deduced polypeptides of Le-RCD1-LikeA and B

The amino acid sequences of Le-RCD1-likeA and B were submitted to the NCBI database for sequence homology searches. This was performed using the BLASTP program against the non redundant protein database (Altschul et al., 1997). Le-RCD1-likeA shared 35% amino acid identity (score 380 bits; E value 7e-104) and 52% similarity with At-RCD1. Le-RCD1-likeB shared 38% identity and 57% similarity at the amino acid level with At-RCD1 (score 297 bits; E value: 1e-78). BLASTP searches also showed 34% and 38% identity between the amino acid sequences derived from Le-RCD1-likeA and B and At-SRO1, respectively.

InterProScan database searches for functional domains indicated the presence of PARP (poly(ADP-ribose) polymerase) and a conserved globular domain called the WWE domain in the Le-RCD1-likeA and B polypeptides. PARP domains are present in all members of the RCD-SRO gene family; however the WWE domain is only found in RCD1 and SRO1. The amino acid sequences of Le-RCD1-likeA and B were further screened for nuclear localization signals (NLS) using Predict NLS database. The polypeptide of Le-RCD1-likeB contains one NLS, at amino acids 57-79. The amino acid sequence of Le-RCD1-likeA was found to have no NLS motif using Predict NLS database (Figure 2-2). However, the presence of one weak NLS was predicted in the polypeptide of Le-RCD1-likeA at amino acids 51-56 by comparing its N-terminal amino acid sequence with the characterized NLS motifs of RCD1 that direct it to the nucleus (Katiyar-Agarwal et al., 2006). An amino acid sequence alignment
between the Le-RCD1-likeA and B polypeptides and the RCD1 and SRO1 polypeptides from Arabodopsis is shown in Figure 2-2.

2.5 Gene expression analysis

2.5.1 Characterization of JWL-26 and Le-RCD1-like gene expression in salt-treated roots

Northern blot analyses was performed to examine the effect of salt stress on the expression of genes corresponding to JWL-26, Le-RCD1-likeA and B. Total RNA was isolated from tomato roots exposed to 170 mM NaCl for 0, 0.5, 2, 8 and 24. In salt treated roots, the transcript level of the gene corresponding to JWL-26 increased at 2 h before declining at 24 h (Figure 2-3). Northern blot analysis also revealed the transient and weak up-regulation of Le-RCD1-likeB 8 h following a salt treatment. Constitutive expression of Le-RCD1-likeB was observed in unstressed control plants. The gene corresponding to Le-RCD1-likeA showed no expression during salt stress or in control plants (Figure 2-3). No expression was detected for genes corresponding to JWL-26, Le-RCD1-likeA and B in salt treated shoots (data not shown).

2.5.2 Characterization of At-RCD1 and At-SRO5 expression under salt stress

Transcript accumulation of At-RCD1 and At-SRO5 was investigated in Arabidopsis plants following exposure for 24 h to 250 mM NaCl. Northern blot analysis showed that a salt treatment increased the transcript level of At-RCD1 in root and shoot tissues by approximately 2-fold. In roots the expression level of At-RCD1 was higher than in the shoots (Figure 2-4-A). RNA blot analysis also
Figure 2-2: Alignment of the deduced amino acid sequences of Le-RCD1-likeA and B with the amino acid sequences of At-RCD1 and At-SRO1. Similar amino acids are shaded in grey and unshaded residues represented non-identical amino acids. The consensus amino acid sequence is given at the bottom of the alignment. The WWE and PARP conserved amino acid domains are underlined. The NLS motifs of RCD1 and Le-RCD1-likeB are highlighted in yellow. No precise match to the consensus NLS sequence was found in SRO1 and Le-RCD1-LikeA due to the high stringency parameters of PredictNLS program. The weak NLS motifs of SRO1 and Le-RCD1-likeA are highlighted in pink and red, respectively. Alignment was constructed using MacVector 7.2.
Figure 2-3: Expression of genes corresponding to JWL-26, Le-RCD1-likeA and B in salt-treated Ailsa Craig (AC) tomato roots.
The AC plants were grown in an aerated hydroponic system with MS nutrient solution. RNA was isolated from the roots of six-week-old tomato plants following exposure to MS media (Control) or 170 mM salt (+Na) for 0, 0.5, 2, 8 or 24 h and subjected to Northern blot analysis. The blots were probed with JWL-26, Le-RCD1-likeA, or Le-RCD1-likeB and then with 18S rRNA. The plot shows the normalized expression level of JWL-26 and Le-RCD1-likeB. This experiment was independently performed three times with similar results.
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<th>Control</th>
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<td></td>
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<tr>
<td>18s rRNA</td>
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<td>18s rRNA</td>
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### Relative Expression Level

- **Le-RCD1-likeB**
- **JWL-26**

![Graph](image61.png)

- x-axis: Time (0C, 0.5C, 2C, 8C, 24C, 0Na, 0.5Na, 2Na, 8Na, 24Na)
- y-axis: Relative expression level (0 to 1.4)
Figure 2-4: Expression analysis of *At-RCD1* and *At-SRO5* in salt-treated Arabidopsis roots and shoots.

RNA was isolated from roots and shoots of Arabidopsis that were exposed to nutrient medium without salt for 24h (-Na) or to nutrient medium with salt for 24h (+Na). The individual blots were probed with At-RCD1 (A) and At-SRO5 (B) and then with an ubiquitin probe. The plots show normalized *RCD1* and *SRO5* levels. This experiment was independently performed three times with similar results.
revealed a 3.5-fold increased expression of genes corresponding to At-SR05 24 h following a salt treatment in root tissues. In shoots, the expression level of At-SR05 showed no changes during salt stress as compared with control plants (Figure 2-4-B).

To gain information about other members of the Arabidopsis RCD1-SRO gene family, 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). A search using the GENEVESTIGATOR digital northern tool, with SR02 (AT1G23550) and SR05 (AT5G62520) as query showed that SR05 and SR02 are stress responsive in roots. Within the digital northern database, SR03 (AT1G70440) and SR04 (AT3G47720) probe sets are not present. Therefore, no information was gained about the expression pattern of these genes using this search tool. Northern blot analyses was also performed to determine the expression of At-SR02 and At-SR04 during salt stress. To this end, after hybridization with RCD1 and SRO5 probes, the same membranes were hybridized with SR04 and SR02 probes, respectively. Interestingly, At-SR02 and At-SR04 did not show any root or shoot expression during salt stress or control conditions (data not shown).
2.6 Discussion

2.6.1 Identification of Le-RCD1-like A and B cDNA clones

A major objective of this chapter was to isolate and characterize Le-RCD1-like genes in tomato. Two cDNA clones that encode Le-RCD1-like polypeptides (Le-RCD1-like A cLEN4A3, and Le-RCD1-like B cTOD6A15) were identified using At-RCD1 as a query against the TIGR database. Le-RCD1-like A shares 35% and Le-RCD1-like B shares 38% amino acid identity with Arabidopsis RCD1. RCD1 mediates hormone signalling pathways involved in the regulation of several stress-responsive genes and ROS-dependant programmed cell death in Arabidopsis (Overmyer et al., 2000, 2003). RCD1 carries a conserved WWE domain, predicted to be involved in protein–protein interactions, and a putative PARP (poly ADP-ribose polymerase) domain (Ahlfors et al., 2004). The PARP domain binds NAD⁺ and transfers the ADP-ribose-moiety of NAD⁺ to target proteins. This suggests that RCD1 and SRO proteins are involved in ADP-ribosylation, which is a reversible post-translational modification involved in the regulation of a variety of biological processes including DNA repair, transcriptional regulation and apoptosis (Smith, 2001). However, the conserved zinc-finger domains, which typically exist in classical PARP are not present in RCD1. Therefore, the RCD1 PARP domain most likely has functions distinct from the classical PARP (Ahlfors et al., 2004). Some members of the PARP protein family can modify their targets by the addition of a single ADP-ribose unit. Mono-ADP-ribosylation, which is catalysed by mono-ADP-ribosyltransferases is involved in the regulation of protein activity. Ahlfors et al. (2004) hypothesised
that RCD1 may be a mono-ADP-ribosyltransferases. An intriguing connection is that some bacterial toxins such as the cholera toxin induces the activity of G-proteins by ADP-ribosylation. In plants G-proteins are involved in ABA signalling and regulation of an oxidative burst in plant-pathogen interactions (Assmann, 2002).

The RCD1 protein interacts with several transcription factors, including DREB2A (drought-responsive element binding 2A), which binds to DRE/CRT (dehydration-responsive element/C-repeat) cis-acting elements to regulate the expression of genes involved in the response to drought and low-temperature (Ahlfors et al., 2004). Furthermore, work by Katiyar et al. (2006) has shown that the plasma membrane-localized Na⁺/H⁺ antiporter polypeptide (SOS1) interacts through its C-terminal cytosolic tail with RCD1 to regulate the accumulation of ROS during salt stress. Such an interaction may either regulate the transport of ROS and/or reductant across the cell membrane or affect the expression of ROS-scavenging genes, which are regulated by RCD1 and SOS1 (Katiyar et al. 2006). The altered expression of SOD genes in the rcd1 and sos1 mutants may explain the increased tolerance of these mutants to methyl viologen (MV) and the higher sensitivity to H₂O₂.

Le-RCD1-LikeA and B contain the WWE and PARP conserved domains, which share 59 and 64% amino acid similarity to the WWE and 67 and 71% similarity to the PARP domains of RCD1 protein. This suggests a similar function for RCD1 and the Le-RCD1-likeA and B proteins. The domain composition of Le-RCD1-LikeA and B suggest that, like RCD1, these proteins also interact with
other proteins through their N-terminal WWE protein–protein interaction domain and/or their C-terminal region. The presence of a PARP signature in Le-RCD1-LikeA and B suggests that these proteins are involved in ADP ribosylation. Therefore, we can hypothesize that like RCD1, Le-RCD1-LikeA and B may be involved in the regulation of a variety of processes such as protein activity and salt-induced ROS production during salt stress.

Nuclear localization signals (NLSs) are necessary for correct targeting of proteins to the nucleus (Boulikas 1994). RCD1, SR01, and Le-RCD1-likeB carry NLS sequences and, therefore a nuclear localization for these proteins is predicted. Weak NLSs exist in Le-RCD1-LikeA. Interestingly, Katiyar et al. (2006) showed that the RCD1 protein resides in the nucleus under normal conditions, but is exported to the cytoplasm during salinity and H2O2 stress to protect the cells against oxidative damage. In the nucleus, RCD1 interacts with transcription factors such as DREB2A to coordinate changes in gene expression and in cytosol it interacts with SOS1 both of these events increase salt-stress tolerance (Katiyar et al. 2006). This suggests a dual function for RCD1 and potentially for Le-RCD1-LikeA and B in coordinating the response to salt and oxidative stress.

2.6.2 Gene expression analysis of Le-RCD1-likeA and B, JW-L-26, At-RCD1 and At-SRO5 during salt stress

A key regulatory mechanism to drive the plants response to a stress condition is to alter the global expression of numerous genes (Shinozaki and Dennis 2003). Many genes respond to salt stress, and the proteins encoded by
these genes are thought to play a role in protecting plants against stress. The expression of the genes corresponding to JWL-26, Le-RCD1-likeA and B putative members of the tomato RCD-SRO gene family was examined together with At-RCD1 and At-SRO5 in salt-treated plants using northern hybridization analyses. JWL-26 shares 65% similarity to the At-SRO5 protein. In addition, At-SRO5 was the only member of the truncated RCD-SRO family that is expressed in roots and is stress-responsive in roots (Figure 2-4). As such, SRO5 may be the Arabidopsis ortholog of JWL-26. Constitutive expression of SRO5 was detected in control roots and that expression was up-regulated following a salt treatment. The level of the SRO5 transcript was clearly higher in salt treated roots than in the shoots. Borsani et al. (2005) showed that the increased expression of SRO5 during salt stress affected the expression of the gene encoding Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH), a stress-related gene. The 3'UTR of SRO5 and P5CDH overlap in a cis-antisense manner, which results in the cleavage of P5CDH mRNA via generation of nat-siRNAs. The degradation of P5CDH results in reduced proline degradation thereby increasing the level of proline in the cell, which is an osmoprotectant that confers salt-stress tolerance (Borsani et al., 2005).

Expression of JWL-26 rapidly and transiently increased in salt treated tomato roots but remained unchanged in shoot tissue. The rapid induction of JWL-26 suggests that this gene is involved in the immediate response to salt stress. Rapid changes of gene expression in salt treated roots have been reported in other studies (Gulick and Dvorak, 1992; Botella et al., 1994; Galvez et
al., 1993; Moons et al., 1997b; Kawasaki et al., 2001), and is thought to be driven by the osmotic component of salt stress (Galvez et al., 1993).

*RCD1* is constitutively expressed in stems, leaves, buds and young flowers of wild-type Arabidopsis, whereas, low transcript levels are detected in old flowers, siliques and roots (Ahlfors et al. 2004). Katiyar et al. (2006) found that *RCD1* is constitutively expressed in seedlings and the expression level is strong in the vasculature, the root tip and the epidermis. To my knowledge, the work in this thesis is the first to describe the salt responsive nature of RCD1, whereby *RCD1* is up-regulated by salt in both roots and shoots. Roots contain higher levels of RCD1 mRNA than shoots. The accumulation of RCD1 during salinity stress may confer tolerance either by regulation of the activity of transcription factors or by protecting the cell against oxidative stress.

In tomato, *Le-RCD1-LikeB* was weakly and transiently up-regulated in salt treated tomato roots and was constitutively expressed in roots but was not expressed in shoot tissue. The expression of *Le-RCD1-LikeB* during salt stress may regulate the expression of tolerance-related transcription factors or genes that encode ROS scavengers. The level of the *Le-RCD1-likeA* transcript did not change during salt stress compared with control plants (Figure 2-3). Therefore, this gene may not play a role during salt stress.
CHAPTER 3: FUNCTIONAL ANALYSIS OF JWL-26 IN SALT-STRESSED PLANTS

3.1 Introduction

The mechanisms of plant adaptation to environmental stress have been investigated in a large number of plant species (Zhu et al. 2003). The major focus over the last few decades has been to identify and isolate stress-responsive genes whose expression is required for survival during stress conditions. Such efforts have been used as means for understanding the mechanisms that impact environmental stress tolerance (Krich et al. 2004). Plants are exposed to various stressors including pathogens, UV exposure, air pollution, salt, drought and high temperature. Since plants have a limited capacity to avoid unfavourable changes in their environment, they require flexible means of adaptation to change in environmental conditions (Arora et al. 2002), and the response and nature of their adaptations are very complex and highly variable. Some of these responses include changes in gene expression, generation of secondary metabolites, changes in the structure of membranes and changes in metabolic processes (Arora et al. 2002). A common response to different environmental stresses including salt is the production of reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen, superoxides and hydrogen peroxide (Apel and Hirt 2004, Katiyar-Agarwal et al. 2006). ROS can cause oxidative damage to proteins, DNA and lipids and their cytotoxic properties explain the development of sophisticated ROS-scavenging

As described in Chapter 2, BLAST-based homology searches revealed similarity between the tomato polypeptide JWL-26 and SRO5 at the amino acid level. Furthermore, like SRO5, JWL-26 contains a PARP signature and has sequence identity with the C-terminal protein interaction domain. The tomato Le-RCD1-likeA and B share similarity with RCD1. Like RCD1, Le-RCD1-likeA and B carry a WWE domain and a PARP signature. Such a relationship between these proteins suggests a common in function.

Reverse and forward genetic approaches together with molecular and physiological analyses were carried out to characterize whether Le-RCD1-likeA and B, JWL-26 and SRO5 proteins are involved in the processes by which their function limits salt stress-induced ROS production.

3.2 Research objectives

The objectives of the work in this chapter were:

1. To characterize the role of Le-RCD1-LikeA and B, JWL-26 and SRO5 in salt stressed plants by generating and analyzing transgenic lines over-expressing these genes.
2. To assess the extent of ROS production and scavenging in wild-type, lines underexpressing SRO5 or P5CDH genes, and transgenic plants overexpressing JWL-26 or SRO5 genes in response to salinity stress.

3.3 Material and methods

3.3.1 Materials

Seeds of the rcd1 mutant were kindly provided by Jaakko Kangasjärvi at the University of Helsinki, Finland. The T-DNA insertion mutants for At-SR05 (SALK-064137, 017099, 040029,140025, 084641 and 130345) were obtained from the Arabidopsis Biological Resource Center (ABRC). The Arabidopsis P5CDH cDNA clone was obtained from TAIR. The sro5 knock out line was obtained from the Max Planck Institute for Plant Breeding Research (GABI-Kat).

3.3.2 Experimental treatments

Arabidopsis seeds were gas-sterilized with NaClO and HCl for 2 hr and sown on 1% (w/v) agar plates containing 1/2 MS (Murashige and Skoog) medium supplemented with 1% (w/v) sucrose. The plates were incubated at 4°C for 3 days for stratification and then transferred to growth chambers and incubated at 23°C under a 16-h/8-h light/dark regime.

(a) Root elongation measurements

Seeds were grown on ½ MS media alone or supplemented with 75 mM NaCl in Petri dishes (90mm dia. x 15mm H). Three replicates with 10 seeds each were established for each treatment. Root measurements were recorded with a ruler 6 days after germination.
(b) Seed germination assay

Seeds were germinated on ½ MS media with or without 150 mM NaCl. The seeds were considered as germinated when the radicles protruded from the seed coat. After five days, the number of germinated seeds was recorded and the percent germination was calculated.

(c) Salt tolerance assay

Seeds were allowed to germinate on ½ MS media with or without 150 mM NaCl. After 10 days, the number of green seedlings was recorded and the percent survival was calculated.

(d) Paraquat resistance assay

Seeds were allowed to germinate on ½ MS media with or without 0.2 μM methyl viologen (paraquat). After 10 days, the effect of paraquat on seedling growth and development was visually recorded by taking pictures with a digital camera.

Statistical analyses were performed using ANOVA (analysis of variance) and Student's t test (JMP software, version 6.0, SAS Institute Inc., Cary, NC, USA).

3.3.3 RNA extraction

RNA extraction was performed as described in section 2.3.4.
3.3.4 Northern hybridization analyses

Northern hybridization analyses were performed as described in section 2.3.6.

3.3.5 Preparation of radiolabelled DNA probes

Radiolabelled DNA probes were prepared as described in Chapter 2 section 2.3.5. The P5CDH cDNA insert was amplified using the following pair of primers 5'-TTTATCAAAGTTGCGGAAGTG-3' (forward) and 5'-CTTGCCACCAAAAGAGTAGC-3' (reverse).

3.3.6 Generation of transformation vectors caring the ORF of Le-RCD1-likeA and B, JWL-26 and SRO5

Total RNA extracted from 6-week-old tomato roots exposed to 170 mM salt for 2 h and from 3-week-old Arabidopsis roots exposed to 250 mM salt for 24 h was used for RT-PCR to isolate the full-length cDNA clones corresponding to JWL-26 and SRO5, respectively. RNA was reverse-transcribed by M-MuLV reverse transcriptase (Fermentas) from an anchored oligo-dT primer as described in Chapter 2 (section 2.3.2). JWL-26 and SRO5 cDNAs were then directly amplified by PCR with the following pair of primers: 5'-CACCGTTTCTCGTATAAAACAATGTC-3' (JWL-26 forward), 5'-TGAAATAGAACAAGACACC-3' (JWL-26 reverse), 5'-CACCCATTCGTTCTAAATTCC-3' (SRO5 forward), 5'-CATTTTGGAGTTGGTAAACAG-3' (SRO5 reverse). Re-amplified cDNA products were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen) and then transformed into Escherichia coli TOP10 (Invitrogen) by the heat-shock
method and selected on agar plates containing 50 μg/ml kanamycin as described in section 2.3.2. Positive clones were further confirmed by PCR using the same primers used to amplify the original cDNAs. The nucleotide sequence of positive cDNA clones was determined using the M13 reverse and forward universal primers.

The ORF of Le-RCD1-like A and B were transferred from the entry vectors into the Gateway destination vector pK2GW7 and the coding regions of JWL-26 and SRO5 were transferred from the entry vectors into the Gateway destination vector pB2GW7 using the LR clonase reaction (Invitrogen). The ligation reaction comprised 2 μL LRClonase II enzyme mix, 1 μL Entry clone (50-150 ng), 1 μL (150 ng) destination vector (pB2GW7 or pK2GW7). The mixture was brought to 10 μL with TE buffer (pH 8.0). The reaction was incubated at 25 °C for 1 hour. The ligation reaction was terminated by adding 2μg Proteinase K solution to each sample, followed by incubation at 37°C for 10 min.

### 3.3.7 Transformation of One Shot® chemically competent E. coli

One μL ligated plasmid DNA was transformed into a vial of One Shot® OmniMAX™ 2 T1 Phage-Resistant cells (Invitrogen) and mixed gently. The reaction was incubated in ice for 30 min and subsequently heat-shocked at 42°C without shaking. The transformed cells were immediately transferred to ice, followed by adding 250 μl of LB medium. The cultures were then incubated at 37°C for 2 h with shaking (250 rpm). Aliquots of bacterial culture (50-100 μL) were spread on pre-warmed agar plates containing 100 μg/ml spectinomycin, and incubated at 37°C overnight. Positive clones were confirmed using PCR with
the same primers used to amplify the original cDNAs. The orientation of the transgenes within pK2GW7 and pB2GW7 destination vectors was determined using PCR with forward primers that annealed to the NPTII (5'-CGTCAAGAGGCCGATAGAAG-3') and Bar (5'-CGAGTTGAGATGAATATGAG-3') genes in the destination vectors and the reverse primers used to amplify the original cDNAs.

3.3.8 Transformations of Agrobacterium tumefaciens via electroporation

Aliquots (100 µL) of competent cells were chilled on ice and approximately 5-10 ng of expression vectors (Le-RCD1-likeA/pK2GW7, Le-RCD1-likeB/pK2GW7, JWL-26/pB2GW7 and SRO5/pB2GW7) were added to the competent cells and carefully mixed in a pre-cooled electroporation cuvette and left for 30 minutes. The cuvettes were then subjected to electroporation at 2500 V, 400 Ω and 25 µF in Electro Cell Manipulator (BTX, San Diego, California). One ml LB medium was added to the transformed cells and further incubated for 2 hr at 28 °C and shaking at 250 rpm. 200 µL aliquots of the cells were plated on LB media containing 100 µg/ml spectinomycin selectable agent for two days at 28 °C. The positive clones were confirmed using PCR with the same primers used to amplify the original cDNAs.

3.3.9 Agrobacterium-mediated transformation of Arabidopsis plants

The rcd1 mutant was transformed via Agrobacterium tumefaciens-mediated transformation with Le-RCD1-likeA/pK2GW7 or Le-RCD1-likeB/pK2GW7 using the floral dip method (Clough and Bent, 1998). Wild-type and the sro5 knock-out line were transformed with JWL-26/pB2GW7 or
SRO5/pB2GW7 expression vectors. Briefly, the plants were allowed to grow to the stage of inflorescence. Agrobacterium carrying the constructs were cultured at 28 °C in 100 ml LB/Spectinomycin up to OD600= 0.6. Bacterial pellets were resuspended in 50 ml inoculation medium (5% sucrose and 250 μL Triton X-100) in magenta boxes (to enable the rcd1 plants to be immersed in the solution for transformation). The plants were inverted and immersed in the solution with gentle shaking for 1 min. The plants were then placed back in the trays and covered with plastic bags for 3 days. After 3 days the covers were removed and plants were allowed to grow to harvest the first generation of seeds (T1).

3.3.10 Extraction of genomic DNA from Arabidopsis plants

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

3.3.11 Screening for transformed plants

After transformation, T1 seeds were collected, gas-sterilized and sown on 0.8% agar, ½ MS nutrient medium containing 50 mg/L kanamycin (Le-RCD1-likeA/pK2GW7 and Le-RCD1-likeB/pK2GW7) or 10 mg/L bar (phosphinothricin)
herbicide (JWL-26/pB2GW7 and SRO5/pB2GW7). After two weeks of growth, transgenic seeds were able to germinate and produce green leaves in the presence of the selectable agent while non-transformed plants failed to grow and turned yellow. The presence of Le-RCD1-likeA/pK2GW7 and Le-RCD1-likeB/pK2GW7 constructs in the rcd1 host plants was checked by PCR amplification using NPTII primers (forward: 5'-CGTCAAGAAGGCGATAGAAG-3' and reverse: 5'-TATGACTGGGCACAACAGAC-3'). The presence of SRO5 and JWL-26 in the sro5 knock-out and wild-type transgenic plants were also determined by PCR using gene specific primers designed to amplify the original SRO5 or JWL-26 cDNAs. Positive plants were transferred to soil and allowed to grow to collect T2 seeds. To assess the expression of Le-RCD1-likeA and B, SRO5 and JWL-26 in the transgenic plants, RNA was isolated form individual transgenic lines and subjected to Northern hybridization analyses.

3.3.12 PCR screening for a knockout mutant carrying SRO5 allele

To isolate an sro5 knock out two T-DNA insertion lines within the promoter (SALK-064137 and 017099), two within the 5'UTR (SALK-040029 and 140025) and two within the 3'-UTR (084641 and 130345) of SRO5 were identified in the SIGnal database (http://signal.salk.edu/cgi-bin/tdnaexpress) and obtained from Ohio State University. Primer sequences specific for each line together with the T-DNA-specific primer (LBA1) were obtained from the SIGnal database to amplify the junction of the SRO5::T-DNA insertion and to select an homozygous line. The PCR-based screening was performed using the following primers:

5'-GACACGACCGACACACGTAAC-3' (SALK-064137-RP)
5'-GAGCTACAGACAAACGCATGG-3' (SALK-064137-LP)
5'-AAGAAGAATCGCAGCTTCCAG-3' (SALK-040029-RP)
5'-TGCCTTTTCTTAACAAGACTTAATTG-3' (SALK-040029-LP)
5'-CTCGTCAGCAAAGATCGATC-3' (SALK-140025-RP)
5'-TTGAATCAAGTTTTCTTG-3' (SALK-140025-LP)
5'-CATTTGGATTGCAATGATAACAAGTC-3' (SALK-017099-RP)
5'-GCTCATGTTTCGAAACAGAGC-3' (SALK-017099-LP)
5'-TCTTTGAGGTGATTTCCAC-3' (SALK-084641-RP)
5'-AGGGAACTCAGTGAACTGGGAC-3' (SALK-084641-LP)
5'-TCACATCATCAAAGCTTG-3' (SALK-130345-RP),
5'-GAGGATGCACGCTCATCTAAC-3' (SALK-130345-LP)
5'-TGGTTTCACGTA GTGGCCATCG-3' (LBA1)

3.3.13 In vivo detection of H2O2 in salt-treated roots

Seeds were germinated on half-strength MS (1.5% w/v sucrose), 0.8% agar plates under 16/8 h light/dark cycle (80 µmol/s/m²) at 22 °C. After 7-d, seedlings were transferred to half-strength MS liquid medium with or without 150 mM NaCl for 12 h. The salt treated and untreated seedlings were then incubated with 10 µM 2,7-dichlorofluorescein diacetate for 5 min. The seedlings were then washed thoroughly with half-strength MS liquid medium for 1 min and viewed under an epi-fluorescent microscope (Nikon Eclipse-E6000). Images were obtained with a digital camera.
3.3.14 In vivo detection of H₂O₂ using DAB staining

To localize the generation of H₂O₂ in leaf tissue during salt stress DAB staining was performed. Seeds were germinated in half-strength MS (1.5% w/v sucrose), 0.8% agar plates under 16/8 h light/dark cycle (80 μmol/s/m²) at 22 °C. After 14-d, seedlings were transferred to half-strength MS liquid medium with or without 150 mM NaCl. After 12 h shoot tissues were cut and placed in 1 mg/ml 3,3’-diaminobenzidine (DAB)-HCl (Sigma) at pH 3.8. Samples were then incubated in room temperature for 4 h in dark and cleared by boiling the leaves in 80 % (v/v) ethanol for 2 h. The accumulation of H₂O₂ was observed as brown colouration in the leaves (Thordal-Christensen 1997). Images were obtained with a digital camera.

3.4 Results

3.4.1 Functional characterization of Le-RCD1-like genes in rcd1 plants

As previously described Le-RCD1-likeA and B share 35% and 38 % amino acid sequence identity with Arabidopsis RCD1, respectively. An alignment of these amino acid sequences showed that Le-RCD1-likeA and B share 59 and 64% similarity to the WWE domain and 67 and 71% similarity to the PARP domain of RCD1, respectively. This suggests a resemblance in the function of Arabidopsis RCD1 and its potential tomato orthologues. To elucidate the extent of functional overlap between Arabidopsis and tomato putative RCD1 polypeptides, I examined whether Le-RCD1-likeA and B can rescue the Arabidopsis rcd1 mutant. To this end transgenic plants were generated to over-express Le-RCD1-like genes in the rcd1 background. Transformed plants were
screened in the presence of kanamycin and the insertion of the transgene in the host plants was confirmed by PCR in the T2 generation (Figure 3-1).

Northern blot hybridization was performed to analyze the expression of Le-RCD1-LikeA and B in the transgenic lines. RNA was isolated from five T2 plants from rcd1-35S::Le-RCD1-LikeA and rcd1-35S::Le-RCD1-LikeB lines. The results indicated significant gene silencing of the transgene in T2 generations of rcd1-35S::Le-RCD1-LikeA and rcd1-35S::Le-RCD1-LikeB transgenic lines (Figure 3-2). When the membranes were stripped and re-probed with RCD1 no degradation of the RCD1 transcript was observed (data not shown). The expression of the transgene in different individual lines ranged from weak to non-detectable. Therefore, among the transgenic lines those that showed some degree of transgene expression (rcd1-35S::Le-RCD1-LikeA line 18 and rcd1-35S::Le-RCD1-LikeB line three) were selected for phenotypic analysis.

3.4.1.1 Phenotypic analysis of rcd1 transgenic lines: response to methyl viologen (paraquat)

The ozone-sensitive Arabidopsis rcd1 mutant is paraquat (methyl viologen) tolerant (Ahlfors et al., 2004). To examine whether Le-RCD1-likeA and B can rescue the rcd1 mutant, the effect of methyl viologen on growth of transgenic plants was examined. Seeds of rcd1, wild-type, rcd1-35S:: Le-RCD1-LikeA and rcd1-35S:: Le-RCD1-LikeB were directly plated on medium containing 0.2µM methyl viologen. The transgenic plants were found to be as resistance to paraquat as the rcd1 mutant. Wild-type plants were sensitive to methyl viologen and failed to grow on media containing 0.2µM methyl viologen (Figure 3-3).
Figure 3-1: PCR amplification of the NPTII gene in \textit{rcd1-35S:: Le-RCD1-LikeA} and \textit{rcd1-35S:: Le-RCD1-LikeB} transgenic plants. Independent transformed lines were screened on ½ MS media containing 50 mg/L kanamycin. Genomic DNA was extracted from kanamycin-resistant lines and used for amplification of the NPT II gene. Amplification of a 700 bp fragment corresponding to the NPT II gene was observed in \textit{rcd1-35S:: Le-RCD1-LikeA} (A) and \textit{rcd1-35S:: Le-RCD1-LikeB} (B) transgenic plants, whereas no amplification was observed in the \textit{rcd1} and non-transformed lines. M, 1kb DNA marker. The PCR products were run on a 1% agarose gel at 80 V for 45 min.
Figure 3-2: Expression of Le-RCD1-likeA and B in rcd1 transgenic plants. Total RNA was extracted from two-week-old seedlings. Northern blot analysis was carried out using $^{32}$P labelled probes corresponding to the full length ORF of Le-RCD1-likeA (A) and Le-RCD1-likeB (B) sequences. The first lane indicates RNA from the rcd1 host plant. The numbers indicate PCR-positive rcd1 transgenic lines. Arrows indicate the transcript location of Le-RCD1-likeA and B in the rcd1 transgenic plants. Ethidium bromide staining of ribosomal RNA is shown at the bottom.
Figure 3-3: The Effect of methyl viologen (paraquat) on the growth of wild-type, rcd1, and transgenic plants.

Wild-type, rcd1, rcd1-35S:: Le-RCD1-LikeA and rcd1-35S:: Le-RCD1-LikeB were grown for 10 days on agar medium with or without 0.2 μM methyl viologen.
Root growth was examined in wild-type, \( rcd1, \ rcd1-35S::Le-RCD1-LikeA \) and \( rcd1-35S::Le-RCD1-LikeB \). Wild-type seedling root length was greater than that of \( rcd1 \) (see Figure 3-3). No significant difference in seedling root length was observed between \( rcd1 \) and \( rcd1-35S::Le-RCD1-LikeA \) and \( rcd1-35S::Le-RCD1-LikeB \) (data not shown).

No further experiments were performed on these lines. Although the data suggest that Le-RCD1-likeA and B do not rescue the \( rcd1 \) mutant and that they may not be involved in the same functional processes as RCD1, it is possible that a high level of expression of \( Le-RCD1-likeA \) and \( B \) is necessary.

3.4.2 Characterization of the function of JWL-26 and SRO5 in transgenic plants during salt stress

Functional analyses of JWL-26 was performed together with SRO5 since both genes are salt-regulated and may perform similar functions in salt-stressed plants. However, the increased expression of \( SRO5 \) during salt stress affects the expression of the gene encoding \( \Delta1\)-pyrroline-5-carboxylate dehydrogenase (\( P5CDH \)), a stress-related gene. The 3'UTR of \( SRO5 \) and \( P5CDH \) overlap in a cis-antisense manner, which results in the cleavage of \( P5CDH \) mRNA via generation of small interfering RNAs (siRNAs). The degradation of \( P5CDH \) results in reduced proline degradation thereby enhancing the level of proline in the cell, which is an osmoprotectant that confers salt-stress tolerance (Borsani et al., 2005). It was necessary to explore SRO5 function independently of its effect on \( P5CDH \) expression and thereby independently of any enhancement of salt-tolerance caused by maintaining proline levels. To achieve this, transgenic
plants were generated in which the SRO5 ORF lacking the 3'-UTR was overexpressed using the 35S promoter. This control would allow us to assess the impact of SRO5 in complementing the sro5 knockout line for comparison with JWL-26.

3.4.2.1 Molecular analysis of an sro5 knockout line

To isolate an sro5 knock out line genomic DNA isolated from SALK lines carrying T-DNA insertions within the promoter (SALK-064137 and 017099) and the 5'UTR (SALK-040029 and 140025) of SRO5 was subjected to two PCR rounds. The first PCR round was done to characterize the T-DNA-tagged SRO5 allele using the primers located in SRO5 and the primer located in the left border (LB) of the T-DNA, these amplify a 800 bp SRO5::T-DNA fragment. The second PCR used primers specific for each line to identify the wild-type allele, amplifying a 1000 bp region of genomic DNA. SALK-017099, 040029 and 140025 lines failed to amplify the junction of the SRO5::T-DNA insertion but amplified the wild-type allele. SALK-064137 line failed to amplify the wild-type allele but did amplify the T-DNA-tagged SRO5 and was identified as homozygous for the T-DNA insertion and selected for further studies (Figure 3-4). This line carries a T-DNA insertion in the promoter region of SRO5.

The expression of SRO5 was examined using northern blot analysis in both SALK-064137 and wild-type plants treated with 150 mM NaCl for 6 h. This treatment up-regulates SRO5 mRNA in roots (Figure 2-4-B). The level of the SRO5 transcript was higher in SALK-064137 (Figure 3-5-A) than in wild-type in
Figure 3-4: Isolation of a homozygous line for the T-DNA insertion in SALK-064137.

(A) Diagram showing the position of the T-DNA in the promoter of SRO5 in SALK-064137. The T-DNA insertion is marked by a triangle. The arrows indicate the position of the primers in the SRO5 locus and the T-DNA insertion. (B) PCR screening to isolate a homozygous line. Genomic DNA was isolated from individual plants of SALK-064137 and subjected to the PCR screening test. Gene-specific and T-DNA-specific primers were used to detect the presence of the T-DNA in SALK-064137 and isolate homozygous lines for the T-DNA insertion. A 0.8 kb fragment was amplified in the homozygous lines of SALK-064137, which is designated with asterisk.
Figure 3-5: Expression analysis of SRO5 and P5CDH in seedlings of wild-type, SALK-064137, SALK-130345 and SRO5 knock out line.

RNA was isolated from 10-day-old seedlings with or without 6 hr exposure to 150 mM NaCl and subjected to northern blot analysis. The blots were probed with SRO5, or P5CDH and then with ubiquitin. Salk-37 and Salk-45 refer to SALK-064137 and SALK-130345, respectively. A. T-DNA insertion within the promoter of SRO5 resulted in the over-expression of SRO5. B. Loss of function of P5CDH in SALK-130345. C. T-DNA insertion within the second exon of SRO5 reduced the expression of SRO5. Arrows indicate the transcript location of SRO5 in the sro5 knock-out-line.
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the presence and absence of salt. Therefore, this line was selected as an SRO5 over-expression line for further experiments. The expression of P5CDH was also examined in this line and was similar to that in wild-type plants.

To find an SRO5 knock out line two other T-DNA insertion lines within the 3'UTR of SRO5 (SALK-084641 and 130345) were identified in the SIGnal database and ordered from Ohio state university. PCR screening was applied to amplify the junction of the SRO5::T-DNA insertion and to select an homozygous line. The SALK-084641 line failed to amplify the junction of the SRO5::T-DNA insertion but amplified the wild-type allele. The SALK-130345, which amplified the T-DNA-tagged SRO5 was identified as homozygous for the T-DNA insertion (Figure 3-6) and subjected to expression analysis. Northern blot analysis showed a similar or slightly elevated level of the SRO5 transcript in the SALK-130345 line (Figure 3-5-B). In SALK-130345 the expression of P5CDH was absent. Thus, the SALK-064137 line overexpresses SRO5 with normal P5CDH and SALK-130345 has a normal SRO5 but reduced P5CDH expression (Figure 3-5-A and B). SALK-130345 hereafter is called p5cdh knock-out line.

Finally, to find a knock out line for SRO5 a T-DNA insertion line was obtained from the Max Planck Institute for Plant Breeding Research (GABI-Kat). The homozygous knock out line carries a T-DNA insertion in the second exon of the SRO5 gene. This T-DNA insertion interrupts the coding sequence of the SRO5 gene and therefore leads to a non-active SRO5 protein. Figure 3-7 shows the genomic structure of the SRO5 gene and the position of the T-DNA insertion. Expression analysis was performed to examine the expression of SRO5 in the
Figure 3-6: Isolation of a homozygous line for the T-DNA insertion in SALK-130345.

(A) Diagram showing the position of the T-DNA in the 3’ UTR of SRO5 in SALK-130345. The T-DNA insertion is marked by a triangle. The arrows indicate the position of the primers in the SRO5 locus and the T-DNA insertion. (B) PCR screening to isolate a homozygous line. Genomic DNA was isolated from individual plants of SALK-130345 and subjected to the PCR screening test. Gene-specific and T-DNA-specific primers were used to detect the presence of the T-DNA in SALK-130345 and isolate homozygous lines for the T-DNA insertion. A 0.8 kb fragment was amplified in the homozygous lines of SALK-130345, which is designated with asterisk.
Figure 3-7: Localization of the T-DNA inserts in the SRO5 locus. Diagram showing the position of the T-DNA in the second exon of *SRO5* in GABI-Kat line. The T-DNA insertion is marked by a triangle.
GABI-325B05
GABI-325B05 line. Northern blot analysis showed a weak expression of SRO5 in this line although a small and large transcript was detected (Figure 3-5-C). Therefore, this knock out mutant was considered to be a good candidate to study the role of SRO5 during salt stress. The expression of P5CDH was also examined in GABI-325B05 line and was similar to that in wild-type plants (Figure 3-5-C). The GABI-325B05 line hereafter is called sro5 knock-out line.

3.4.2.2 Over-expression of JWL-26 and SRO5 in wild type and the SRO5 knockout line

The ORF of SRO5 and JWL-26 were overexpressed in wild-type Arabidopsis via Agrobacterium tumefaciens-mediated transformation to generate WT-35S::SRO5 and WT-35S::JWL-26, respectively. They were also constitutively expressed in an sro5 knock-out line (sro5-35S::SRO5 and sro5-35S::JWL-26). The insertion of transgenes in the host plants was checked by PCR amplification analyses in the T2 generation of the transgenic plants (data not shown).

The expression of SRO5 and JWL-26 was analysed in different individual transgenic lines including WT-35S::SRO5, WT-35S::JWL-26, sro5-35S::SRO5 and sro5-35S::JWL-26. RNA was extracted from individual plants from each of the transgenic lines and subjected to Northern blot analysis. Northern blot analysis showed that the SRO5 and JWL-26 transgenes were constitutively expressed in wild-type transgenic plants (Figure 3-8). The WT-35S::JWL-26 line eight and WT-35S::SRO5 line four were selected for further analysis.
Figure 3-8: Expression of JWL-26 and SRO5 transgenes in wild type Arabidopsis plants.

Wild-type plants were transformed with JWL-26 (A) or SRO5 (B). RNA was isolated from 10-day-old seedlings and subjected to northern blot analysis. The blots were probed with SRO5 or JWL-26. Lanes 1 to 8, contain RNA from individual transgenic lines; Lane 9, contains RNA from wild type plants. Ethidium bromide staining of ribosomal RNA is shown at the bottom.
Likewise, the SRO5 and JWL-26 genes were constitutively expressed in the sro5 knock-out line (Figure 3-9). The sro5-35S::SRO5 line two and sro5-35S::JWL-26 line five were selected for further experiments. Over-expression of these genes had no effect on the expression level of P5CDH in wild-type and the sro5 knock-out line (data not shown).

3.4.2.3 Phenotypic analyses of plants with altered expression of SRO5 and JWL-26

3.4.2.3.1 Root growth of wild-type, sro5, p5cdh and plants overexpressing SRO5 and JWL-26 under Salt Stress

Root growth, in the presence of salt, was examined in wild-type, WT-35S::SRO5 and WT-35S::JWL-26, SALK-064137, p5cdh, sro5, sro5-35S::SRO5 and sro5-35S::JWL-26 so as to assess salt tolerance. In the absence of salt there were differences in root growth between genotypes. The seedling root length of SALK-064137, sro5 and p5cdh was shorter than that of wild-type. The length of sro5 and p5cdh seedling roots was similar whereas those of SALK-064137 were shorter (Figure 3-10-A). Wild-type transgenic plants overexpressing SRO5 and JWL-26 had shorter roots when compared with wild-type plants but WT-35S::SRO5 had shorter roots than WT-35S::JWL-26 (Figure 3-11-A). The length of sro5 seedling roots was similar to that of sro5-35S::SRO5 and sro5-35S::JWL-26 and they all exhibited shorter root lengths than wild-type (Figure 3-12-A).

Salt reduced root growth of all genotypes. In the presence of salt, the seedling root growth of SALK-064137, p5cdh, and sro5 was shorter than that of wild-type. Root growth of salt-stressed seedlings did not differ between SALK-064137, p5cdh, and sro5 (Figure 3-10-A). Wild-type transgenic plants
Figure 3-9: Expression of *JWL-26* and *SRO5* in an *sro5* knock-out line. The *sro5* knock-out line was transformed with *JWL-26* (A) or *SRO5* (B). RNA was isolated from 10-day-old seedlings and subjected to northern blot analysis. The blots were probed with *SRO5* or *JWL-26*. Lanes 1 to 8, contain RNA from individual transgenic lines; Lane 9, contains RNA from the *sro5* knock-out plants. Ethidium bromide staining of ribosomal RNA is shown at the bottom.
Figure 3-10: Effect of salt on the growth and germination of wild-type, SALK-064137, sro5, and p5cdh roots.

Seeds were germinated on ½ MS media with or without 75 mM NaCl. Root length was measured 6 days after germination. A. Root length for each genotype. B. Root growth for each genotype relative to growth on media without salt. C. Seed germination analysis. Seeds were germinated in the presence of 150 mM salt. Germination percentage was scored 5 days after stratification. Log transformed data were analyzed by ANOVA. Bars represent mean +/- standard error. Bars denoted with the same letter are not significantly different (student’s t-test P<0.05).
A

![Graph showing root length (mm) for different genotypes.]

- WT
- Salk-064137
- p5cdh
- srt5

B

![Graph showing log relative growth for different genotypes.]

- WT
- Salk-064137
- p5cdh
- srt5

C

![Graph showing seed germination (%) for different genotypes.]

- WT
- p5cdh
- Salk-064137
- srt5
Figure 3-11: Effect of salt on the growth and germination of wild-type, WT 35S::SRO5, and WT-35S::JWL-26.

Seeds were germinated on ½ MS media with or without 75 mM NaCl. Root length was measured 6 days after germination. A. Root length for each genotype. B. Root growth for each genotype relative to growth on media without salt. C. Seed germination analysis. Seeds were germinated in the presence of 150 mM salt. Germination percentage was scored 5 days after stratification. Log transformed data were analyzed by ANOVA. Bars represent mean +/- standard error. Bars denoted with the same letter are not significantly different (student's t-test P<0.05).
Figure 3-12: Effect of salt on the growth of wild-type, sro5, sro5-35S::SRO5, and sro5-35S::JWL-26. Seeds were germinated on ½ MS media with or without 75 mM NaCl. Root length was measured 6 days after germination. A. Root length for each genotype. B. Root growth for each genotype relative to growth on media without salt. C. Seed germination analysis. Seeds were germinated in the presence of 150 mM salt. Germination percentage was scored 5 days after stratification. Log transformed data were analyzed by ANOVA. Bars represent mean +/- standard error. Bars denoted with the same letter are not significantly different (student's t-test P<0.05).
overexpressing SRO5 and JWL-26 had shorter root length than wild-type plants but WT-35S::SRO5 had shorter roots than WT-35S::JWL-26 (Figure 3-11-A). The sro5 transgenic lines showed the same root length as sro5 in the presence of salt and they all exhibited shorter root lengths than wild-type (Figure 3-12-A).

Relative root growth was examined to determine root growth sensitivity to salt stress. SALK-064137, which has elevated expression of SRO5 was less sensitive to salt relative to the wild-type plants. The sro5 and p5cdh knock-out lines showed the same sensitivity to salt as wild-type (Figure 3-10-B). The wild-type transgenic plants over-expressing SRO5 and JWL-26 also exhibited reduced sensitivity to salt relative to wild-type (Figure 3-11-B). Overexpressing JWL-26 in sro5 did not alter root growth sensitivity to salt whereas over-expressing SRO5 in sro5 increased root growth sensitivity to salt (Figure 3-12-B).

The role of SRO5, JWL-26 and P5CDH in seed germination in response to salt stress was also examined. In the absence of salt, 100% of seeds germinated for all genotypes. Approximately, 97% of wild-type seeds germinated in the presence of 150 mM salt. The p5cdh knock-out line germinated at the same level as wild-type in the presence of salt. Seeds of salk-064137 germinated at lower level relative to wild-type as did those of sro5 knock-out line. Seeds of sro5 germinated at lower percentage relative to Salk-064137 (Figure 3-10-C). Overexpressing SRO5 and JWL-26 affected germination efficiency and the germination percentage of WT-35S::SRO5 and WT-35S::JWL-26 was lower than that of wild-type plants (Figure 3-11-C). However, over-expressing SRO5 or JWL-26 in sro5 improved germination efficiency, with SRO5 promoting
germination to a greater extent relative to JWL-26 compared to sro5 (Figure 3-12-C).

3.4.2.3.2 The effect of SRO5 and JWL-26 on plant survival during salt stress

To analyse the tolerance of different lines to salt, the response of plants was monitored in ½ MS media containing 150 mM NaCl. Figure 3-13-A shows seedling phenotype of wild-type, sro5, Salk-064137, p5cdh and transgenic plants in ½ MS-salt media. To test the role of SRO5 and JWL-26 in plant salt tolerance, the survival rate of the plants was determined 10 days after exposure to the stress. The treatment caused seedling death in all genotypes. Approximately 70% of wild-type plants survived in the presence of salt. The highest survival rate (88%) was observed in the p5cdh knock-out line. The survival rates of the wild-type transgenic lines overexpressing SRO5 and JWL-26 (WT-35S::SRO5, WT-35S::JWL-26) were significantly higher than that of the wild-type plants. Salk-064137 also had a higher survival rate relative to wild-type plants. The survival rate of the sro5 knock-out line was significantly lower than wild-type plants. Overexpressing SRO5 or JWL-26 in the sro5 knock-out line improved the survival rate, with JWL-26 promoting the survival rate to a greater extent relative to SRO5. The survival rate of sro5-35S::SRO5 was similar to that of wild-type plants under salt stress (Figure 3-13-B).

3.4.2.3.3 ROS detoxification effects of SRO5 and JWL-26 and salt-induced oxidative stress tolerance of the transgenic plants

The role of SRO5 and JWL-26 in ROS scavenging was examined by analysing the accumulation of H$_2$O$_2$ in root and shoot tissues of wild-type, sro5,
Figure 3-13: The response of plants to salt stress.
Seeds were gas-sterilized with NaClO and HCl for 2 hr and placed on 0.8% agar, ½ MS nutrient medium with or without 150 mM NaCl. The photographs (A) represent the phenotypes of wild-type, Salk-064137, sro5, p5cdh and transgenic plants (WT-35S::SRO5, WT-35S::JWL-26, sro5-35S::SRO5 and sro5-35S::JWL-26) 10 days after exposure to salt. The survival rates (B) were determined by counting the number of green seedlings on the media containing salt. Bars represent mean +/- standard error. Bars denoted with the same letter are not significantly different (student's t-test P<0.05).
A

Control

WT

Salt

WT-35S::SRO5

WT-35S::JWL-26

SALK-064137
Salk-064137, p5cdh, WT-35S::SRO5, WT-35S::JWL-26, sro5-35S::SRO5 and sro5-35S::JWL-26 under salinity stress. Salt stress induced the accumulation of H$_2$O$_2$ in the roots of all genotypes. As shown in Figure 3-14 the accumulation of H$_2$O$_2$ in sro5 was higher than that of the wild-type. Likewise, H$_2$O$_2$ levels in p5cdh were higher than wild-type in the upper parts of roots although lower than in sro5 after exposure to salt. Lower levels of H$_2$O$_2$ were produced in sro5 plants over-expressing SRO5 and JWL-26 compared to sro5. In fact, sro5-35S::SRO5 and sro5-35S::JWL-26 showed a similar level of H$_2$O$_2$ accumulation as wild-type plants. Salt stress induced an increased accumulation of H$_2$O$_2$ in WT-35S::SRO5, WT-35S::JWL-26, but the levels of H$_2$O$_2$ accumulation was lower in the wild type transgenic lines as compared with wild-type plants. Salk-064137, which has high expression of SRO5 also showed reduced level of H$_2$O$_2$ when compared with wild-type plants.

The accumulation of H$_2$O$_2$ was further monitored in the shoot tissue using a diaminobenzidine (DAB) staining method. No H$_2$O$_2$ was detected in control plants (data not shown). Salt stress induced an increased accumulation of H$_2$O$_2$ in all genotypes. The sro5 knock-out line accumulated higher levels of H$_2$O$_2$ than wild-type. The wild-type transgenic lines overexpressing SRO5 and JWL-26 accumulated lower levels of H$_2$O$_2$ than wild-type in response to salt stress. H$_2$O$_2$ levels in sro5-35S::SRO5 were lower than sro5. The sro5 knock-out line overexpressing JWL-26 (sro5-35S::JWL-26) and p5cdh showed a similar level of H$_2$O$_2$ accumulation as sro5 knock-out line (Figure 3-15). This experiment was repeated for the second time but the obtained results were not consistent.
Figure 3-14: \( \text{H}_2\text{O}_2 \) production in salt-stressed Arabidopsis seedlings.

Wild-type, Salk-064137, SRO5 knock-out, p5cdh, and transgenic plants (WT-35S::SRO5, WT-35S::JWL-26, sro5-35S::SRO5 and sro5-35S::JWL-26) were germinated in half-strength MS (1.5\% w/v sucrose) 0.8\% agar plates under 16/8 h light/dark cycle (80 \( \mu \)mol/s/m\(^2\)) at 22 \(^\circ\)C. After 7-d, seedlings were transferred to half-strength MS liquid medium with or without 150mM NaCl for 12 h. The salt treated and untreated seedlings were then incubated with 10 \( \mu \)M 2,7-dichlorofluorescein diacetate for 5 min. ROS production was detected in an epifluorescent microscope 5 min after addition of the dye. Images were obtained with a digital camera at identical exposure times. The scale bar (1mm) applies to all images. This experiment was independently performed for two times.
Figure 3-15: In vivo detection of H₂O₂ accumulation in shoots of plants exposed to salt stress through DAB staining method.

Wild-type, Salk-064137, sro5 knock-out, p5cdh, and transgenic plants (WT-35S::SRO5, WT-35S::JWL-26, sro5-35S::SRO5 and sro5-35S::JWL-26) were germinated in half-strength MS (1.5% w/v sucrose) 0.8% agar plates under 16/8 h light/dark cycle (80 μmol/s/m²) at 22 °C. After 14-d, seedlings were transferred to half-strength MS liquid medium with or without 150mM NaCl for 12 h. The salt treated and untreated seedlings were then placed in 1mg/ml 3,3'-diaminobenzidine (DAB)-HCl for 4 h under dark conditions. The level of H₂O₂ accumulation correlates with the intensity of brown stain in the leaves. Images were obtained with a digital camera (Canon, PowerShot S3 IS) using the automatic settings.
3.5 Discussion

Transgenic plants are a powerful tool to study the function of novel genes, in particular to analyse the physiological function of an unknown gene (Shinozaki et al. 2003). These tools involve transferring a stress-inducible gene in order to evaluate whether it can enhance the level of tolerance against a specific stress (Holmberg and Buelow, 1998). Several stress-inducible genes that encode proteins involved in osmolyte biosynthesis and ROS scavenging have been over-expressed in transgenic plants to generate stress tolerant phenotypes using the constitutive CaMV 35S promoter to drive high levels of expression. In this research, transgenic plants were generated to study the role of the SRO5 and JWL-26 genes in salt stress. In addition, knock-out lines lacking the genes encoding SRO5 (Similar to RCD-One 5) or P5CDH (Δ1-pyrroline-5-carboxylate dehydrogenase) were studied to provide greater insight into the role of SRO5 and JWL-26 in salt stress tolerance. These reverse and forward genetic approaches provide information with which to evaluate the functional involvement of these genes in response to salt stress. Using the same approach, Katiyar-Agarwal., 2006 found that RCD1 is involved in scavenging salt-induced ROS in Arabidopsis.

3.5.1 SRO5 and JWL-26 contribute to salt tolerance

Over-expressing SRO5 or JWL-26 genes in wild-type and in an sro5 knockout line conferred increased tolerance to salt stress as shown by survival on media containing salt (Figure 3-13). Salk-064137, which has an increased level of SRO5 expression, was also more tolerant to salt stress than wild type.
The sro5 knock-out line was less tolerant of salt than wild-type. The results indicate that over-expression of the SRO5 and JWL-26 genes can promote salt tolerance in transgenic Arabidopsis plants. Salk-064137 and the transgenic plants overexpressing SRO5 and JWL-26 contained a normal level of P5CDH expression and were more salt tolerant too. Thus, over-expression of these genes had no effect on the expression level of P5CDH and consequently the enhanced tolerance was not influenced by proline.

The accumulation of proline is a common response to abiotic stresses. Proline acts as a compatible osmolyte, a protective agent for membranes and enzymes and as a radical scavenger (Hare and Cress, 1997). P5CDH is essential for the degradation of proline (Deuschle et al., 2004). It was previously reported that the p5cdh knock-out accumulates more proline than wild type under control conditions and slightly more under salt stress (Borsani et al., 2005). The p5cdh knock-out is more tolerant to salt and this may be related to its higher accumulation of proline (Borsani et al., 2005). Here, the p5cdh knockout line also exhibited more tolerance to salt compared with the wild-type and sro5 plants.

SRO5 belongs to the RCD1-SRO gene family, which plays an important role in regulating hormone signalling pathways in Arabidopsis (Ahlfors et al., 2004). JWL-26 shares the highest amino acid sequence similarity with SRO5 and, like SRO5, contains a PARP signature and has sequence identity with the C-terminal protein interaction domain. Both SRO5 and JWL-26 were expressed in salt-stressed roots and a similar function was predicted for these proteins.
When over-expressed, SRO5 and JWL-26 clearly increased salt tolerance but they reduced seed germination efficiency of wild-type Arabidopsis plants under salt. It is possible that the negative effect of SRO5 and JWL-26 on seed germination in wild-type transgenic plants arise, at least in part, from disrupting the hormone balance such as ethylene. Ahlfors et al. (2004) demonstrated that mutation in RCD1 affects hormone-related processes. This was confirmed by microarray analysis, which revealed up-regulation of genes involved in ethylene biosynthesis, including ACC oxidase, in rcd1, which correlates with the higher basal level of ethylene in rcd1. The sro5 knock-out line germinated very poorly relative to wild-type and in the sro5 line overexpressing SRO5 or JWL-26 germination was improved although it was less than wild-type. These results suggest that an optimum level of these proteins is needed for proper function and increasing or decreasing the expression of these genes results in aberrant phenotypes.

P5CDH is ubiquitously expressed in all tissues, especially in pollen (Nakashima et al., 1998). Pollen grains accumulate high levels of proline, which supposedly protect the pollen grain during desiccation or serve as a source of energy during pollen germination (Schwacke et al., 1999). However, pollen germination and pollen tube growth were unaffected in p5cdh-1 (Deuschle et al. 2004). Work by Deuschle et al. (2004) showed that the germination rate was reduced in the p5cdh mutant. They suggested that proline degradation may be important for seed maturation or germination (Deuschle et al. 2004). Here, we have observed that germination was not affected by the reduced expression of
P5CDH, which suggests that proline may not be involved in seed germination processes. It is possible that the level of proline in the p5cdh knock-out line used in this research was different from the line used by Deuschle et al. 2004.

Plants over-expressing SRO5 and JWL-26 had shorter roots than wild-type in the presence or absence of salt. Reduced seedling root length of wild-type transgenic plants may be associated, in part, with the hypothetical role of SRO5 and JWL-26 in regulating hormone signalling pathways. As showed by Fujibe et al. (2006) overexpression of RCD1 in wild-type resulted in a phenotype intermediate in size between rcd1 and wild-type, whereas over-expression of RCD1 in the rcd1 mutant fully complemented the phenotype of the rcd1 mutant (Fujibe et al., 2006). Overexpressing SRO5 and JWL-26 in the sro5 knock-out line did not affect root growth and the sro5 knock-out line had a shorter root length relative to wild-type. The sro5 knock-out line accumulated higher levels of ROS than wild-type (Borsani et al., 2005) and, since exogenous H2O2 inhibits root growth (Chi Lin and Huei Kao, 2001), this may explain its reduced root growth. However, overexpressing JWL-26 or SRO5 reduced ROS levels without any recovery of root growth. Thus, an optimal level of RCD1-SRO proteins and their related orthologs may be required for a proper function. However, the obtained results require further analysis to determine the reasons for the negative impact of overexpression of JWL-26 and SRO5 genes on seedling root growth. The p5cdh knock-out line also had reduced root length. Seedling development was inhibited by externally supplied proline in ProDH (Pro-dehydrogenase)-antisense lines (Mani et al., 2002; Hare et al., 2003) and proline
accumulation is associated with root growth inhibition (Lin and Kao, 2001). Therefore, root growth of p5cdh may be inhibited by proline.

Over-expressing SRO5 and JWL-26 reduced salt sensitivity of root growth. The sro5 knock-out line exhibited the same salt sensitivity as wild type indicating that SRO5 does not contribute to the reduction in root growth caused by salt stress. It is possible that the loss of SRO5 in the sro5 knock-out line is masked by compensation from other members of the RCD1-SRO gene family. The p5cdh knock-out line showed the same sensitivity as wild-type to salt. This indicates that accumulation of proline does not reduce the sensitivity of root growth during salt stress. The wild-type transgenic plants over-expressing SRO5 and JWL-26 were more salt tolerant than wild-type as root growth was less sensitive to salt stress. Salk-064137, which has high expression of SRO5 was also less sensitive in terms of root growth in response to salt. Thus, accumulation of SRO5 and JWL-26 reduced the sensitivity of root growth to salt, possibly, in part, by inhibiting ROS accumulation in roots. Reduced sensitivity to salt results in better root growth and facilitates water uptake by roots by growing away from the saline environment.

When SRO5 was expressed in the sro5 knock-out line root growth was more sensitive to salt stress than that of either sro5 or the wild-type plants. These plants were also less tolerant of salt as gauged by their survival on salt even though ROS was reduced in the roots. In many cases, transgenes are not expressed at the same level or with the same regulatory controls as the endogenous genes, due either to variation in sites of chromosome integration or
absence of upstream or downstream regulatory controls (Vitart et al. 2001). Since the ORF of SRO5 was introduced into the sro5 knock-out line without any upstream or downstream elements, this may affect its function. Expression of JWL-26 in the sro5 knock-out line did not impact root growth and salt survival was enhanced in this line. At this point, it is not clear why JWL-26 did not alter the sensitivity of the sro5 knock-out line during salt stress and this requires further studies.

3.5.2 Transgenic plants over-expressing SRO5 and JWL-26 had reduced levels of ROS during salt stress

ROS are unavoidably generated in plants as by-products of aerobic metabolic processes such as respiration and photosynthesis (Asada, 1994 and Apel 2004). Oxidative stress is a consequence of many abiotic stresses, including salt and it disrupts the function of organelles with high oxidizing metabolic activity or with sustained electron flows such as chloroplast and mitochondria (Arora et al. 2002 and Apel 2004). ROS are detoxified by enzymatic and non-enzymatic scavenging mechanisms (Apel et al., 2004). The main enzymatic components to protect cells against oxidative stress are superoxide dismutase, glutathione reductase, and glutathione peroxidase (Tertivanidis et al., 2004 and Apel 2004). It has been previously reported that over-expression of ROS scavenging enzymes including superoxide dismutase, catalase and glutathione S-transferase (GST) increased salt tolerance (Borsani et al. 2000 and Noctor and Foyer 2002). The correlation between elevated SRO5 and JWL-26 expression in salt-stressed roots and the reduced H$_2$O$_2$ levels
detected in transgenic plants over-expressing these genes suggest that they may be involved in protection against salt-induced oxidative damage. Furthermore, the transgenic plants over-expressing *JWL-26* or *SRO5* were more tolerant to salt stress.

Borsani et al., (2005) demonstrated that up-regulation of the *SRO5* transcript during salt stress regulates the expression of *P5CDH*, through formation of a nat-siRNA. The 3'UTR of SRO5 and P5CDH overlap in a cis-antisense manner, which results in the cleavage of P5CDH mRNA via generation of nat-siRNAs. The degradation of *P5CDH* results in a reduction of proline breakdown thereby enhancing the level of proline in the cell, which is beneficial to salt-stress tolerance but, in parallel causes the accumulation of ROS (Borsani et al. 2005). Deuschle et al. (2001) has shown that P5CDH is required to protect plant cells against proline toxicity. Down-regulation of *P5CDH* results in the accumulation of its substrate P5C, which causes the generation of ROS (Borsani et al., 2005, Deuschle et al., 2001 and Nomura and Takagi, 2004). The mechanism underlying P5C’s toxicity has not been revealed in detail yet. It is suggested that reaction of P5C with various cellular compounds triggers oxidative damage (Nomura and Takagi, 2004). Borsani et al. (2005) has shown that *sro5* and *p5cdh* knock-out plants accumulate high levels of ROS in response to salt stress when compared with wild-type plants and that *sro5* had higher levels of ROS than *p5cdh*. I observed similar results. Therefore, it is hypothesised that the SRO5 protein might be involved in counteracting the accumulated ROS to balance the loss of P5CDH activity. This is supported by intercellular localization
of SRO5, which is predicted to be a mitochondrial protein (Borsani et al., 2005). Proline is catabolized in the mitochondria, and the P5CDH protein is located on the matrix side of the inner mitochondrial membrane (Deuschle et al., 2001 and Nomura and Takagi, 2004). Furthermore, SRO5 belongs to the RCD1-SRO gene family and RCD1 is involved in ozone-induced oxidative stress tolerance (Ahlfors et al., 2004).

Here, the involvement of SRO5 and JWL-26 in oxidative stress tolerance was confirmed by over-expression of these genes in wild-type Arabidopsis and an sro5 knock-out line. Visualization of H$_2$O$_2$ in roots of salt-stressed plants demonstrates a role for SRO5 and JWL-26 in scavenging ROS. The reduced ROS in plants with elevated expression of SRO5 and JWL-26 may explain, in part, why they were more resistant to salt stress than wild-type and sro5.

The data presented here contribute to our understanding of the function of SRO5 and JWL-26 in ROS detoxification and suggest that SRO5 and JWL-26 may be involved in oxidative stress detoxification pathways involved in salt stress tolerance. Involvement of both SRO5 and JWL-26 in ROS detoxification indicates that, at least in part, there is a functional overlap between these proteins and JWL-26 may be the tomato ortholog for SRO5.
3.6 Summary

Application of differential display-polymerase chain reaction (DD-PCR) to isolate novel salt-responsive genes from tomato roots resulted in the identification of JWS-26. (Wei et al., 2000). A full length cDNA clone (JWL-26) was isolated from a tomato cDNA library constructed with mRNA isolated from salt-treated tomato roots. JWL-26 shares sequence similarity with several Arabidopsis proteins belonging to the RCD1 (Radical-induced Cell Death 1) - SRO (Similar to RCD-One) gene family. The JWL-26 amino acid sequence is most closely related to SRO5 and, like SRO5, contains a PARP signature and amino acid sequence identity with the C-terminal protein interaction domain.

Putative tomato orthologs for RCD1 were obtained based on sequence homology searches. Like RCD1, the putative tomato orthologs for RCD1, Le-RCD1-likeA and B, contain the WWE and PARP conserved domains. Salt treatments had no effect on the expression of genes corresponding to Le-RCD1-likeA, suggesting that it does not play a role during salt stress. Le-RCD1-LikeB was constitutively expressed and that expression was weakly and transiently up-regulated in salt treated tomato roots indicating potential involvement of this gene in salt stress. In tomato JWL-26 was expressed exclusively in salt stressed roots. In Arabidopsis, SRO5 and RCD1 expression was responsive to salt stress and both genes were predominantly expressed in roots. The salt responsive nature of Le-RCD1-LikeB, JWL-26, SRO5 and RCD1 suggest a role for the proteins encoded by these genes during salt stress.
To elucidate the extent of functional overlap between Arabidopsis and tomato putative RCD1-SRO polypeptides, Le-RCD1-likeA and B were over-expressed in the rcd1 mutant. JWL-26 and SRO5 were over-expressed in wild-type Arabidopsis and in an SRO5 knock-out line. Over-expression of Le-RCD1-like genes in the rcd1 background resulted in a significant gene silencing of the transgene and phenotypic analysis of the transgenic lines with some degree of transgene expression showed no difference in the paraquat-resistant nature of the rcd1 mutant. Over-expressing SRO5 and JWL-26 in an sro5 deficient genotype was preceded by a search for an sro5 knock-out line. Various SALK lines were examined among which a knock-out for SRO5 was not identified but lines with altered SRO5 and P5CDH expression were found. A T-DNA induced gene knock-out for SRO5 was obtained from the Max Planck Institute for Plant Breeding Research (GABI-Kat), which carries a T-DNA insertion in the second exon of the SRO5 gene and was used as a recipient line for over-expressing SRO5 or JWL26.

SRO5 and JWL-26 contribute to salt tolerance. Over-expression of SRO5 or JWL-26 improved survival, decreased root sensitivity to salt, and reduced levels of ROS during salt stress. However, overall the root length of transgenic plants was shorter than wild-type indicating a negative impact of SRO5 and JWL-26 on root growth. The sro5 knock-out line had shorter roots and accumulated higher levels of ROS than wild-type, which may, in part, explain its shorter root length. Over-expressing SRO5 did not recover root length in sro5 and lowered salt tolerance but did decrease ROS. Thus, SRO5 did not compensate or
complement the *sro5* mutant. Data suggest that an optimum level of these proteins is needed for proper function and is consistent with fact that SRO5 and JWL-26 may have a role in regulating hormone signalling pathways. Both SRO5 and JWL-26 are involved in oxidative stress detoxification pathways involved in salt stress tolerance. Involvement of both SRO5 and JWL-26 in ROS scavenging indicates that, at least in part, there is a functional overlap between these SRO5 and JWL-26 and that JWL-26 may be the tomato ortholog for SRO5.
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