The Role of SCARECROW-LIKE 3 in Salt Tolerance During Germination and Seedling Development in *Arabidopsis thaliana*

by

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Abstract

Salinity is a stress factor that results in reduced crop productivity. As much as three hectares of arable land is lost due to salinity per minute. With an increasing world population and shrinking arable land, world crop production must increase by at least 20% in the next two decades to avoid global food crisis according to the Food and Agriculture Organization. It is imperative to investigate and develop salt tolerance in plants so that saline land can be reclaimed for agricultural use. Much study has been done to understand the mechanism of salt stress response in plants; however, most of the intermediates involved in the stress signaling process have not been identified. We investigated the role of a GRAS family member called SCARECROW-LIKE 3 (SCL3) in salt tolerance in *Arabidopsis thaliana*.

We used mutant alleles of SCL3, each having a T-DNA insertion at different positions of the gene to investigate the mutant phenotype and RNA expression pattern. By exposing mutants to high NaCl concentrations, we demonstrated that the mutants exhibited a delay in germination and stunted root length phenotype when compared to wild-type. Using reverse transcription PCR (RT-PCR), we observed a change in the transcription levels of the mutant alleles when compared to WT during salt stress. We also observed an absence of the C

terminal of the RNA transcript in two mutants which correlated with the severity of the mutant phenotypes.

Our work indicates that SCL3 is involved in salt tolerance during both germination and during seedling development.

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Introduction

Plants undergo a large array of stresses and their ability to appropriately detect and respond to these stresses determines the extent of the plant's health and yield. Plants are generally exposed to two types of stresses; biotic stresses (for example insect, bacteria or pathogen attacks) and abiotic stresses (for example drought, ionic stress, humidity, loss of soil fertility and nutrients, and pH imbalance). While biotic factors typically depress crop yield by only about 10%, abiotic stresses have been documented to reduce the yield by as much as 50% (Boyer, 1982; Bray et al., 2000).

Salt stress is a major abiotic stress factor that results in reduced crop development and productivity. It affects every stage of plant development from seed germination to maturity. Plants, therefore, have developed a number of different mechanisms to cope with salt stress, many of which involve complex multiple interaction pathways. Much research has been done to understand the mechanism of salt stress detection and salt stress response. *Arabidopsis thaliana*, a model plant with a smaller genome and a quicker life cycle than most of the higher plants was used to study the process of salt tolerance.

Plant roots play a large role in sodium exclusion mechanisms to maintain low sodium ion concentrations in shoots under salt stress. Endodermal casparian bands, found in roots, are highly effective to exclude charged solutes such as

sodium (Steudle, 2000). Casparian bands do this by acting as a barrier, allowing a build up of a sodium gradient between the root and shoot (Steudle, 2000).

Abscisic acid (ABA) and gibberellic acid (GA) are regulators of plant development (Zhu, 2002; Brian, 2008). Both ABA and GA have been indicated to be involved in stress responses in plants. It has been shown that stress responsive genes are regulated by both ABA dependent and ABA independent pathways (Zhu, 2002). Studies also indicate that the repression of GA is a key event for the detection of salt stress. Some transcription factors that are repressed by GA are liberated to effect downstream factors involved in the response to salt (Dill et al., 2004; Kim et al., 2008).

The GRAS family [GA INSENSITIVE (GAI), REPRESSOR of GAI (RGA) and SCARECROW (SCR)] is a large family of transcription factors. The defining member of this family, SCARECROW (SCR) is required for proper radial patterning of the root and shoot (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000) Mutant *scr* roots are shorter than wild-type (WT) roots. In addition, *scr* roots have a single ground tissue layer in place of the cortex and epidermis found in WT (Di Laurenzio et al., 1996).

Some of the GRAS family genes, those with no known function, were called SCLs (SCR-LIKE) based on the presence of the VHIID domain and high sequence homology to the SCR carboxyl terminus (Fig. 1; Pysh et al., 1999; Bolle, 2004). The function of many of these SCLs of is still unknown. Structural analysis suggests that SCL members may function as transcription factors in early developmental processes. This is based on the structural similarity of

leucine heptad motifs I and II of SCL proteins to leucine zippers which are known motifs in transcription factors like the lac repressor (Alberti et al., 1993; Bolle, 2004).

The focus of this thesis is to elucidate the function of one of the SCLs called SCL3. SCL3 is expressed predominantly in the root endodermis and has an expression pattern similar to SCR (Pysh et al., 1999). We took a reverse mutational based genetic approach using several *SCL3* alleles, each containing a T-DNA insertion in a different part of the *SCL3* gene. We compared the phenotypes of each of the alleles to WT. This work analyzed four different SCL3 alleles; *scl3-1, scl3-5, scl3-3,* and *scl3-4* to investigate the function of SCL3. Our results indicate that SCL3 is involved in salt stress response based on the salt sensitivity exhibited by the mutants when compared to WT.

Literature Review

Significance of Arabidopsis

Arabidopsis thaliana is a small, dicotyledonous flowering plant from the mustard plant family *Brassicaceae* and is commonly known as thale cress. In the last two decades, *Arabidopsis* has been used as a model organism because it possesses many qualities that make it easy to grow and study. It has a small genome of 125Mb with 5 chromosomes making it ideal for genetic mapping and sequencing. It is the first plant to have its entire genome sequenced (TAGI, 2000). A large number of mutant lines and genomic resources are available (Koncz, 1992; Martinez-Zapater et al., 1998; www.arabidopsis.org). Transgenics can be easily generated by *Agrobacterium* transformation (Clough and Bent, 1998; Zhang et al., 2006). Mature plants are small and can be grown in a small confined space. Arabidopsis has a short life cycle that can be completed in 6-8 weeks and one plant can yield up to 10,000 seeds. It is self-pollinating which makes homozygous mutant collection easy. It is also well suited for microscopy as young seedling and roots are relatively translucent (Moreno et al., 2006). Conservation of basic mechanisms within the plant kingdom has been documented, allowing the results of research from a simpler plant such as *Arabidopsis* to be applied to more complex food and cash crops (Peng et al.,

1999).

There are two main methods presently utilized for investigating the function of genes and the relationship between genotype and phenotype. The first and more traditional method is forward genetics in which an organism of interest shows a mutant phenotype compared to wild type. The phenotype is mapped to discover the location of the mutated gene causing this phenotype. This approach is useful for identifying new loci involved in specific pathways where clear phenotypic differences can be observed between at least two alleles. The other more modern technique is to utilize reverse genetics. In this case, a gene is selected without knowing the precise function of that gene. The gene is altered by mutational insertion, deletion or substitution. The resulting mutants are analyzed for phenotypic differences from WT, which in turn can suggest a function for that gene. In this study, we employed reverse genetics utilizing T-DNA insertion lines of *Arabidopsis thaliana* to investigate relationship between the genotype and phenotype of a putative transcription factor SCL3.

The Effect of Salt on Plants

Many complex regulatory networks exist in plants to sense the presence of nutrients and regulate gene expression in order to adapt to environmental conditions (Wang et al., 2003; Hong-Bo et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Sodium in low concentration is a micro nutrient for some plants and is essential for growth (Brownell, 1965). It is desirable as a way to absorb water and sustain turgor in plants (Pardo and Quintero, 2002). High salinity,

however, is one of the most significant abiotic stresses that can limit plant growth and lead to plant death. An influx of sodium dissipates the membrane potential and facilitates the uptake of chlorine down the chemical gradient. Sodium is toxic to cell metabolism and has deleterious effects on some enzyme functions due to imbalance of the hydrophobic-electrostatic forces that maintain protein structure (Niu et al., 1995; Serrano et al., 1999). High sodium levels lead to the reduction in photosynthesis and the production of reactive oxygen species (Yeo, 1998). Excess salt can lead to cell wall damage, accumulation of electron dense proteinaceous particles, plasmolysis, cytoplasmic lysis and damage to the endoplasmic reticulum (Garcia et al., 1997; Khan et al., 1997; Sivakumar et al., 1998). Excess sodium also interferes and directly competes with the binding of potassium, calcium and magnesium (Serrano et al., 1996).

Salt stress is a major factor that results in reduced crop development and productivity (Bray et al., 2000). It affects every stage of plant development from seed germination to maturity (Jamil et al., 2006). Salinity is a major agricultural problem, particularly to lands that are irrigated because of NaCl contamination in poor quality irrigation water. There is over 680 million acres of irrigated agricultural land and it is estimated that as much as a third of that irrigated agricultural land is threatened by salinity (Allen et al., 1994). High salinity is not a recent phenomenon. It has been recorded that humans have not been able to keep a civilization in one location for more than 1000-2000 years because of the destruction of the resources in the area which includes salinity buildup resulting from human activity (Ashaf, 1994). It has been suggested that increasing salinity

contributed to the breakup of the Sumerian Empire in Mesopotamia by 1700BC (Jacobsen and Adams, 1958). Investigating the mechanism of salt stress detection and signaling is crucial in finding solutions that may be applied to real world food problems.

There are some plants with a natural tolerance to high salinity called halophytes. These plants adapt to high salt environments by altering their energy metabolism (Winicov et al., 1997). However, most food crops are glycophytes that are sensitive to salt. In order to adapt to salinity, glycophytes have complex mechanisms of identifying salt stress and responding to salt stress in a protective manner. Salt tolerance is a multigenic trait hence plants have devised different pathways to deal with salt stress (Flowers, 2004).

An early response to salt stress is to exclude it from the cytoplasm. Another response is to store salts in the vacuoles. This is done by vacuolar Na^+/H^+ antiporters like the NHX that are powered by vacuolar H^+ ATPases and pyrophosphatases (Liu and Zhu, 1998; Xiong and Zhu, 2002; Zhu, 2002). Cells, being finite in size, are limited in the number of vacuoles they can contain. Therefore, the intake of sodium must not exceed the rate of sodium collection in the vacuoles for homeostasis to be achieved. Another method of response is to allow salt build up outside the cell in intercellular spaces. This however, can lead to dehydration due to the creation of an osmotic gradient, pulling water outside the cell (Volkmar et al., 1998). Plants also use potassium as a balancing charge to counteract the level of sodium. The use of ion pumps, ion channels and carrier proteins are major ways in which plants deal with salt stress. It has been noted

that a Na^+/H^+ antiporter has been found in salt tolerant plants but absent from salt sensitive plants (Maathius and Amtmann, 1999). The plasma membrane antiporter that pumps sodium to the apoplast is encoded by SOS1 in *Arabidopsis thaliana* (Shi et al., 2000; Zhu, 2002). This pump functions in a pathway known as the salt overly sensitive (SOS) pathway. This SOS pathway has a pivotal regulatory function in salt tolerance in *Arabidopsis thaliana* (Yokoi et al., 2002). The SOS pathway is triggered by a rise in cytoplasmic Ca2+ due to the presence of excess Na^+ (Zhu, 2000). A calcium binding protein encoded by SOS3 senses the rise in Ca^{2+} and binds to and activates a serine/threonine protein kinase encoded by SOS2 (Liu and Zhu, 1998; Xiong and Zhu, 2002). SOS2 targets the expression of several downstream genes that act in ionic homeostasis like Na+/H+ antiporter SOS1 and a vacuole Na^+/H^+ exchanger NHX (Zhu, 2002).

Plant roots also play a role in sodium efflux and have solute exclusion mechanisms to maintain low sodium ion concentrations in shoots and prevent toxic sodium from reaching the shoots under salt stress. Casparian bands are bands of tissue containing suberin and lignin which line the endodermis along the radial and transverse walls (Bonnett, 1968). Suberin is hydrophobic and prevents the passage of water and aqueous solutes. Endodermal casparian bands are highly effective to exclude charged solutes such as sodium by acting as a barrier, blocking substances from entering the apoplast. Hence, they prevent the entry of salts that are not transported by the protoplasts (Cronk and Fennessy, 2001). Casparian bands thus build up a sodium gradient between the root and shoots (Steudle, 2000).

Phytohormones effect on plant response to salt stress

Abscisic acid has a role in seed dormancy, leaf transpiration and stress tolerance. Evidence indicates that there are several abscisic acid signaling mechanisms with many downstream targets that are involved in the protection of a plant from abiotic stress (Tuteja, 2007). Arabidopsis mutants have been isolated that are involved in abscisic acid metabolism or sensitivity (Koorneef et al., 2004). Abscisic acid signaling mutants include ABA insensitive mutants (abi) (Koorneef et al., 1984). ABI1 is a negative regulator of ABA signaling, as indicated by increased sensitivity of mutants to ABA in seeds (Gosti et al., 1999). ABI1 and ABI2 have overlapping functions during seed development and other developmental stages (Leung and Giraudat, 1998). ABI3, ABI4, and ABI5 act together to control seed development and ABA response (Soderman et al., 2000). Previous data have indicated that several genes in the ABA signaling pathway are involved in the delay and inhibition of seed germination caused by salt stress (Yuan, 2007; Kim et al., 2008). ABI3 and ABI5 both play roles during seed germination and early seedling development under stress (Yuan, 2007). This indicates that ABA is involved in mediating a salt stress response in Arabidopsis.

Gibberellic acid is required for seed germination and is involved in stem growth and elongation (Debeaujon and Koornneef, 2000). It has been shown that GA represses some transcription factors that are involved in salt stress signaling during seed germination like DELLA proteins RGA, GAI and RGL2 and NAC

factor NTL8 (Dill et al., 2004; Zentella et al., 2007; Kim et al., 2008). Salt stress down regulates the production of GA, hence inducing these transcription factors via a GA repression pathway. The downstream effect is a GA-dependent delay in seed germination. It is has been reported that adding exogenous gibberellic acid alleviates the effects of low levels of NaCl on a dwarf red kidney beans which are very sensitive to salt (Niehman and Bernstein, 1959). GA increased the stem length, fresh and dry weight of the plant, the yield and the total leaf area of the dwarf red kidney bean plant (Niehman and Bernstein, 1959). However at much higher salt concentration, growth was so stunted that GA was not able to completely alleviate the effects of salt stress (Niehman and Bernstein, 1959). The only noticeable improvement to the plant was an increase in stem length (Niehman and Bernstein, 1959). This evidence indicates that GA is involved in the adequate response of a plant to salt stress. The growth suppression caused by salt may be partially or completely reversed in early stages of salt stress with GA treatment but is irreversible at high salt concentration (Niehman and Bernstein, 1959).

GRAS family Involvement in the Response to Salt Stress by plants

GRAS is a family of gene products containing several conserved domains that suggest their function as transcription factors (Pysh et al., 1999). The name of the family is an acronym derived from the names of the initial three founding members with known function: GA INSENSITIVE (**G**AI), REPRESSOR of GAI (**R**GA) and SCARECROW (**S**CR). There are a total of 33 genes that belong to

this family in *Arabidopsis thaliana*. All the members of this family have extensive sequence similarity within their carboxyl-termini, particularly in five characteristic motifs; the leucine heptad repeat (LHR I), the VHIID motif, the leucine heptad repeat (LHR II), the PFYRE motif and the SAW motif (Figure 1). The SAW motif and the VHIID motif are the most highly conserved motifs, present in all members of the GRAS family, suggesting these regions are required for activity of the GRAS proteins (Pysh et al., 1999). The presence of the two LHR regions that may mediate protein-protein interactions suggests that these gene products may function as multimers (Hurst, 1994; Bernstein et al., 1995). Evidence suggests that GRAS family is an ancient family of genes and this family has been conserved across the plant species in both dicotyledonous and monocotyledonous plants (Bolle, 2004). There have been SCR genes orthologs identified from plants as diverse as maize (*Z. mays),* rice (*O. sativa)* peas (*P. sativum)* and pine *(P. sylvestris*) (Lim et al., 2000; Sassa et al., 2001; Kamiya et al., 2003; Laajanen et al., 2007). SCR homologs from different plants, in addition to showing strong sequence homology, also have similar expression patterns, at least in roots, suggesting conservation of SCR function (Lim et al., 2000; Kamiya et al., 2003).

Based on the homology within the carboxyl-terminal as well as additional homology within the entire protein, the GRAS family is further divided into several subfamilies (Pysh et al., 1999; Bolle 2004). One of the subfamilies, the DELLA proteins, contains five highly conserved members in *Arabidopsis thaliana*. At least three of these genes seem to encode negative regulators of GA signal

transduction pathways (Bolle, 2004). These proteins, GAI, RGA, and RGL2 (RGA-LIKE), have a 5 amino acid N terminal motif called DELLA (Bolle, 2004; Tyler et al., 2004). These DELLA proteins have been shown to respond to salt stress and inhibit cell proliferation and expansion that promotes cell survival (Archard et al., 2006). These proteins work by a GA dependent pathway, activating target genes that are down-regulated by GA repressors (Zentella et al., 2007). Majority of these genes are regulatory proteins which encode GA signaling repressors (Zentella et al., 2007). These regulatory proteins then cause repression of GA (Zentella et al., 2007).

SCR and SHR are members of the GRAS family that are essential for proper radial patterning in the root and shoot. They do this by controlling some key cell divisions throughout the life of the plant (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000; Helariutta et al., 2000). Mutations in these genes lead to a cell layer deletion in roots between the epidermis and pericycle (Scheres et al., 1995; Di Laurenzio et al., 1996). This arises from a failure in specific cell divisions in the embryo and later in the meristem. These cell divisions are necessary to generate and maintain the normal and constant number of cell layers in *Arabidopsis thaliana*. The *scr* mutant roots have a single cell layer in place of the two that are present in WT (Di Laurenzio et al., 1996). In *scr*, this single ground tissue derived cell layer has the characteristics of both the cortex and the endodermis (Di Laurenzio et al., 1996; Wysocka-Diller et al., 2000). In *shr* mutants however, the mutant layer lacks the characteristics of the endodermis. Therefore, shr roots do not have casparian strips (Scheres et al.,

1995).

The GRAS sequences of unknown function were named SCLs (SCR-LIKE sequences; Pysh et al., 1999). These sequences encode proteins that have the carboxyl-termini motifs homologous to other GRAS family proteins (Pysh et al., 1999). Some SCL genes have been found to be regulated by phytohormone auxin during early stages of adventitious root formation (Sanchez et al., 2007). The transcripts, mRNAs, of three SCL members (SCL6-II, SCL6-IV, AND SCL6-III) have been shown to have complementary sequences to miRNA and may be targeted by miRNA fragments in Arabidopsis (Llave et al., 2002). Similarly in rice, *O. sativa,* it was found that a miRNA has complementary sequences to four SCL genes. In addition, this miRNA has been found to target these SCL genes (Llave et al., 2002). It has been noted that most of the targets of plant miRNA encode members of transcription factor families that have been implicated in plant developmental patterning or cell differentiation (Rhoades et al., 2002). An interesting point to note is that SCL proteins, being members of the GRAS family, possess the two leucine heptad repeat motifs. Heptad motifs facilitate protein-protein interactions or/and protein-DNA interactions. A study of the sigma 54 factor showed that the mutation of any leucine in the leucine heptad repeat region caused the inability of the whole transcription complex to transition from a closed complex to an open complex and to recognize promoters, both necessary for transcription. This caused a fall in mRNA production of the target genes (Hsieh et al., 1994). The leucine heptad motif forms an amphiphatic alpha helix (Plevin et al., 2005).This is similar to a leucine zipper structure, which also

contains two alpha helical regions containing leucine motifs. Leucine zippers have been well established to be associated with transcription factors that directly interact with DNA to modulate transcription (Landschultz, 1988). Another interesting point is that miRNAs are known to target leucine zipper transcription factors for scission (Llave et al., 2002; Rhoades et al., 2002). As SCLs are also targeted by miRNA, it is possible that the leucine heptad repeat region may be the site for miRNA scission. There are three possible effects of miRNA processing in plants. One effect is to cause the inactivation of mRNA by shearing (degradation of) the transcript. The second effect is to create two or more mRNA transcripts with novel characteristics. The third effect of miRNA processing is to activate transcripts by cutting off a repressive element on the transcript (Llave et al., 2002).

A well-known transcription factor, the Lac repressor, contains a leucine heptad repeat region that causes it to form homomeric four helical bundles (Alberti et al., 1993). Heterozygous mutants with mutations within the heptad regions show a semi dominant phenotype. This semi-dominant phenotype has been speculated to be caused by subunit poisoning of the multimeric complex by the mutant monomers (Alberti et al., 1993). It has been shown that substitutions of leucine or isoleucine residues located within the heptad repeats of the human immunodeficiency virus type I virus renders the virus noninfectious (Chen et al., 1993). This mutant virus also inhibited the infectivity of wild-type virus by forming dysfunctional hetero-oligomers that impair the early steps of viral replication (Chen et al., 1998). This indicates that leucine heptads are involved in protein-

protein and protein-DNA interactions that are necessary for the function of transcription factors that work as multimeric protein complexes. Even though the first leucine heptad repeat subunits are not in phase (Pysh et al., 1999), it is plausible that this motif may assemble in another conformation which allows an interaction with other structures.

Very little is known about the functions of most SCLs. This project focuses on studying the function of one *Arabidopsis* SCLs, SCL3. According to quantitative RT-PCR experiments by Lee et al., the strongest SCL3 expression is found in flowers and young seedlings with lower expression levels found in the root and leaves (Lee et al., 2008). Microarray data from the GENEVESTIGATOR database indicates that the highest level of SCL3 expression is in the roots and young seedlings with lower levels in flowers and leaves (http://www.genevestigator.ethz.ch/at/). RNA gel blot analyses indicate that the strongest SCL3 analysis is found in roots and siliques with lower levels found in shoots (Pysh et al., 1999). RNA in situ hybridization revealed a tissue specific endodermal root expression pattern of *SCL3* that is similar to the endodermal root expression pattern of *SCR* (Pysh et al., 1999). Using microarray transcription profiles from young *shr* root tips in an induction experiment, *SCL3* was identified to be positively regulated by SHR (Levesque et al., 2006). Based on ChIP-PCR binding assays, it has been shown that *SCL3* is also a direct target of the *SCR* protein (Cui et al., 2007). This suggests that SCL3 and SCR genes may be in the same pathway or may be regulated by each other. It also suggests that SCL3 may also be involved in the specification of the endodermal layer of

the root.

The SCL3 gene is found on chromosome 1 and is 2192 base pairs long. Its protein product is made of 482 amino acids (Figure 2a). It has a single 241 bp intron in the 5' untranslated region (5' UTR) of the gene (Figure 3a).

SCL3 expression has been suggested to be down regulated by GA but up regulated by DELLA proteins, RGA and GAI (Curtis et al., 2005, Zentella et al., 2007). Microarray data obtained from seedlings imbibed in water or 2 μM GA showed that SCL3 expression was down regulated (Zentella et al., 2007). Microarray data was obtained from *rga-17* seedlings showed that SCL3 was up regulated (Zentella et al., 2007). *rga-17* mutants produce RGA proteins that are lacking the DELLA motif and are, therefore, resistant to degradation. This indicated that SCL3 is up regulated by RGA (Zentella et al., 2007). SCL3 has also been found to be regulated by brassinosteroids. By exposing a brassinosteroids-deficient mutant *det-2* to 10nM brassinolide, microarray analysis obtained from the *det-2* showed that SCL3 was up-regulated (Goda et al., 2004). mRNA expression data obtained from *scl3-1* and *scr* mutants imbibed in 200mM NaCl for 12 hours using RT-PCR showed that SOS1 transcription level was reduced in *scl3-1* but increased in *scr*. This indicates that both SCR and SCL3 are involved in the SOS pathway by modulating the expression of SOS1 under salt stress (Yuan, 2007). SCL3 up regulates SOS1 while SCR down regulates SOS1. SOS1 encodes a Na^+/H^+ antiporter that has been shown to be important for *Arabidopsis* in salt tolerance (Shi et al., 2000). In addition, SCR may be involved in down-regulation of two other members of the SOS pathway; SOS2

which encodes a serine threonine protein kinase and SOS3 which encodes a protein that acts as a Ca sensor (Liu and Zhu, 1998; Zhu, 2002). It has been found that SOS2 and SOS3 are required for the activation of SOS1 (Quintero et al., 2002). It has also been noted that plants exhibit higher salt tolerance when there is an overexpression of SOS1, SOS2 and SOS3 (Yang et al., 2009). SOS2 and SOS3 transcription levels were increased in *scr* mutant background compared to WT, suggesting that SCR also down regulates SOS2 and SOS3 (Yuan, 2007). The relationship of SCL3 and SCR with these three SOS genes suggests that SCL3 may play a pivotal role in the protection of plants from salt toxicity through the SOS pathway. A yeast two hybrid screen using VHA-B1(vacuolar ATPase subunit B1) as bait revealed that SCL3 also interacts with VHA-B1 in the nucleus while VHA-B1 is associated to HXK1 (hexokinase 1) and RPT5B (a 19S regulatory particle triple A-ATPase; Cho et al., 2006). VHA-B1 is an isoform of the B subunit of V1 complex in vacuolar ATPases (Sze et al., 2002). V-ATPases power vacuole Na^+/H^+ exchanger NHX by generating proton motive force by pumping H^+ into vacuoles during salt stress (Zhu, 2002).

Previous work done on three T-DNA insertion lines that contain T-DNA insertions within the *SCL3* transcription unit indicated that SCL3 may have a role in salt tolerance in Arabidopsis. Two mutants (*scl3-1* and *scl3-5*) showed a strong salt sensitive phenotype while one mutant had a weak salt sensitive phenotype (*scl3-3*; Yuan, 2007). In order to confirm the function of SCL3, I re-tested these mutants and obtained a new T-DNA insertion line that contained a T-DNA insert within the *SCL3* coding region (*scl3-4*; Fig. 3a; http://www.arabidopsis.org/abrc/).

I studied the phenotypes of each insertion line compared to wild-type under salt stress during germination and early seedling development. Previous work also suggested that one of the T-DNA insertional mutants, *scl3-1*, showed complete loss (null) or reduced levels of gene expression (Lee et al., 2008). I isolated mRNA from all insertion lines and checked the expression level of *SCL3* in all insertional mutant lines.

Results

SCL3 is important for conferring salt resistance during salt stress in seedlings

Four T-DNA insertion lines were analyzed for seedling phenotypes. Each of these lines contains a T-DNA insert in a different position within SCL3 transcription unit. The position of each insert is indicated in Figure 3a. All of these lines were indistinguishable from WT when grown on MS plates with or without sucrose. It was previously shown that *scl3-1* and possibly *scl3-5* have shorter roots than WT under salt stress conditions (Yuan, 2007).

Seeds from potentially heterozygous *scl3-1* line were tested for post germination response to salt stress. WT and *scl3-1* seeds were germinated on agar plates with 3% sucrose. Three day old seedlings were then transferred to 110mM NaCl agar plates with 3% sucrose. The roots of these plantlets showed different phenotypes under salt stress when observed after 12 days (Figure 4). Some seedlings had roots similar in length to WT (phenotype 1); some seedlings had shorter roots than WT (phenotype 2) and some seedlings had a very stunted root length when compared to WT (phenotype 3). The phenotype 2 showed root length of intermediate length between phenotype 1 and phenotype 3, suggesting that *scl3-1* mutation is incompletely dominant therefore, phenotype 2 represents

heterozygous plants.

Genomic DNA was extracted from individual plants and was tested, using PCR for the presence of the T-DNA insertion (Figure 5). The primer set SCL3 forward-485 and SCL3 reverse-1659 tested for the presence of the WT allele. The primer set of LBb1.3 and SCL3 reverse-1659 tested for the presence of the mutant allele and the T-DNA insertion within *SCL3* gene. DNA from WT plants resulted in an amplification of a 1170 bp fragment with the primer set of SCL3 forward-485 and SCL3 reverse-1659. DNA from plants with the phenotype 1 only produced a wild type fragment of 1170bp with SCL3 forward-485 and SCL3 reverse-1659. DNA from plants with phenotype 2 produced two fragments, an 1170bp fragment with SCL3 forward-485 and SCL3 reverse-1659 and a 1000bp fragment with LBb1.3 and SCL3 reverse-1659. DNA from plants with phenotype 3 only produced a mutant fragment with LBb1.3 and SCL3 reverse-1659 (Figure 5). This indicates that plants with phenotype 1 are homozygous WT (+/+); plants with phenotype 2 are heterozygous (-/+) and plants with phenotype 3 are homozygous mutants for the *scl3-1* allele (-/-).

To further confirm that the mutation in SCL3 was responsible for NaCl sensitivity, I analyzed the phenotypes of three other *scl3* alleles. *scl3-5* (salk_023428) has a T-DNA insertion in the 3' UTR at position 2177 of the gene. This insertion is within the predicted polyadenylation signal sequence (Figure 3b). This insertion could interfere with normal processing of the 3" end of the transcript and could affect transcript stability. *scl3-3* (salk_099576) has a T-DNA insertion in the 3' UTR at position 2010 (Figure 3b). The insert may affect the

length of the 3' UTR by changing the position of the polyadenylation signal with respect to the stop codon. This could also affect transcript stability. *scl3-4* (sail_435_C08) has a T-DNA insertion in the coding region at position 629 of the gene, which if transcribed would lead to a truncated protein (Figure 2c).

All mutant seedlings had similar phenotypes to that of WT seedlings when grown on control plates. Compared to WT, *scl3-5* and *scl3-3* showed very little salt sensitivity when transferred to 110mM NaCl plates and observed after 12 days (Figure 6 and Figure 7 respectively). The difference of root length in 110 mM of salt of *scl3-5* and *scl3-3* compared with WT based on a t-test was not significant (Table 1). *scl3-4* mutant seedlings; however, showed a clear sensitivity to salt compared to WT (Figure 8). The difference of root length in 110 mM of salt of *scl3-4* compared with WT based on a t-test was significant (Table 1).

Sequence analyses of the insertion point in the mutant *scl3-1* indicates that the translation of the mutant *scl3-1* transcript would produce a truncated protein made up of only the first 102 of the normal 482 amino acids (Figure 2b). The *scl3-1* would also contain 8 new amino acids at its carboxy-terminus encoded by the T-DNA (Yuan, 2007). Sequence analyses of the insertion point in the mutant *scl3-4* indicates that the translation of this transcript would lead to the production of a peptide containing only the first 45 of the normal 482 amino acids (Figure 2c). The *scl3-4* protein would contain 23 new amino acids at the carboxy-terminus that are encoded by the T-DNA (Figure 2c).

These results suggest that SCL3 has an effect on conferring salt tolerance to plants during seedling development. The two *SCL3* alleles, *scl3-1*

and *scl3-4*, that both contain T-DNA insertions within the coding region of the gene show hypersensitivity to NaCl during seed germination. This hypersensitivity may be explained by the sequence data which shows that both mutant products, if expressed, would have truncated proteins that are missing essential motifs involved in transcription modulation. The other two alleles, *scl3-3* and *scl3-5* show slight or no hypersensitivity to NaCl when compared to WT.

SCL3 is involved in seed germination under salt stress

scl3 mutants showed different degrees of sensitivity to salt during seedling development. We investigated the effect of salt stress during seed germination. The initiation of germination was demonstrated by radicle emergence from the seed coat. Germination of all *scl3* mutant seeds had reached over 95% by 36 hours on the control agar plates with no sucrose or salt (Figure 11). This pattern matched WT which typically took 36 hours for 100% germination. On plates containing 150mM NaCl, all seeds experienced a delay in germination (Figure 12). All *scl3* mutants and WT showed a one-day delay of germination and started germinating by the 48-hour mark. The changes in germination were very subtle by 72-hour mark. WT seeds achieved a germination percentage of 50% while the germination percentages of *scl3-1* and *scl3-5* seeds were 47%. *scl3-3* seeds had a germination percentage of 54% and *scl3-4* seeds had a percentage of 51%. By day 7, 89% of the WT seeds had germinated. *scl3-1, scl3-5* and *scl3-3* seeds had a germination percentage similar to wild-type seeds with 90%, 90%, and 96% germination percentages respectively by the day-7. Only *scl3-4* seeds

showed a lower germination percentage of 73% by day 7 on 150 mM NaCl.

When higher concentrations of salt were used, 200mM NaCl, the differences in germination rates between WT and the *scl3* mutants were more pronounced (Figure 13). All of the *scl3* mutants and WT seeds started germinating on day 3. By day 5, WT seeds had outpaced the mutants in germination with 37% of the WT seeds germinated. *scl3-1* and *scl3-4* seeds only achieved a germination percentage of 23% and 28% respectively. *scl3-5* and *scl3-3* seeds had a weaker phenotype and exhibited germination percentages of 35% and 34% respectively. By day 7, 61% of WT seeds had germinated while only 35% of *scl3-1* seeds germinated. *scl3-5* and *scl3-3* seeds had germination percentages of 45% and 54% germination rates respectively. *scl3-4* had only 41% of its seeds germinate by day 7 (Figure 13).

These results suggest that SCL3 has an effect on conferring salt tolerance to seeds during germination. The two *SCL3* alleles, *scl3-1* and *scl 3-4*, that both contain T-DNA insertions within the coding region of the gene show hypersensitivity to NaCl during seed germination.

Transcript sizes and mRNA level correlate to the observed salt sensitive phenotype

mRNA levels of all four *scl3* mutant alleles were analyzed and compared to WT to see if the phenotypes of the *scl3* mutants could be explained on the molecular level. Total RNA was obtained from twelve day old seedlings of WT, *scl3-1*, *scl3-5*, *scl3-3*, and *scl3-4* that were grown at the same time and in the

same conditions. Using a Thermo Scientific Verso™ 1-Step reverse transcription kit, we checked for the expression level of SCL3 using CBP20 as an internal control after 35 cycles of PCR.

We noted that when using SCL3 forward-1177 primer and SCL3 reverse-1659 primer set; there was a lack of amplification for RNA obtained from *scl3-1* and *scl3-4* (Figure 14). There was amplification for RNA obtained from WT, *scl3-5*, and *scl3-3*. There was amplification for control CBP20 primer set for RNA obtained from WT and all *scl3* mutants. However, using SCL3 forward-39 and SCL3 reverse-484 primer, there was amplification for RNA obtained from all the *scl3* mutants and WT (Figure 15). The RNA levels of all *scl3* mutants were comparable to WT. This indicates that all the *scl3* mutants produce mRNA transcripts but some part of the 3' end of the transcript is not being transcribed in *scl3-1* and *scl3-4* mutants. This may be due to an early termination of transcription in *scl3-1* and *scl3-4* due to the T-DNA insertions. This may explain why these two allele mutants show the strongest salt sensitive phenotypes during germination and seedling development.

My data suggests that the phenotypes observed are correlated to the presence or absence of complete transcription of the mRNA product. The two *SCL3* alleles, *scl3-1* and *scl 3-4*, that both contain T-DNA insertions within the coding region of the gene and show strong hypersensitivity to NaCl during seed germination and seedling development, lack amplification of mRNA past the 1177bp, suggesting early transcription termination of the mRNA. This indicates that SCL3 is involved in salt tolerance in the seedling and germination stage.

scl3-3 and *scl3-5* that both contain T-DNA insertions outside the coding region in the 3' UTR of the gene and show weak or no hypersensitivity to NaCl during seed germination and seedling development, have complete transcription of the coding region. The RNA levels of *SCL3* in *scl3-3* and *scl3-5* are comparable to WT which indicates that *SCL3* transcript stability is not affected by the T-DNA insertions within the 3' UTR.

scr3 mutants are resistant to germination delay salt during seed germination

scr3 mutants showed some degree of resistance to salt during seed germination. We investigated the effect of salt stress during seed germination. The initiation of germination was demonstrated by radicle emergence from the seed coat. Germination of all *scr3* mutant seeds had reached over 95% by 36 hours on the control agar plates with no sucrose or salt (Figure 16). This pattern matched WT seeds which typically took 36 hours for 100% germination. However, on plates containing 150mM NaCl, the *scr3* mutant seeds exhibited a slight increase in germination frequency when compared to WT seeds. WT seeds show only 10% germination rate compared to 33% rate for *scr3* at 48 hours. WT seeds achieved a germination percentage of 50% while the germination percentage of *scr3* seeds was 61% by day 3. By day 7, WT had matched *scr3* in germination frequency with WT achieving a germination percentage of 89% while *scr3* had a germination percentage of 87%.

When higher concentrations of NaCl were used, 200mM NaCl, the *scr3*

mutant seeds exhibited an increase in germination frequency when compared to WT seeds. It took WT seeds 72 hours to start germinating showing a germination percentage of 3% as compared to a germination percentage of 12% by *scr3* seeds. WT seeds achieved a germination percentage of only 16% while the germination percentage of *scr3* seeds was 40% by day 4. By day 7, WT seeds had a germination percentage of 61% while *scr3* seeds had a germination percentage of 65%**.**

These results indicate that *scl3* seeds experience shorter delay of seed germination than WT under salt stress conditions.

Discussion

SCR and SHR are members of the GRAS family which are essential for proper radial patterning in the root and shoot. (Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000). They are both found in the root endodermis where they associate and regulate transcription of genes (Bolle, 2004; Cui, 2007). Mutations in these genes lead to a cell layer deletion in the roots causing abnormal layer patterns. Phenotypically, these mutants have stunted roots when compared to WT. Given these strong characteristics, it is possible that they would adversely affect plant's ability to respond to salt stress. However, previous work suggests that *shr1* and *scr3* mutant seedlings are not more salt sensitive than WT (Yuan, 2007). My data indicates that, compared to WT, *scr3* is more salt resistant than WT during germination. *scr3* had a better germination frequency than WT under salt stress. This indicates that SCR is involved in salt sensitivity or salt perception in *Arabidopsis thaliana*.

The exclusive endodermal root expression pattern of *SCL3* is similar to the expression pattern of *SCR* (Pysh et al., 1999). SCL3 is expressed in the leaves, siliques, flowers and roots (Pysh et al., 1999; Lee et al., 2008). SCL3 has been documented to be down regulated by GA but up regulated by DELLA proteins (Curtis 2005, Zentella et al., 2007). Both SCR and SCL3 have been suggested to be involved in the salt overly sensitive pathway and by affecting the expression of

SOS1 which codes for a Na^+/H^+ antiporter. SCR may down-regulate *SOS1* expression while SCL3 appears to have the opposite effect and up-regulates its expression (Yuan, 2007). SCR may also down-regulate the expression of two other members of the SOS pathway; *SOS2* which encodes a serine threonine protein kinase important for salt tolerance and *SOS3* which encodes a protein that acts as a Ca^{2+} sensor (Yuan, 2007). It has been found that SOS2 and SOS3 are required for the activation of SOS1. It has also been noted that plants exhibit higher salt tolerance when there was overexpression of SOS1, SOS2 and SOS3 (Shi et al., 2002; Yang et al., 2009). Also, SCL3 has been found to directly interact with the B1 subunit of V1 complex of V-ATPases (Cho et al., 2006). V-ATPases catalyze the ATP-dependent translocation of H^+ from the cytoplasm to the vacuole to repair the ion imbalance caused by vacuolar Na^+/H^+ exchangers sequestering Na^+ in the vacuole (Liu and Zhu, 1998). Vacuolar Na^+/H^+ exchangers are directly regulated by SOS2 (Zhu, 2002). The relationship of SCL3 and SCR with these three SOS genes and V-ATPases indicates that SCL3 may play a pivotal role in the protection of plants from salt toxicity through the SOS pathway and also by a direct interaction with vacuolar Na^+ uptake system.

My study shows that SCL3 is involved in the conferring of salt resistance to plants during germination and during seedling development. Previous study indicated that *scl3* mutants have no obviously visible morphological phenotypes under non-stress conditions when compared to WT (Lee et al., 2008). However, *scl3* mutants showed salt sensitive phenotypes of varying degree during germination and seedling development. Salt sensitivity was measured by

prolonged delay in germination compared to WT and shorter roots than WT during seedling development. The level of salt sensitivity was dependent on the location of the T-DNA insertion within the SCL3 gene. We found that *scl3* mutants with T-DNA insertions within the coding region (that is, *scl3-1* and *scl3-4*) produced more severe salt sensitive phenotypes during germination and seedling development than WT. These mutants had shorter roots during seedling development and longer delay in germination than WT. *scl3* mutants with T-DNA insertions in the 3' UTR (that is, *scl3-5* and *scl3-3*) had little or no phenotype during germination and seedling development compared to WT. Because all of the *scl3* mutants and WT roots grew at the same rate under normal conditions, this suggests that SCL3 is not involved in root growth in the absence of salt stress. However, previous work has indicated that the endodermis is the main tissue involved in GA-dependent regulation of root growth, which is dependent on the degradation of DELLA protein GAI (Ubeda-Tomas et al., 2008). Since *SCL3* is exclusively expressed in the root endodermis and may be regulated by both DELLA proteins and GA, this suggests that SCL3 may be involved in this GA-regulated root growth pathway and may be one of the downstream targets of GAI.

The location of the T-DNA insertion in *scl3-5* and *scl3-3* within the 3' UTR suggested that these mutants would exhibit salt sensitive phenotypes only if transcript abundance was affected. Previous study indicated that *scl3-5* exhibited a mild salt sensitive phenotype while *scl3-3* had little or no salt sensitive phenotype when compared to WT under salt stress. It was hypothesized that the

T-DNA in *scl3-5* but not in *scl3-3* could interfere with the polyadenylation of the *SCL3* mRNA transcript and would lead to transcript instability and/or the mRNA transcript's inability to leave the nucleus (Yuan, 2007). My results indicate that both *scl3-5* and *scl3-3* have little or no perceivable phenotype under salt stress compared to WT during germination and seedling development. I also found that *scl3-5* and *scl3-3* mRNA transcript level was comparable to WT indicating that the T-DNA insertions in the two alleles do not affect transcript stability. It is possible that the cleavage factors involved in recruiting poly A polymerase utilized other upstream elements to catalyze polyadenylation. It is also possible that the T-DNA border may provide a cryptic polyadenylation signal for use in polyadenylation.

It has been reported that *scl3-1* mutant does not express SCL3 gene (Lee et al., 2008). However, my results indicate that *scl3-1* contains WT levels of abnormal SCL3 transcripts. This difference in the results stems from the differences in primer design used in RT-PCR. Lee et al., 2008 designed primers that spanned a region of the SCL3 gene that is downstream of the T-DNA insertion point in the *scl3-1* allele. This spanned the 3' end of the transcript which I have shown to be absent in *scl3-1* and *scl3-4*, probably due to the termination of transcription within the T-DNA insert. I demonstrated the presence of WT levels of *SCL3* transcripts in all *scl3* alleles by using primers that span the 5' region of the gene. I also show that the transcript levels are comparable to WT, indicating that there is no reduction of transcription level and/or no significant changes in transcript stability.

My results show that *scl3-1* mutants have a stronger phenotype under salt stress than *scl3-4* even though *scl3-4* would produce a shorter protein than *scl3-1* if the abnormal transcripts were translated (Fig 2b and 2c). *scl3-1* had a lower germination frequency and shorter roots during seedling development than *scl3-4*. *scl3-1* mRNA level was comparable to *scl3-4* mRNA level indicating that the phenotypes observed were probably due to differences in the proteins. *scl3-4* has only 45 normal amino acids of the 482 originally found in SCL3 while *scl3-1* has 102 amino acids. *scl3-4*, but not *scl3-1*, is missing a stretch of amino acids including the LXXLL motif that marks the beginning of the first heptad repeat (LHRI, Bolle, 2004). The LXXLL motif has been indicated to be involved in the ligand-dependent binding of co-activators to nuclear pore receptors (Heery, 1997). This LXXLL motif of the co-activator associates with the activation function region of nuclear receptor ligand binding domain. The nuclear receptor then activates transcription directly by recruiting RNA polymerase II pre-initiation complex or indirectly by removing chromatin repression using intrinsic histone acetyltransferase activity (Plevin, 2005). This indicates that the SCL3 protein in *scl3-4* is completely unable to bind to these receptors and alter their function in any way. Therefore, *scl3-4* probably represents a null allele. In contrast, *scl3-1* may represent a neomorph that may be able to bind to nuclear receptors but since it is missing all the other domains including VHIID motif and LHRII, it may not be able to function properly. This may hamper the activity of the other proteins complexes bound to this mutant SCL3 causing a subunit poisoning and preventing transcription modulation. This conclusion is also supported by the fact

that *scl3-1* is semi-dominant and has a salt sensitive phenotype in heterozygotes.

Materials and Methods

Plant materials and growth methods

Two ecotypes of *Arabidopsis thaliana* were used in this study: Columbia (Col-0) and Wassilewskija (Ws-2). Various Arabidopsis mutants were obtained from Arabidopsis stock center:- s*cr3* (CS3997), *scl3-1* (salk_002516), *scl3-5*(salk_023428), *scl3-3*(salk_099576), and *scl3-4*(sail_435_C08). (Arabidopsis Resource Center OSU; (http://www.arabidopsis.org/abrc/)

Germination assays

Seeds were surface sterilized in 6.25% sodium hypochloride for 3 minutes and rinsed thoroughly in sterile distilled water. Seeds were then planted on 0.5X Murashige and Skoog (MS) medium pH5.8 (Caisson Laboratories), solidified with 0.8% agar (Fisher Scientific). Control plates had no NaCl and test plates containing varying concentrations of NaCl as noted in the text. Arabidopsis seeds were stratified for 3 days at 4ºC in the dark and then placed in a growth chamber with 70% humidity and under 18 hour light and 6 hour dark cycles at 23ºC to facilitate germination. Germination was based on radicle emergence from the seed coat and was scored every 12 hours. Each plate contained 90 to 110 seeds. Each experiment was done in triplicate and in each experiment, a different batch

of seeds were used. In each experiment, wild type and mutant seeds were collected at the same time from same age plants grown under the same conditions. While the time course of germination differed in each experiment, the trend remained consistent for all experiments. Data shown here is from one of the experiments. Seed germination data was analyzed using Microsoft Excel 2000.

Root growth assays

Seeds were surface sterilized in 6.25% sodium hypochloride for 3 minutes and rinsed thoroughly in sterile distilled water. Seed were planted on 0.5X MS media 3% sucrose, 0.8% agar. Arabidopsis seeds were stratified for 3 days at 4ºC in the dark and then placed in a growth chamber with 70% humidity and under 18 hour light and 6 hour dark cycles at 23ºC to facilitate germination. Three-day-old seedlings were transferred to 0.5MS media containing 0.8% agar and 110mM NaCl concentration via sterile forceps. The root length was marked for each seeding. The root lengths were measured at 12 days after transfer to salt plates. Root length was measured using a Kodak ID Image ROI Analysis Software. Root data was analyzed using Microsoft Excel 2000.

DNA Isolation and PCR

Genomic DNA was extracted from seedlings of all four *scl3* mutants, *scr3* mutants and wild type Columbia as described by Edwards 1991. For DNA extractions, 600µl of lysis buffer (10mM Tris-HCl pH8, 25mM EDTA pH8 and

0.5% SDS) and ground plant tissue, frozen with liquid nitrogen were placed in an eppendorf tube. The tubes were incubated for about 30 minutes at 55°C. The samples were cooled to room temperature and each was treated with 2µl RNase A at 5mg/ml to degrade RNA and incubated at 37°C for one hour. Proteins were then precipitated by addition of 200µl of 5M ammonium acetate. Tubes were centrifuged at 14,000 rpm for five minutes. The supernatant containing DNA was transferred to new tubes containing an equal amount of isopropanol. Tubes were again centrifuged at 14,000 rpm for 5 minutes. The DNA pellet was then washed with 70% ethanol and air dried. The pellet was then resuspended in 75µl TE buffer (10mM Tris-HCL pH8, 1mM EDTA). The quality and quantity of DNA was analyzed using a NanoDrop 1000 spectrophotometer.

PCR analysis of T-DNA insertions was performed using the following primers depending on each T-DNA insertion point:

SALK T-DNA left border primer LBb1.3 of Pbin-Prok2 for SALK lines

ATTTTGCCGATTTCGGAAC,

SAIL T-DNA left border1 - GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

SAIL T-DNA left border 3 - TAGCATCTGAATTTCATAACCAATCTCGATACAC

SCL3 forward primer-485 - GCCTTCAATGGTGGCTATGT

SCL3 forward primer-1177 - TTGAGGAAGCAGAGAAACTCG

SCL3 forward primer-39 - TGGATAGAGAGATAAAGATTGAGAGA

SCL3 reverse primer-1659 - CCGAAGAGCATCTTCTCCAC

SCL3 reverse primer-2685 - GCCAAAGTTTGGCTGGTCTCT

SCL3 reverse primer-484 - ATTGAAGGCCAAAAGCTTGA

Thermocycler conditions were as follows: stage 1 10 cycles with 1 minute at 94ºC, 1 minute at 52ºC and 2 minutes at 72ºC. Stage 2 has 30 cycles with 20 seconds at 94ºC, 30 seconds at 52C and 2 minutes at 72ºC. Stage 3 has one cycle of 10 minutes at 72ºC.

After confirmation of T-DNA insertion in *scl3-4* using PCR, a PCR amplified product with the following primers was sequenced (Genomics and sequencing Laboratory, Auburn, University) SAIL T-DNA left border1- GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC 3' paired with SCL3 forward primer-485 - GCCTTCAATGGTGGCTATGT. The thermocycler conditions were the same as above. T-DNA primers were designed by http://signal.salk.edu/tdnaprimers.thml and the other primers were designed using the program http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.

RNA Isolation and Reverse Transcription PCR

For mRNA analysis, 12 day old seedlings were grown in 3% sucrose at 23ºC. Total RNA was extracted using the Onate-Sanchez protocol (Onate-Sanchez, 2008) or TRI Reagent from Ambion.

Total RNA was treated with a Turbo-DNA free kit (Ambion) and tested with reverse transcription PCR for DNA contamination using Thermo Scientific Verso^{TM} 1-Step RT-PCR Kit. The quality and quantity of RNA was analyzed using a NanoDrop spectrophotometer. The purified RNA was used as a template in a one step reverse transcription PCR reaction using primers as follows:
SCL3 forward primer-485 - GCCTTCAATGGTGGCTATGT

SCL3 forward primer-1177 - TTGAGGAAGCAGAGAAACTCG

SCL3 forward primer-39 - TGGATAGAGAGATAAAGATTGAGAGA

SCL3 reverse primer-1659 - CCGAAGAGCATCTTCTCCAC

SCL3 reverse primer-2451 - GCCAAAGTTTGGCTGGTCTCT

SCL3 reverse primer-484 - ATTGAAGGCCAAAAGCTTGA

Control CBP20 forward - ATGGCTTCTTTGTTCAAGGAGC

Control CBP20 reverse - TTAAGATCTTCTCTTCCGATCATC

SCR forward-639 - TCCTTCTCCTCCACAACAGCAACA

SCR reverse-1808 - TGCTGTTCCACGACATGTCTCTCT

The thermocycler conditions were as follows: stage 1 was 20 minutes at 50ºC; stage 2 was 5 minutes at 95ºC, stage 3 had 10 cycles with 1 minute at 94ºC, 1 minute at 52ºC and 2 minutes at 72ºC. Stage 3 has 25 cycles with 20 seconds at 94ºC, 30 seconds at 52ºC and 2 minutes at 72ºC. Stage 4 has one cycle of 10 minutes at 72ºC. Reverse transcription PCR were carried out in a 25ul reaction using gene specific primers in concentrations determined for each set of primers.

Genomic detection was done using Kodak Image Station 440 CF with UV illumination and quantified using Kodak ID Image Analysis Software.

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Figure 1: A schematic diagram of the highly conserved C terminal region of the GRAS family of proteins - the leucine heptad repeat (LHR I), the VHIID motif, the leucine heptad repeat (LHR II), the PFYRE motif and the SAW motif

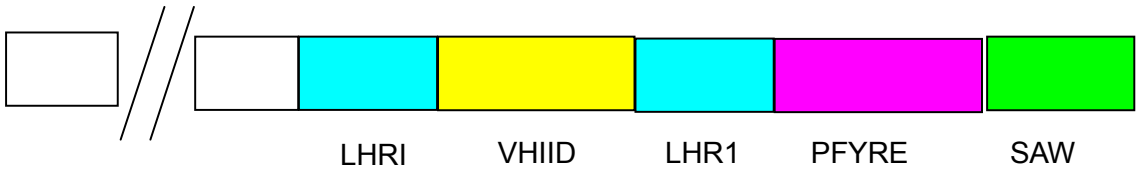


Figure 2. Figure of the amino acid sequence of SCL3 and *scl3* mutants. LHR I motif is in light blue highlight, VHIID motif is in yellow highlight, LHRII motif is in light blue highlight. New amino acids are in italics and underlined. Leucine heptad residues are in bold. Conserved VHIID residues are in red font. LXXLL motif is in dark blue font. (a) SCL3 (b) *scl3-1* (c) *scl3-4*

(a)

M V A M F Q E D N G T S S V A S S P **L** Q V F S T M S **L** N R P
T L **L** A S S S P F H C L K D **L** K P E E R G **L** Y **L** I H **L** **L** **L** T
C A N H V A S G S **L** Q N A N A A **L** E Q **L** S H **L** A S P D G D T
M Q R I A A Y F T E A **L** A N R I **L** K S W P G **L** Y K A **L** N A T
Q T R T N N V S E E I H V R R **L** F F E M F P I **L** K V S Y **L** **L**
T N R A I **L** E A M E G E K M **V** **H** **V** **I** **D** L D A S E P A **Q** W L A
L L Q A F N S R P E G P P H **L** **R** **I** **T** **G** V H H Q K E V L E Q M
A H R L I E E A E K L D I P F Q F N P V V S R L D C L N V E
Q L R V K T G E A L A V S S V L Q L H T F L A S D D D L M R
K N C A L R F Q N N P S G V D L Q R V L M M S H G S A A E A
R E N D M S N N N G Y S P S G D S A S S L P L P S S G R T D
S F L N A I W G L S P K V M V V T E Q D S D H N G S T L M E
R L L E S L Y T Y A A L F D C L E T K V P R T S Q D R I K V
E K M L F G E E I K N I I S C E G F E R R E R H E K L E K W
S Q R I D L A G F G N V P L S Y Y A M L Q A R R L L Q G C G
F D G Y R I K E E S G C A V I C W Q D R P L Y S V S A W R C
R K Stop

(b)

M V A M F Q E D N G T S S V A S S P **L** Q V F S T M S **L** N R P
T L **L** A S S S P F H C L K D **L** K P E E R G **L** Y **L** I H **L** **L** **L** T
C A N H V A S G S **L** Q N A N A A **L** E Q **L** S H **L** A S P D G D T
M Q R I A A Y F T E A **L** *G L R V N M R L* Stop

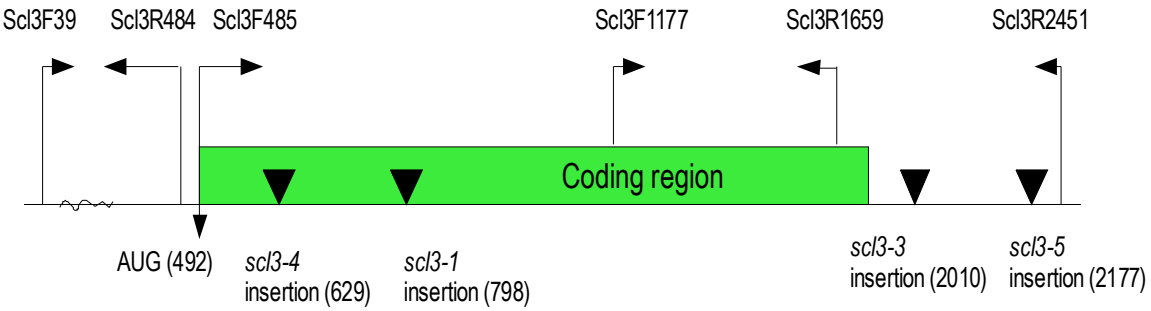
(c)

M V A M F Q E D N G T S S V A S S P **L** Q V F S T M S **L** N R P
T L **L** A S S S P F H C **L** K D **L** *I D E T N N K L T L R Q L N N*
T L R T F L M Y Stop

Figure 3: (a) A schematic diagram of SCL3 locus showing the T-DNA insertions and the primers used for this work. The T-DNA insertion sites are indicated by closed triangles. Arrowheads denote position and orientations of primers used for PCR and RT-PCR reactions. Wavy lines indicate introns.

(B) SCL3 3' UTR highlighting *scl3-5* and *scl3-3* T-DNA insertions. Stop codon is highlighted in purple, Far Upstream Element is highlighted in yellow, Near Upstream Element is highlighted in green, and cleavage site is highlighted in light blue.

(A)



(B)

TGAATGATATATTACAGTTTGTCTTCTATTTTGGTTATGAGCAGAGTCCCTTTC TTTTTTGTATACATGG **(*scl3-3* insertion)** GGACACAATCTTAGTTGTTTTGT GATGGTGACTTTCTGTCTCTTTATGCTATTTTGGCTTAAATGCTTCTACTGCCT CTGCATGTAAAGCCTTTGTGTGTTGGTTCAATTTGGTCTGGTGTGGGTGTAAT ACCAAACCAAATCCAATTTGAGCTGAAGATAACTAAT **(*scl3-5* insertion)** TT GATGATCGGTTCAAG

Figure 4. Representative, 15 day old, seedling phenotypes from heterozygous line of *scl3-1* allele grown on 110mM NaCl. (a) WT Columbia, (b) +/+ WT, (c) -/+ heterozygous *scl3-1*, (d) -/- homozygous mutant *scl3-1*

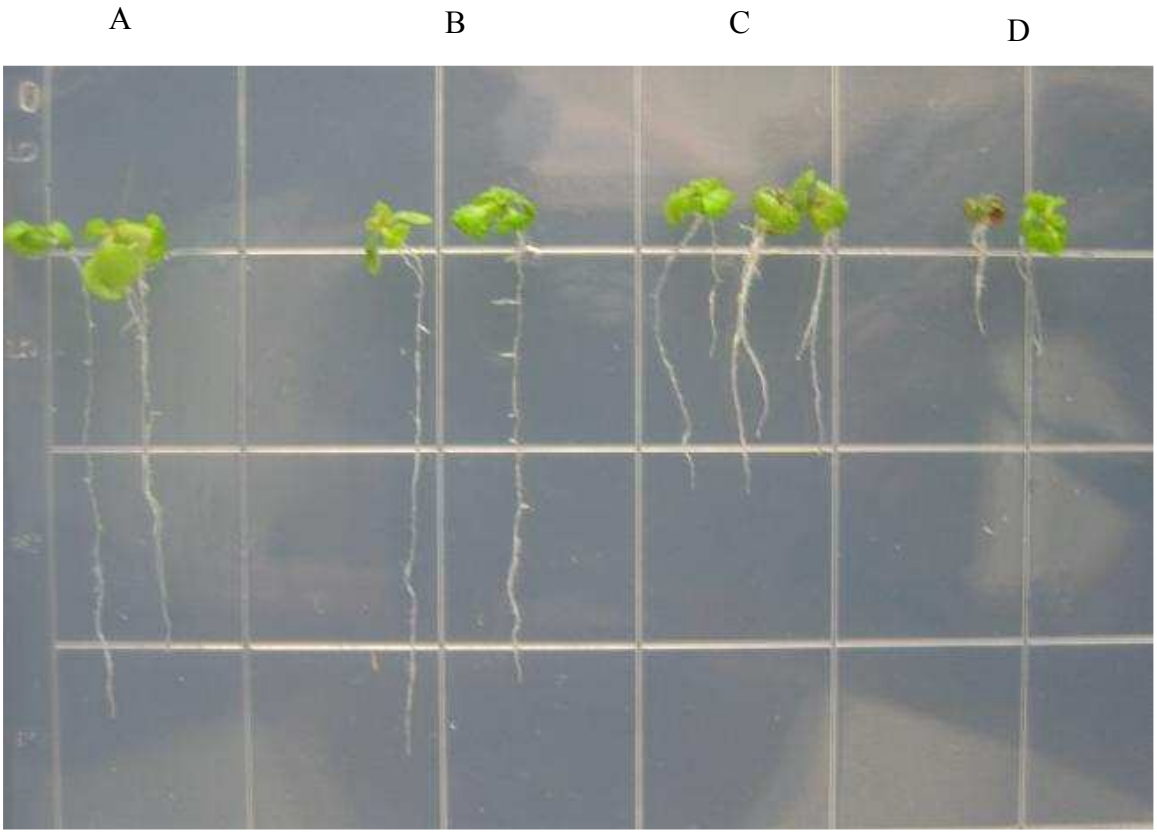


Figure 5: PCR amplification of *scl3-1* mutants using primers SCL3 forward primer-485, SCL3 reverse primer-1659, and SALK T-DNA left border primer LBb1.3.

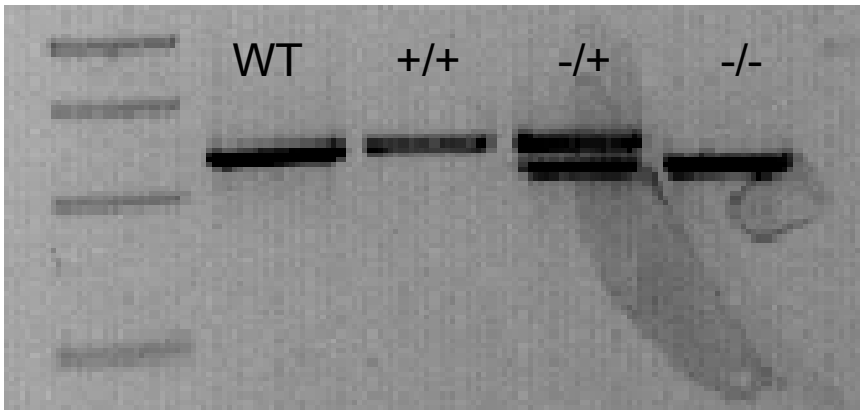


Figure 6. Representative, 15 day old, seedling phenotypes from homozygous line of *scl3-5* allele grown on 110mM NaCl. (a) WT Columbia, (b) -/- homozygous mutant *scl3-5*

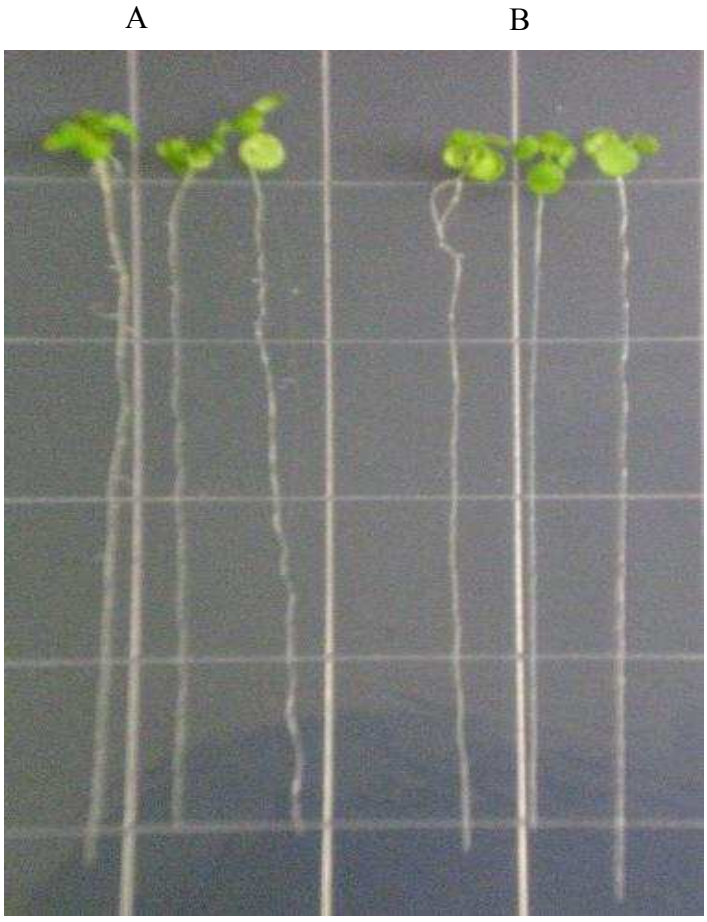


Figure 7. Representative, 15 day old, seedling phenotypes from homozygous line of *scl3-3* grown on 110mM NaCl. (a) WT Columbia, (b) -/- homozygous mutant *scl3-3*

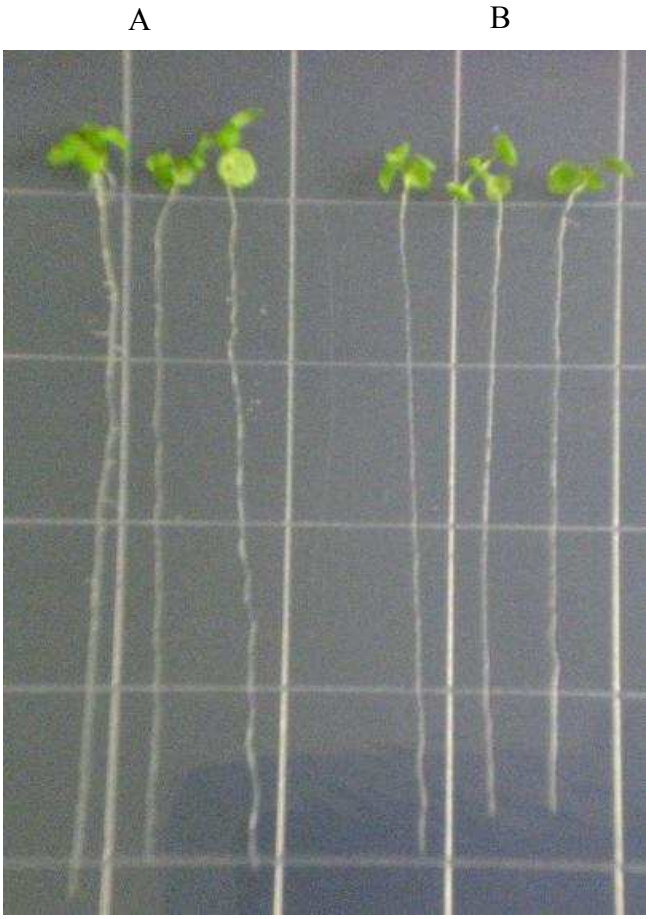


Figure 8. Representative, 15 day old seedlings phenotypes from homozygous line of *scl3-4* grown on 110mM NaCl. (a) WT Columbia, (b) -/- homozygous mutant *scl3-4*

A B

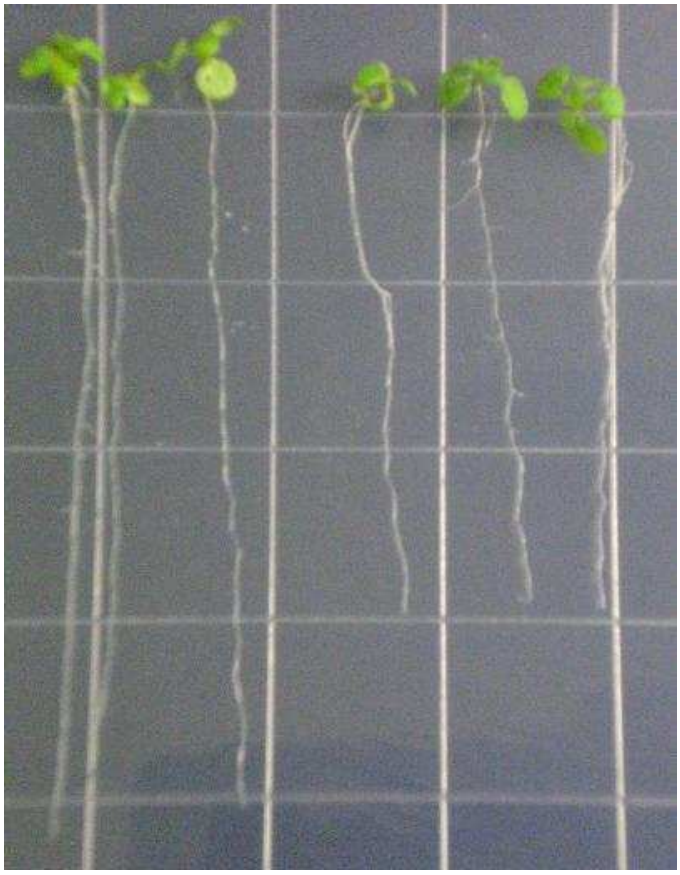


Table 1. Root length of 15 day old WT and *scl3* mutants. Seeds were germinated without salt and 3-day-old seedlings were transferred to 110mM NaCl for 12 days.

Mutant	**Mean Root length (in mm)**		**P Value**	**Standard deviation**	**Standard error**	**Confidence Interval**
	Mutant (n)	**WT (n)**				
scl3-1	9.4 (188)	22.21 (261)	P<0.0001	5.923	0.432	10.87 – 14.18
scl3-5	22.1 (329)	23.27 (152)	P=0.2883	11.334	0.6248	-0.9 – 1.16
scl3-3	25.2 (304)	26.97 (132)	P=0.0707	11.712	0.6717	-5.3 – 0.21
scl3-4	26.0 (262)	33.20 (138)	P<0.0001	11.289	0.6974	4.95 – 9.44

Figure 9. Root length of 15-day old WT and *scl3-1*. Seeds were germinated without salt and 3-day-old seedlings were transferred to 110mM NaCl for 12 days.

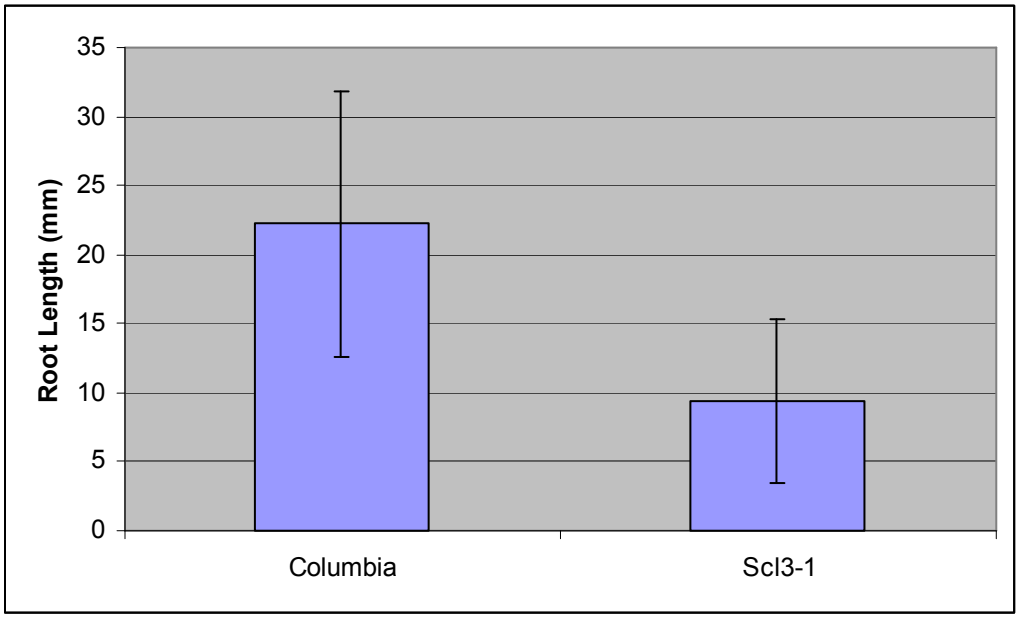


Figure 10. Root length of 15-day old WT and *scl3-4*. Seeds were germinated without salt and 3-day-old seedlings were transferred to 110mM NaCl for 12 days.

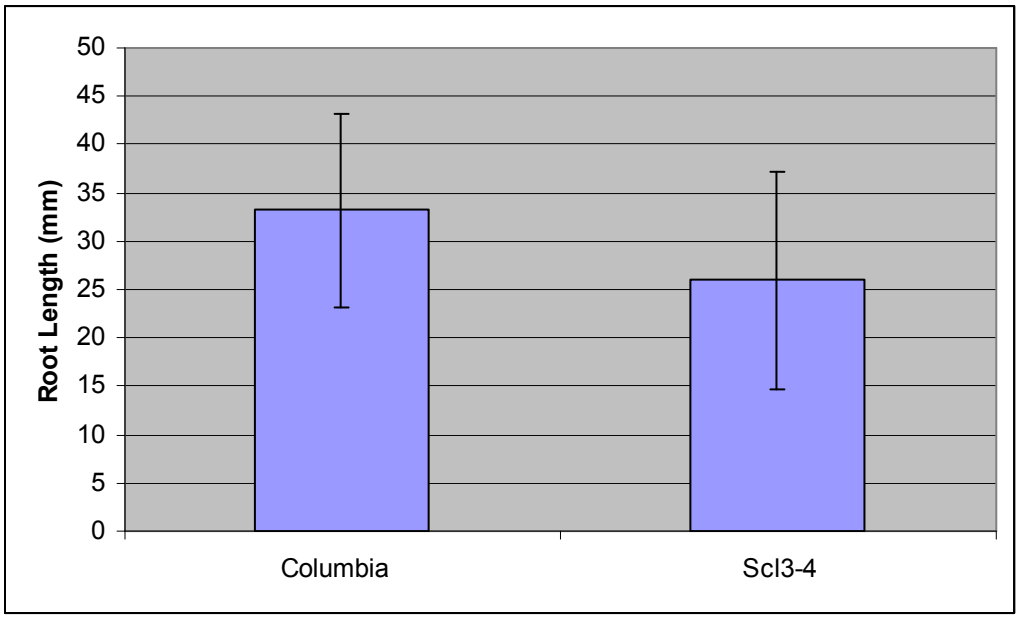


Figure 11. Time course of WT and *scl3* mutants seed germination on 0.5MS agar plates. Points on the Z axis are in hours after transfer of plates to the growth chamber.

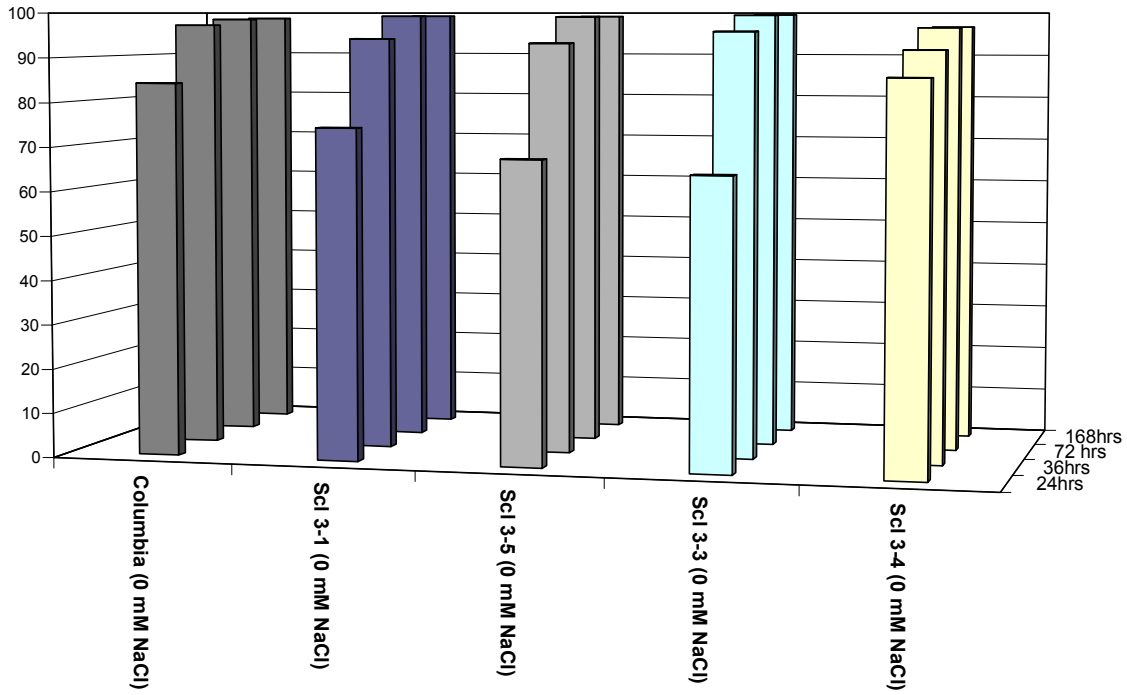


Figure 12. Time course of WT and *scl3* mutants seed germination on 0.5MS agar plates containing 150mM NaCl. Points on the Z axis are in hours after transfer of plates to the growth chamber.

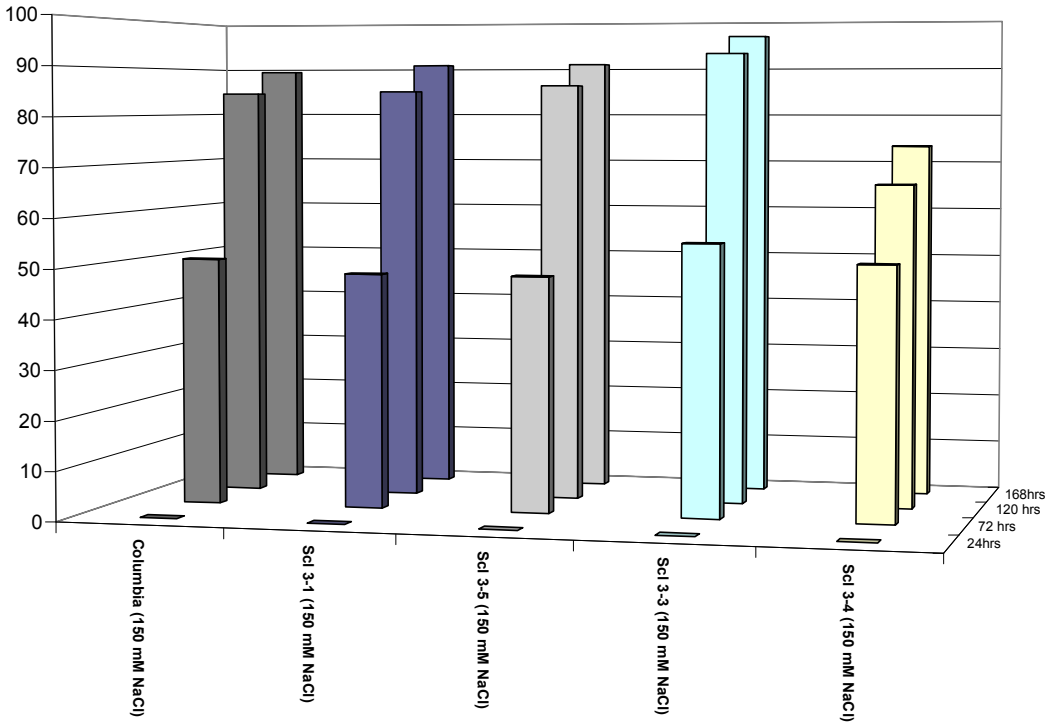


Figure 13. Time course of WT and *scl3* mutants seed germination on 0.5MS agar plates containing 200mM NaCl. Points on the Z axis are in hours after transfer of plates to the growth chamber.

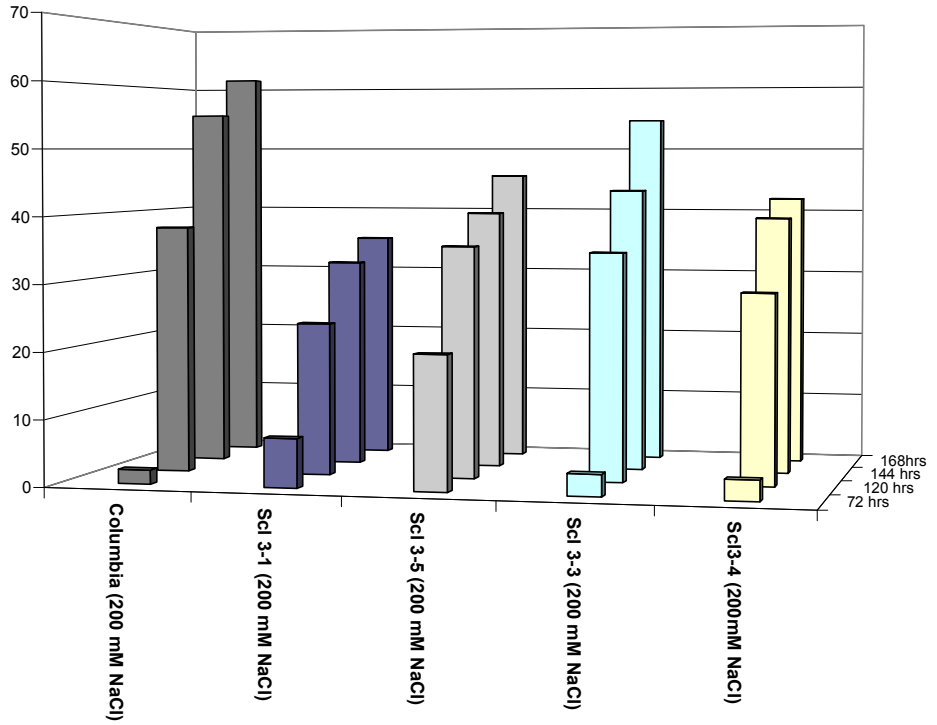


Figure 14: RT PCR test for the presence of SCL3 complete mRNA transcript. SCL3 forward primer1177 and SCL3 reverse primer 1659 were used for one reaction and Control CBP20 forward and Control CBP20 reverse primers were used as a control.

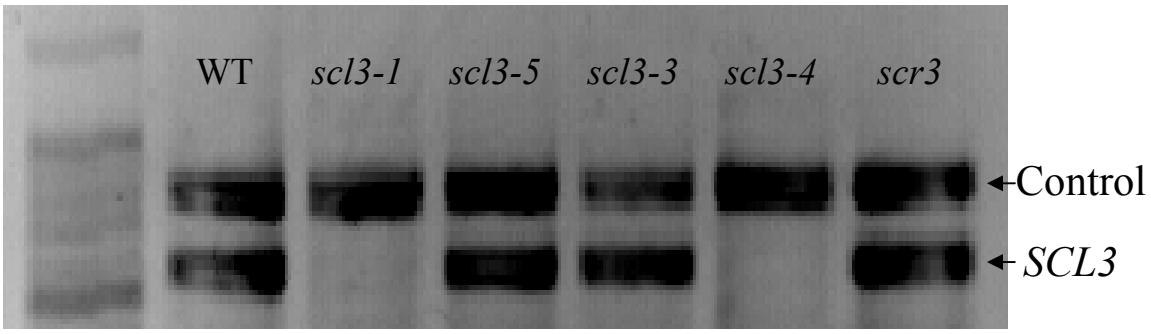


Figure 15: RT PCR test for the presence of SCL3 mRNA transcript. SCL3 forward primer39 and SCL3 reverse primer 484 were used for one reaction and Control CBP20 forward and Control CBP20 reverse primers were used as a control.

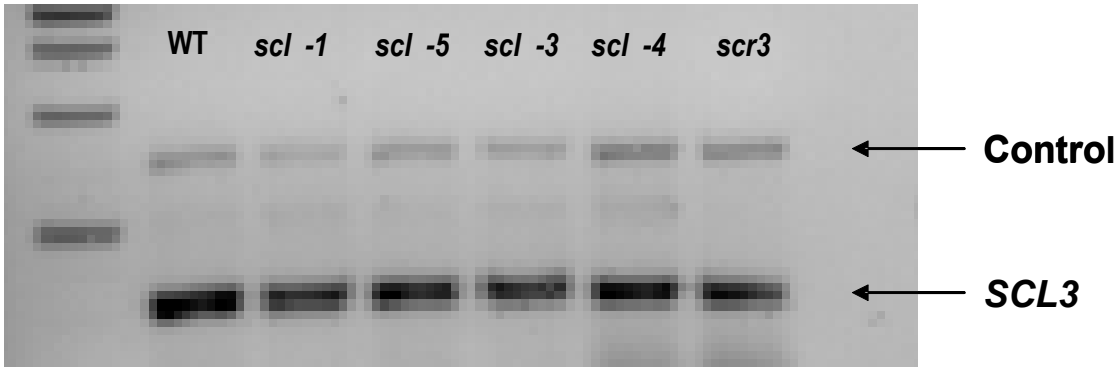


Figure 16. Time course of WT and *scr3* seed germination on 0.5MS agar plates containing varying concentrations of NaCl. Points on the Z axis are in hours after transfer of plates to the growth chamber.