THE ROLE OF PLASMA MEMBRANE H\(^+\)-ATPase
IN PLANT RESPONSES TO SALT STRESS

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by
Munira Ferdousi

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Abstract

of

THE ROLE OF PLASMA MEMBRANE H\(^+\)-ATPase

IN PLANT RESPONSES TO SALT STRESS

by

Munira Ferdousi

Plant plasma membrane H\(^+\)-ATPases (PM H\(^+\)-ATPases) play a central role in plant physiology. The plant plasma membrane proton pump (H\(^+\) pump) contributes to the control of ion and nutrient uptake and water fluxes which are required for cell growth. PM H\(^+\)-ATPase are regulated by hormones, environmental stresses, and light. PM H\(^+\)-ATPases establish proton electrochemical gradients across the plasma membrane that provide the driving force for the secondary active transport of Na\(^+\) from the cytosol across the plasma membrane and so may play a role in plant responses to salt stress. Excessive salt is toxic for plants and, at high concentrations, leads plant to death. Studies using transgenic tobacco (*Nicotiana tabacum*) plants expressing either wild-type plasma membrane H\(^+\)-ATPase4 (wtPMA4) or a PMA4 mutant generating a constitutively activated enzyme have revealed that overexpression of the PM H\(^+\)-ATPase gene increased the salt tolerance of the plant during germination and seedling growth. Moreover, the tomato (*Solanum lycopersicum*) PM H\(^+\)-ATPase isoform LHA8 expression is induced at significantly higher levels in response to high salt in leaves and roots.
The aim of this study is to investigate the role of the PM H\textsuperscript{+}-ATPase in controlling plant growth and development in response to salt and whether PM H\textsuperscript{+}-ATPase isoforms are regulated in response to salt. The study has utilized previously generated transgenic *Arabidopsis* lines overexpressing the plasma membrane H\textsuperscript{+}-ATPase (OEX lines) and transgenic *Arabidopsis* lines containing construct of the tomato isoform *LHA2* promoter fused to the reporter gene GUS (*LHA2::GUS* lines). Overexpressing lines have demonstrated the physiological effects of overexpression under saline conditions and have tested whether modified activity of the H\textsuperscript{+} pump can enable plants to tolerate salt stress better than wild-type plants. The effect of overexpressing of the H\textsuperscript{+} pump in response to salt was determined by measurement of root lengths and monitoring the number of emerged lateral roots.

*Arabidopsis* seeds were surface sterilized and grown on 0.5 X MS (Murashige and Skoog) media. Three to four days after germination, seedlings were then transferred from starter plates to plates containing 0, 50 100 or 150 mM NaCl and incubated in a growth chamber for four additional days. Primary root length was noted at the time of transfer. Primary root length and the number of lateral roots (laterals) were recorded at two and four days following transfer. The experiment was replicated three to four times, with each replicate including 16-18 *Arabidopsis* seedlings at each treatment.

Primary roots of overexpressing lines of *Arabidopsis* seedlings grown on all concentrations of NaCl and control (0 mM NaCl) plates were significantly (t-test;
P<0.05, ANOVA; P<0.01) longer compared to the wild-type *Arabidopsis* seedlings. In wild-type *Arabidopsis*, lateral root number (LRn) is higher than in OEX lines with increasing concentrations of NaCl (t-test; P<0.05, ANOVA; P<0.01) and the lateral root density (LRd) is increased with increasing amounts of NaCl compared to the overexpressing *Arabidopsis* lines (t-test; P<0.05). In addition, ANOVA analysis indicates that genotype (OEX lines such as 3C and 6G lines and wild-type) and salt concentration have a significant effect on the primary root growth and lateral root number.

*LHA2:*GUS lines have demonstrated the transcriptional regulation of the isoform LHA2 in *Arabidopsis* plant in response to salt. Expression of *LHA2:*GUS constructs were then observed and recorded using differential interference contrast microscopy. GUS activity assays were utilized to quantify the level of GUS expression in treated plants. GUS histochemical analysis and activity assays were conducted on two independently replicated and one pseudoreplicated sets of seedlings.

The expression pattern of *LHA2:*GUS changed during the course of different NaCl concentration. In untreated (control) *Arabidopsis* plants, *LHA2:*GUS expression was condensed in columella cells, epical meristem, vasculature tissue, elongation zone and root cap of the primary root. However, with higher concentration of NaCl treatment the *LHA2:*GUS expression was more condensed in columella cells of root cap. The expression is also significantly decreased in vasculature tissue, apical meristem and elongation zone of the primary root in 100mM and 150mM NaCl concentration.
In leaf, the expression of \textit{LHA2::GUS} is decreased with increasing NaCl concentration. The expression pattern was gradually limited on leaf vasculature system with increasing amount of NaCl. The expression of \textit{LHA2::GUS} was increased in lateral root with increasing amount of NaCl. The \textit{LHA2::GUS} expression was little in root cap, vasculature tissue of young lateral root and mature lateral root at 0mM and 50mM NaCl and high in root cap and vasculature tissue of young lateral root and mature lateral root at 100mM and 150mM NaCl.

Based on this study it appears likely that continuously overexpressing the PM H\textsuperscript{+}-ATPase causes increased primary root length with increasing NaCl compared to wild-type (control) plants. Primary root growth is considered to be a predominant phenotypic characteristic of plants growth and survivability. Therefore, our results suggest that PM H\textsuperscript{+}-ATPase play a critical role in the ability of plants to tolerate salt.

The observation that the expression pattern of \textit{LHA2::GUS} changes in the primary root cap and other areas of the primary root as well as in lateral roots and leaves in response to salt supports the hypothesis that LHA2 is regulated by NaCl. In addition, the activity level of \textit{LHA2::GUS} is also changed with increasing NaCl which provides further support for the hypothesis that LHA2 is modulated by NaCl.

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Dr. Nicholas Ewing

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Date

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INTRODUCTION

Plants use transport systems to move a variety of solutes across the cellular membrane and these systems are composed of transmembrane proteins. P-type ATPases are a large family of membrane transport proteins that utilize the energy of hydrolysis of ATP in the process of active transport of solutes (reviewed in Rosewarne et al., 2007). Members of this superfamily of transmembrane ion pumps are present in biological membranes of all organisms (with the exception of some bacteria and archaea) and are involved in an extensive range of elemental cellular processes which include producing and maintaining both the membrane potential and proton gradients that provide the driving force for the transport of essential micronutrients and a range of other solutes including sugars, amino acids, and hormones. They also contribute to the maintenance of cytosolic and extracellular pH and participate in cell signaling (Axelsen and Palmgren, 2001). The mechanism of transport through P-type ATPases involves a characteristic formation of an aspartyl phosphate intermediate during the catalytic cycle (Duby and Boutry, 2008).

Recent studies have identified a total of 46 P-type ATPases in Arabidopsis and 43 in rice and these ATPases have been classified into five major evolutionary related subfamilies which includes P1B-ATPases (heavy metal ATPases), P2A-ATPases (plasma membrane and ER-type Ca^{2+}-ATPases), P4-ATPases (putative aminophospholipid ATPases), P5-ATPases (a branch with unknown specificity), P3A-ATPases (H^{+}-ATPases in plants and fungi and Na^{+}/K^{+}-ATPases in animals) (Baxter et al., 2003, Rosewarne et al., 2007, Duby and Boutry, 2008, Axelsen and Palmgren,
2001). The amino acid sequence that includes the phosphorylated aspartate residue of all P-type ATPases is very highly conserved (Palmgren, 2001) and it is known as the signature pattern of all the P-type ATPases (Palmgren, 2001, Axelsen and Palmgren, 2001). In addition to the domain that includes the phosphorylated aspartate there a number of very highly conserved domains. Phylogenetic analyses of the conserved regions of all P-type ATPases reveal that Arabidopsis harbors ATPases belonging to all the five major subfamilies (Axelsen and Palmgren, 2001).

P-type H^+-ATPases are generally found in plant and fungal cells, where they are localized in the plasma membrane and transport protons out of the cell and in doing this establish the cell membrane potential and a gradient for protons (Duby and Boutry, 2008). This group consists of approximately 11 to 12 very closely related members (66% identical amino acids) in higher plants. Plasma membrane H^+-ATPases (PM H^+-ATPase) have been examined and shown to have a C-terminal regulatory domain that includes a 14-3-3 binding site and a key phosphorylation site at the penultimate Thr residue, which is a common feature shared by all the members of this family (Axelsen and Palmgren, 2001). The plant PM H^+-ATPase is a functional 100 kDa monomer that can oligomerize to form dimeric and hexameric complexes (Duby and Boutry, 2008, Palmgren, 2001).

The crystallized structure of Arabidopsis thaliana plasma membrane H^+-ATPase isoform 2 (AHA2) has been examined by electron cryo-microscopy that yielded an 8-A resolution projection map and revealed a dimeric protein complex (Jhan et al., 2001, Duby and Boutry, 2008). The PM H^+-ATPase has ten transmembrane domains (TM1-
10) that encompass a small loop between TM2 and TM3 and a large loop between TM4 and TM5. The N- and C-terminal segments of the PM H\(^+\)-ATPase protrude into the cytoplasm. The regulatory C-terminal part of the protein acts as an autoinhibitory domain and is known as the R domain (Duby and Boutry, 2008). The other domains are known as A-, M-, P- and N-domains respectively. The A-domain or actuator domain consists of the N-terminal segment and the small loop. The M-domain corresponds to the membrane segment and P-domain is the phosphorylation domain, which is present in the large loop of the protein. The nucleotide binding domain is referred to as N-domain and located between the two parts of the sequence that forms the P-domain (Duby and Boutry, 2008).

One way in which activation of the PM H\(^+\)-ATPase is regulated is through phosphorylation of the universally conserved threonine residue at the C terminal end and concomitant binding of 14-3-3 proteins to the phosphorylated motif which, in turn, results in displacement of the C-terminal autoinhibitory domain (Olsson et al., 1998, Fuglsang et al., 1999). A crystal structure of the PM H\(^+\)-ATPase and 14-3-3 complex showed the binding of 14-3-3 protein to the phosphorylated peptide containing the last five amino acids of the carboxy terminus of PM H\(^+\)-ATPase (Wurtele et al., 2003). The binding of the 14-3-3 protein requires at least three residues of the carboxy terminal end of the PM H\(^+\)-ATPase (Fuglsang et al., 1999). 14-3-3 proteins are small soluble proteins and are encoded by comparatively large gene families in multicellular eukaryotic organisms (Rosenquist et al., 2001, Alsterfjord et al., 2004). In the Arabidopsis genome there are fifteen genes that encode 14-3-3 isoforms (Alsterfjord et al., 2004). As noted
above, the plant plasma membrane H\textsuperscript{+}-ATPase is also encoded by a large gene family. The large number of 14-3-3 and PM H\textsuperscript{+}-ATPase isoforms present in plant genomes implies that binding of 14-3-3 protein and PM H\textsuperscript{+}-ATPase may be isoform specific (Alsterfjord et al., 2004). The study by Rosenquist et al. (2000) shows large differences in affinity between the nine isoforms of Arabidopsis 14-3-3 protein and a phosphorylated peptide which represents the 14-3-3 binding motif in AHA2. Fusicoccin, a toxin produced by fungus Fusicoccum amygdale, binds to and stabilizes the activated H\textsuperscript{+}-ATPase/14-3-3 complex (Jahn et al., 1997, Oecking et al., 1997, Alsterfjord et al., 2004). In a study, Alsterfjord et al. (2004) exhibit that the main target of the 14-3-3 protein for binding, is the H\textsuperscript{+}-ATPase present in the plasma membrane and the distribution of 14-3-3 isoforms to the plasma membrane is affected by fusicoccin and treatment with fusicoccin increases the binding affinity of 14-3-3 to the PMH\textsuperscript{+}-ATPase. The PM H\textsuperscript{+}-ATPase can also be regulated independently of 14-3-3 proteins. An auxin binding protein which directly increases the activity of PM H\textsuperscript{+}-ATPase was found in rice. Furthermore, auxin binding increases the affinity of the auxin-binding protein for H\textsuperscript{+}-ATPase (Kim et al., 2001, Arango et al., 2003).

The PM H\textsuperscript{+}-ATPase genes can be activated by various abiotic and biotic environmental factors and, as a result, the amount of H\textsuperscript{+}-ATPase might be increased in a condition that requires high levels of active transport of solutes (Arango et al., 2003). It has been shown that blue light induces the binding of 14-3-3 to the guard cell PM H\textsuperscript{+}-ATPase and phosphorylation of the enzyme which precedes the opening of stomatal cells (Kinoshita and Shimazaki, 1999, Kinoshita and Shimazaki, 2002, Alsterfjord et al.).
al., 2004). Other external environmental factors which have been shown to have an affect on the activation of the proton pump are salt and cold acclimation (Gaxiola et al., 2007). However, some factors have been shown to inhibit the pump including fungal elicitors, low temperature and sugar depletion (Gaxiola et al., 2007). Additional factors including high aluminum, high sugar treatments and iron deficiency result in moderate changes of H\(^+\)-ATPase expression (Gaxiola et al., 2007). Although the PMH\(^+\)-ATPase activity is modulated by various physiological signals, the experimental evidence to demonstrate whether those factors change the amount of proteins or gene expression is incomplete (Gaxiola et al., 2007).

Activated PM H\(^+\)-ATPases create electrochemical gradients across the plasma membrane. The proton gradient generated by the pump is used to drive a range of secondary active transporters that couple the movement of protons down their electrochemical gradient to the movement of solutes including ions, sugars, amino acids, and hormones against their electrochemical gradients. In establishing the membrane potential and providing the driving force for secondary active transport, the plasma membrane H\(^+\)-ATPase is a key component in the control of transport processes in the plant, such as root nutrient uptake and xylem and phloem loading (Duby and Boutry, 2008). Moreover, in driving solute transport, the H\(^+\)-ATPase contributes to the control of water flux as solute transport is directly related to the osmotic water movement. By controlling the water fluxes across the cell membrane, the H\(^+\)-ATPase plays a critical role in turgor regulation that regulates the cell size (Morsomme and Boutry, 2000, Gevaudant et al., 2007). The pump is also involved in other important
physiological functions, such as stomata aperture opening, cell elongation and cellular pH regulation (Duby and Boutry, 2008).

In addition, the PM H⁺-ATPase has been proposed to mediate a broad range of physiological responses by maintaining the intracellular and extracellular pH through its translocation of H⁺. These physiological responses play a central role in the growth and development of plants. The most predominant response is auxin-induced growth and it has been stated that auxin-induced activation of the proton pump causes acidification of the cell wall which results in loosening of the wall, thus allowing turgor driven expansion of the cell as described in the acid growth hypothesis (Rayle and Cleland, 1970, Hager, 1971).

Despite the basic function of the PM H⁺-ATPase, which is coupling of ATP hydrolysis to the H⁺ pumping, the large diversity of physiological functions associated with this enzyme suggests that regulatory control of this enzyme is complex (Arango et al., 2003). A number of studies suggest that the PM H⁺-ATPase is regulated at multiple levels in addition to the control by 14-3-3 proteins mentioned above (Arango et al., 2003, Dubby and Boutry, 2007). The presence of multiple genes for coding various isoforms of the PM H⁺-ATPase suggests that the isoforms are regulated at the transcriptional level. An unusual leader sequence in the 5’ untranslated region of PM H⁺-ATPase and an upstream open reading frame that consists of 3 to 13 codons that appears in transcripts of various species, has been shown to contribute to regulation at the translational level (Arango et al., 2003). The H⁺-ATPase is thought to be regulated at both the transcriptional level and posttranscriptional level by auxin, a key growth
hormone, with its activation providing the acidification that causes cell wall loosening as described in the acid growth hypothesis (Rayle and Cleland, 1970, Hager, 1971, Gevaudant et al., 2007, Morsomme and Boutry, 2000).

Furthermore, through the proton gradient it generates, the PM H^+-ATPase energizes the secondary transport of Na^+ from the cytosol across the plasma membrane and so may play a role in the plant response to salt (NaCl) stress. Salinity (salt stress) is one of the major abiotic stresses severely affecting plant growth. Worldwide, salt affected land which is mainly due to natural salinity, accounts for more than 6% of the total land area and close to 20% of irrigated land (Li et al., 2010). Salinity causes plant cell damage by evoking osmotic stress, oxidative stress and ion toxicity, which ultimately results in cell dehydration, reduced turgor pressure, disruption of critical biochemical processes and impaired photosynthetic capacity (Li et al., 2010). Excessive Na^+ in the cytoplasm not only affects critical biochemical processes but also increases plasma membrane injury (Lv et al., 2008). For these reasons, the exclusion of excessive Na^+ which acts as a cytotoxic ion, from the cytosol, is an absolute requirement for the plant.

A range of mechanisms have evolved in plants in response to salt stress that include osmotic adjustment and reducing excessive Na^+ in the cytoplasm (Lv et al., 2008). The response of plants to salt stress involves processes from the cellular to the whole-plant level. Na^+ is transported from the cytosol into the vacuole and across the plasma membrane into the apoplast against its electrochemical gradient by Na^+/H^+ antiporters and this process is energy dependent (Kalampanayil and Wimmers, 2001,
Niu et al., 1993). Moreover, overexpression of a Na\(^+\)/H\(^+\) antiporter increases salt resistance in *Arabidopsis thaliana* and a salt resistance gene that encodes a Na\(^+\)/H\(^+\) antiporter has been identified (Apse et al., 1999, Shi et al., 2000 and reviewed in Kalampanayil and Wimmers, 2001). The activity of Na\(^+\)/H\(^+\) antiporter depends on the pH gradient or proton motive force that is generated by the H\(^+\)-ATPase across the plasma membrane (Niu et al., 1993, Duby and Boutry, 2008). Thus control of ion transport across the plasma membrane and tonoplast by the H\(^+\)-ATPase is likely a critical factor in salt tolerance.

Salt (NaCl) stress induces the expression of proton pumps present in both the tonoplast and the plasma membrane (Pons et al., 2011, Niu et al., 1993, Kalampanayil and Wimmers, 2001). Furthermore, a plasma membrane H\(^+\)-ATPase gene has been identified in rice that is closely linked to a locus responsible for increased salt resistance (Zhang et al., 1999, reviewed in Kalampanayil and Wimmers, 2001). Another study reveals that overexpression of the constitutively activated PMA4 gene present in tobacco (*Nicotiana tabacum*) plant resulted in greater *in vivo* proton pumping activity which in turn results in increased salt tolerance of the plant. Increased salt tolerance has been displayed by the transgenic plants during germination and seedling growth (Gevaudant et al., 2007). Extensive research has provided evidence that salt stress stimulates the accumulation of the PM H\(^+\)-ATPase transcripts in a variety of plants. As an example, accumulation of plasma membrane H\(^+\)-ATPase mRNA is induced in *Atriplex nummularia* cells by NaCl exposure. Moreover, NaCl induction of mRNA accumulation was detected in deadapted cells (cells previously adapted to NaCl) but not
in unadapted cells, indicating that adaptation enhances responsiveness of the PM H\(^+\)-ATPase gene expression to NaCl. This information provides evidence that the PM H\(^+\)-ATPase in \emph{A. nummularia} is regulated by NaCl at least in part by transcriptional or post-transcriptional processes that result in higher mRNA accumulation (Niu \textit{et al.}, 1993).

Plant H\(^+\)-ATPases are encoded by a multigene family as mentioned previously and organized into five subfamilies. The gene expression of several members of the PM H\(^+\)-ATPase in various species have been examined and revealed to have different cell or tissue specificities, sometimes with important overlap among isoforms (Gevaudant \textit{et al.}, 2007, Ewing and Bennett, 1994, Kalampanayil and Wimmers, 2001). There are eleven isoforms (AHA1 through AHA11) present in \emph{Arabidopsis thaliana} (Gaxiola \textit{et al.}, 2007). In \emph{Arabidopsis}, the promoter of the gene AHA3 has been fused to the reporter gene \textit{beta}-glucuronidase and its expression analyzed. The activity of this gene was found to be localized to phloem cells (Dewitt \textit{et al.}, 1991). Also in \emph{Arabidopsis}, transcripts of the gene AHA9 have been shown to be restricted to floral tissues (Houlne and Boutry, 1994). In \emph{Solanum lycopersicum Mill}, (tomato), the family consists of at least 10 differentially expressed genes (Ewing \textit{et al.}, 1990, Ewing and Bennett, 1994, Rosewarne \textit{et al.}, 2007, Kalampanayil and Wimmers, 2001). At least three of the ten tomato PM H\(^+\)-ATPase isoforms (\textit{LHA1, LHA2 and LHA4}) are expressed abundantly in the specific plant tissues (Ewing \textit{et al.}, 1990, Ewing and Bennett, 1994). However, a range of studies provide the evidence that expression of a subfamily is not restricted to a
particular organ and more than one isoforms are expressed within the same cell type (Gevaudant et al., 2001, Ewing and Bennett, 1994, reviewed in Arango et al., 2003).

Moreover, there is experimental evidence that some of these tomato genes are differentially regulated by the application of exogenous sugars, under high salt conditions and in mycorrhizal symbiosis, situations that require greater transport activity (reviewed in Rosewarne et al., 2007). The study done by Mito et al. (1996) has provided the information that both LHA4 and LHA2 mRNAs are induced by the addition of exogenous sugars and this induction appears to be dependent on sugar uptake and metabolism. Examination of one of the isoforms of the tomato PM H^+\text{-ATPase} LHA8 has revealed that LHA8 expression is induced in expanded leaves, unexpanded leaves and roots in response to salt stress. Usually LHA8 is not expressed at detectable levels in roots or expanded leaves and is present at very low levels in unexpanded leaves (Kalampanayil and Wimmers, 2001).

Previous work in Dr. Ewing lab at CSUS has determined the sequence of the LHA2 gene and the complete cDNA that are accessible at Genbank with accession no AF179442, (Bradshaw, Reviewed in Dani, 2007). The LHA2 gene consists of 22 exons, 2.3kb 5’UTR and 842 bp3’UTR (Figure1). The transcriptional start site is located in exon 2 and the stop site is located in exon 22. There are three GH3 auxin response elements (AuxREs) with the sequence TGTCTC present within the LHA2 promoter that have previously been shown to control genes in response to auxin (Ulmasov et al., 1995). In addition, rolB auxin response elements with the sequence ACCTTCA are present in the promoter. These were first identified in the oncogene rolB of the root
inducing bacterial plasmid of Agrobacterium rhizogenes (Baumann et al., 1999, reviewed in Dani 2007). Ro1B AuxREs occur four times in the LHA2 promoter, in which three of the Ro1B elements are inversely oriented. The characteristic feature of these AuxREs is that they cause up-regulation in response to auxin while they are within the gene and removal of these elements cause no upregulation of that gene in the presence of auxin (Ulmasov et al., 1995 and 1997).

Furthermore, Arabidopsis lines overexpressing the PM H\(^+\)-ATPase have been generated through the work in the Ewing lab (Tsuyada, 2007 and lee, 2007). This was accomplished by the insertion of a construct consisting of LHA2 coding region driven by CaMV (Cauliflower mosaic virus) 35S promoter. The LHA2 coding region has been truncated at the regulatory C-terminal end which should cause the protein to be constitutively active as has been demonstrated in studies in which the truncated pump was expressed in yeast (Gevaudant et al. 2007, Zhao et al., 2000). Overexpression of the PM H\(^+\)-ATPase gene caused an overall decrease in the germination rate and increased the growth rate (primary root length) and number of lateral roots of Arabidopsis thaliana (Boyce, 2012). Furthermore, overexpressing lines reveal changes in primary root length and lateral root density compared to the wild-type in response to natural auxin and polar auxin transport inhibitors (Boyce, 2012).
Figure 1: LHA2 gene structure
In addition, the \textit{LHA2::GUS} construct containing transgenic \textit{Arabidopsis} and tomato plants have been generated in which the reporter gene GUS (\textit{beta-glucuronidase}) has been fused to the LHA2 promoter (Ro, 2000 and Kirschke, 2000). The \textit{LHA2::GUS} construct drives GUS expression in similar patterns in both \textit{Arabidopsis} and tomato plants, with expression in shoot and root apical meristems, the root vascular cylinder, developing lateral roots from the first divisions of initiation, hypocotyls, the mesophyll of expanding leaves, hydathodes and throughout the vasculature of the leaf (Ro, 2000). Transformed \textit{Arabidopsis} and tomato plants treated with synthetic auxins as well as natural auxin reveals that the expression of \textit{LHA2::GUS} has broadened to include cortical cells and the entire vascular cylinder in roots. Quantification of GUS activity also shows that \textit{GUS} expression increased significantly with increasing levels of auxin (Ro, 2000, Dani, 2007).

In preliminary studies, the overexpressing (OEX) lines and GUS lines were tested for the effect of salt stress (Ferdousi, unpublished). \textit{Arabidopsis} plants overexpressing the pump showed increased growth and survival compared to wild-type in the presence of salt. \textit{LHA2::GUS} expression was inspected following histochemical detection and appeared to expand in roots and change during the development of leaves in response to increasing salt (NaCl).

The previous studies examining the role of the PM H\textsuperscript{+}-ATPase in plant response to salt along with the preliminary results of this study provide evidence that increased pump activity may enable plants to deal with salt stress more effectively. This study tested that. In addition, we examined the regulation of \textit{LHA2}, in response to salt (NaCl)
stress at the molecular level. This study will help us to understand the molecular mechanism of the physiological responses of plants to salt stress as well as the regulation of the activity of the PM H$^+$-ATPase in the plant. One way to identify physiological roles for the PM H$^+$-ATPase in response to salt is to determine the phenotypic changes that occur in plants in terms of root length, lateral root count and the pattern of expression of the PM H$^+$-ATPase isoform in different tissue types using reporter gene analysis.

**Hypothesis and Objective:**

Two hypotheses have been tested in this study. The first hypothesis is that overexpression of the PM H$^+$-ATPase gene enables the plant to tolerate salt stress better than wild-type plants. The second hypothesis is that the tomato H$^+$-ATPase isoform *LHA2* is regulated by salt during the plant’s response to salt stress.

To test the first hypothesis, *Arabidopsis* lines 3C and 6G that have been modified to overexpress the plasma membrane H$^+$-ATPase will be grown in the presence of different concentrations of salt. To characterize the effect of overexpression of the pump, primary root lengths and the number of emerged lateral roots (laterals) of overexpressing plants will be compared to wild-type plants across the range of salt concentrations. To test the second hypothesis, the expression of the GUS reporter gene driven by *LHA2* promoter will be examined in response to a range of concentrations of salt by histochemical detection and Beta-glucuronidase (GUS) activity assays.
MATERIALS AND METHODS

Two independent *Arabidopsis* lines (6G and 3C) that were previously generated in Dr. Ewing lab were used in this analysis (Figure 2) (Tsuyada, 2007 and Lee, 2007). The lines are each homozygous for the overexpression construct that consists of the Cauliflower Mosaic Virus 35S promoter fused to a truncated LHA2 coding region in the pK2GW7 destination vector that was designated LHA2/pK2GW7 (Figure 2).

The LHA2::GUS construct and transgenic lines expressing this construct were prepared by former graduate students in Dr. Ewing lab (Ro 2000 and Kirschke, 2000). The construct was made through the fusion of 2.3kb of the promoter of the tomato PM H⁺-ATPase gene LHA2 with the reporter gene GUS (β-glucuronidase; Figure 3). Both the reporter gene construct and the overexpression constructs were introduced into *Agrobacterium tumefaciens* by direct transformation (Glover, 1985). The constructs were then transferred into *Arabidopsis thaliana* ecotype Columbia (col-0) through *Agrobacterium*-mediated transformation using the floral dip method as described in Clough and Bent (1998) and independent homozygous single copy lines were generated.

Growth media

Growth media for *Arabidopsis* plants was made according to Murashige and Skoog (1962) and consisted of 0.5x MS media (Murashige and Skoog, 1962), 1% (w/v) Sucrose, 0.5g/L MES (12-N morpholino-ethanesulphonic acid), 0.8% (w/v) Phytagar media, pH 5.8 (pH adjusted with KOH). The sterilized media were then poured into
square culture plates and left overnight under sterile conditions to solidify. The plates were wrapped in sterile plastic bags and stored at 4°C for future use.

**Salt treatment**

In order to prepare media with increasing salt concentrations, NaCl was added to the 0.5 x MS, 1% (w/v) media described above prior to autoclaving to yield final concentrations of 0, 50, 100 and 150 mM.

**Seed sterilization and transfer**

*Arabidopsis* seeds (typically 80-100 seeds per 1.5mL microcentrifuge tube) were incubated in 1ml of 100% ethanol for five minutes. This was repeated five times. The seeds were next incubated for 10 min in sterilization solution consisting of 20% (v/v) commercial bleach and 0.03% Tween (v/v). Following treatment, seeds were washed with 100% ethanol once. Seeds were then washed with sterile water three times and vernalized in sterile water in 1.5 ml tubes at 4°C for four days. After vernalization, *Arabidopsis* seeds were transferred to square plates containing 0.5 x MS, 1% (w/v) sucrose under sterile conditions in a laminar flow hood. The plates were sealed by the application of parafilm and were placed vertically at 22°C under a 16 hour light/ 8 hour dark cycle for 24-36 hours in a growth chamber until they germinated. Four days after germination, seedlings were transferred from starter plates to 0.5 x MS, 1% sucrose plates supplemented with 0, 50, 100, or 150 mM NaCl and to fresh control plates and incubated in growth chamber for four more days. Three replicates each with 16-18 of *Arabidopsis* seedlings at each treatment.
Figure 2: LHA2/p2KGW7 (35S::LHA2 construct)
Figure 3: LHA2::GUS construct
Measuring primary root length and determination of lateral root number

Primary root length was measured at the time of transfer when the seedlings were four days old. Primary root growth and number of emerged lateral roots were recorded at two days and four days following transfer. Primary root growth was calculated by subtracting the initial root length from the final root length. The density of lateral roots was calculated by dividing the number of lateral roots by the primary root length.

Histochemical assay

In order to test response of the LHA2 promoter to salt, seedlings were grown on media supplemented with NaCl as described above and stained to detect the pattern of GUS expression. A qualitative view of the expression pattern of LHA2::GUS constructs was seen under a microscope as a blue staining in various parts of the Arabidopsis plants. The intensity of the staining is proportional to the level of LHA2::GUS expression. Four to six plants were used for staining at each treatment and staining was done at the time when the plants were at two days and four days old following transfer to media containing added NaCl or control plates with no additional NaCl.

Histochemical detection of GUS expression was conducted according to Jefferson’s method (Jefferson et al., 1987). Whole seedlings were placed in 90% acetone and incubated on ice for thirty minutes. After incubation, seedlings were washed with 50mM phosphate buffer (pH 7.0) three times. Arabidopsis seedlings were then incubated in GUS staining solution containing 1mg/ml X-gluc (5-bromo-4chloro-3-indolyl-b-D-glucuronic acid; Bioworld inc.), 100mM ferricyanide, 100mM
ferrocyanide and 50mM phosphate buffer in double distilled water, at 37°C for 2 hours, 16 hours and 24 hours. Following staining, seedlings were rinsed with 50mM phosphate buffer (pH 7.0) and stored at 4°C until they were mounted onto slides.

Mounting on slides

Whole mounts of seedlings were prepared according to the method of Malamy and Benfey (1997) as described below. Parafilm, cut into rectangles, was folded in half. The cutting and removal of the inner part of the rectangles creates a well when placed on a microscope slide into which the plants were placed following the addition of approximately 100 µl of 30% glycerol to the well (Figure 4). Coverslips were applied and sealed in place with Permount. Slides were viewed for GUS expression using Differential Interference Contrast microscope or using an Olympus SZ60 dissecting microscope. Photographs of seedlings were taken with an Olympus DP11 digital camera mounted onto either of the two microscopes.

Beta-glucuronidase (GUS) activity assay

The activity of the p2.3LHA2::GUS gene construct was detected using a fluorometric assay as described by Jefferson et al. (1987) in crude protein extracts from LHA2::GUS containing Arabidopsis treated with a range of salt concentrations. Roots of Arabidopsis were collected and used for isolation of crude protein extractions. A colorless substrate 4-MUG [Methylumbelliferyl-beta-D-Glucuronide, FW-352.3] was added to the extracts and GUS converts the substrate, 4-MUG to the fluorescent product, 4-MU [4- Methylumbelliferone]. The amount of 4-MU produced in the reaction were measured using the Glomax plate reader (Promega Inc.).
Figure 4: Diagram showing mounted seedling on a microscope slide within a parafilm well
The activity of \textit{LHA2::GUS} in crude protein extract was then normalized by determining the amount of total protein present in crude extract. The Bradford protein assay procedure was used to determine the sample protein concentration (Bradford, 1976) in order to calculate the specific activity which is calculated here as \( \mu \text{mol MU} \) produced per minute per \( \mu \text{g} \) total protein. The GUS activity was determined in roots of the plants grown on treatment plates containing 0mM, 50mM, 100mM and 150mM added NaCl.

\textit{Sample preparation}

Root parts were collected from just below the hypocotyl-root junction in order to prepare the sample for GUS activity. After cutting, roots were placed in 1.5mL microfuge tubes and immediately placed on ice until sufficient samples had been collected to conduct assays. Twenty-two to twenty-four \textit{Arabidopsis} roots were pooled with each being treated as a single sample for each experiment. A single sample was divided into two aliquots in two microfuge tubes each containing eleven to twelve \textit{Arabidopsis} roots.

\textit{Fluorometric determination of LHA2::GUS activity}

Pooled root tips were then transferred to 1.5 ml sterilized microfuge tubes containing 0.5ml of GUS extraction buffer and immediately placed on ice. The components of GUS extraction buffer were as follows, 50mM NaHPO\textsubscript{4}, pH 7.0, 10mM Beta-mercaptoethanol, 10mM Na\textsubscript{2}EDTA, 0.1\% (w/v) Sodium lauryl sarcosine, and 0.1\% (v/v) Triton X-100. The root tissue was macerated with an eppendorf pestle in the microfuge tube until homogenized. The resulting mixture was then centrifuged for
fifteen minutes at 14,000 rpm. After centrifugation, the supernatant was collected into a microfuge tube for immediate use or stored frozen at -80°C.

Activity assays were conducted by transferring 10 µl of the plant extract from each sample to wells of a 96-well microtitre plates in triplicates. Following transfer, 100 µl of prewarmed GUS assay buffer (2mM 4-MUG{Methylumbellyferyl beta-D-Glucoronide FW=352.3} in GUS extraction buffer) was added and immediately after adding the GUS assay buffer, 190 µl of stop buffer (0.2M Na₂CO₃ with filtered ddH₂O) was added to each well. This provided the triplicate readings at time zero for each experiment. The plant extract samples were maintained on ice while conducting the assay. The same assay procedure was followed for the thirty minutes and sixty minutes readings (following incubation at 37°C, stop buffer was added at thirty minutes and sixty minutes respectively).

GUS activity converts 4-MUG to 4-MU (4-methylumbelliferone) as described above. The amount of 4-MU produced in the reaction is indicated by the fluorescence reading provided by Glomax plate reader. For each set of measurements, the plate reader and incubator were prewarmed for thirty minutes. Fluorescence was measured and recorded for each well containing reaction mixture samples in black 96-well microtitre plates. The background readings were provided by the fluorescence readings taken at zero minute and were subtracted from the readings at thirty and sixty minutes to get the reading of 4MU.
Determination of protein concentration in crude extracts

In order to determine the sample protein concentration, the Bradford protein assay procedure was used using BSA (Bovine Serum Albumin) as a standard with standard curves generated for each assay with freshly prepared BSA stock solutions.

Generating a BSA standard curve

A 96-well microtitre plate assay procedure was used to generate standard BSA curve and to determine the sample protein concentration in crude plant extracts. In order to prepare the standard curve, BSA standards of 0, 4, 8, 12, 16, 20 and 30 µg were prepared in triplicate by taking 0, 2, 4, 6, 8, 10 and 15 µl aliquots of the 2mg/ml BSA stock solution to a final volume of 200 µl with 40 µl of Bradford reagent (Bio-Rad, Inc) and distilled water. The absorbance at 600 nm of known concentrations of BSA was used to generate a standard curve.

Protein assay of tissue sample

Appropriate volumes for crude protein extract samples were determined to avoid high absorbance readings that may be out of range from the standard curve. Each volume of extract samples was measured into 96-well plates in triplicate. Bradford reagent (40 µl) was added as a colorimetric reagent and double distilled water was added to adjust the final volume of 200 µl. The absorbance at 600nm was measured using the Glomax plate reader. Clear microtitre plates were used for both generating BSA standard curves and for determining the amount of protein present in the sample. Specific GUS activity was calculated at thirty minutes and sixty minutes using the following equation-
Specific activity = (X units MU/Y minutes) x (Z µg protein in sample).

Where, X units MU is the fluorescence reading of samples obtained after subtracting the zero minute values from thirty or sixty minute’s value. Y minutes are the time of incubation of the activity assay (either thirty or sixty minutes). Z µg protein in sample was determined by Bradford protein assay.

**Statistical analysis**

**Salt Treatment**

Two-tailed Student’s t-test and 2-Way ANOVAs with P<0.05 were used to determine whether overexpression of the pump causes increased plant growth on salt compared to wild-type controls.

**Beta-glucuronidase Activity (GUS) Assay**

Two-tailed Student’s t-test with P<0.05 were used to determine significance of the results of the GUS activity assay.
RESULTS

Effects of salt on root growth in *Arabidopsis*

Four day old *Arabidopsis* seedlings were transferred to treatment plates containing 0 mM (control), 50 mM, 100 mM or 150 mM NaCl. The seedlings were grown for four additional days in order to observe changes in primary root growth and lateral root number in response to the salt.

Primary roots of overexpressing lines grown on 0mM NaCl plates were significantly longer compared to the wild-type *Arabidopsis* seedlings at four days post transfer (Figure 5: Student’s t-test; t = 4.20, df = 126, P< 0.01 for 3C line, t= 3.90, df = 126, P< 0.01 for 6G line at 0mM).

Primary roots of overexpressing lines grown on 50mM NaCl containing were significantly longer than wild-type *Arabidopsis* seedlings at four days post transfer (Figure 6: Student’s t-test; t = 2.46, df = 126, P= 0.02, for 3C line and t = 4.56, df = 126, P< 0.01 for 6G line at 50mM).

The primary root length of overexpressing lines grown on 100mM NaCl were significantly greater than the wild-type *Arabidopsis* plants at four days post transfer (Figure 7: Student's t-test; t = 3.03, df = 120, P= 0.03 for 3C line, t = 4.06, df = 126, P< 0.01 for 6G line at 100mM).

Primary roots of overexpressing lines grown on 150mM NaCl containing plates were significantly longer compared to the wild-type *Arabidopsis* seedlings at four days post transfer (Figure 8: Student’s t-test; t = 6.63, df = 120, P= 0.02 for 3C line, t = 7.08, df = 126, P< 0.01 for 6G at 150mM).
In addition, ANOVA analysis across all concentrations of NaCl at four days post transfer data indicates that the difference in primary root lengths between all lines is significant (P< 0.01). For Genotype effect, F-calculated = 13, P=0.006 and the critical region for the F value at V₁ (Genotype) =2, V₂ (NaCl concentration) =3 degrees of freedom at the 0.05 significance level is 5.143. Therefore, the null hypothesis that the means are all equal and genotype has no effect on primary root length is rejected.

Increasing the amount of NaCl caused a significant reduction (Figure 5, 6, 7 and 8) in root length in both the OEX lines and wild-type Arabidopsis (ANOVA: P<0.01). For NaCl concentration; F-calculated = 165, P< 0.01, critical region for F value at V₁ (Genotype) =2, V₂ (NaCl concentration) =3 degrees of freedom and 0.05 significant level is 4.757. Therefore, the null hypothesis that the means are all equal and NaCl concentration has no effect on primary root length is rejected.

Two-way ANOVA analysis for average root growth (primary root length) shows that the primary root growth of OEX lines per day was significantly greater than wild-type Arabidopsis plant (Figure 9: Genotype effect, F-calculated=39.12, df= 2, 3, P< 0.01. NaCl concentration, F-calculated=370.79, df= 2, 3, P< 0.01).

Lateral root number (LRn) of wild-type Arabidopsis plants on 0mM, 50mM and 100mM concentration of NaCl were significantly higher than overexpressing Arabidopsis lines (Figure 11: Student’s t-test, t= 3.22, df= 94, P= 0.03 (2 days) for 3C at 0mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at 0mM, t= 3.54, df= 94, P< 0.01 (2 days) for 3C at 50mM, t= 4.12, df= 94, P< 0.01 (2 days) for 6G at 50mM, t=4.54, df= 94, P< 0.01 (2 days) for 3C at 100mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at 100mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at 100mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at 100mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at 100mM, t= 2.45, df= 94, P< 0.01 (2 days) for 6G at
100mM, $t= 4.21$, df= 94, $P< 0.01$ (2 days) for 3C at 150mM, $t= 3.92$, df= 94, $P< 0.01$ (2 days) for 6G at 150mM, Figure 13: $t= 2.141$, df= 94, $P= 0.035$ (4 days) for 3C at 0mM, $t= 3.12$, df= 94, $P< 0.01$ (4 days) for 6G at 0mM, $t= 4.939$, df= 94, $P< 0.01$ (4 days) for 3C at 50mM, $t= 4.253$, df= 94, $P< 0.01$ (4 days) for 6G at 50mM, $t= 4.101$, df= 94, $P< 0.01$ (4 days) for 3C at 100mM, $t= 5.606$, df= 94, $P< 0.01$ (4 days) for 6G at 100mM, $t= 2.71$, df= 94, $P< 0.01$ (4 days) for 3C at 150mM, $t= 5.980$, df= 94, $P< 0.01$ (4 days) for 6G at 150mM NaCl). Lateral root density (LRd) of wild-type Arabidopsis plants on 0mM, 50mM and 100mM concentration of NaCl was significantly higher than overexpressing Arabidopsis lines (Figure 12: t-test, $t= 2.64$, df= 94, $P< 0.01$ (2 days) for 3C at 0mM, $t= 3.38$, df= 94, $P< 0.01$ (2 days) for 6G at 0mM, $t= 3.04$, df= 94, $P< 0.01$ (2 days) for 3C at 50mM, $t= 3.44$, df= 94, $P< 0.01$ (2 days) for 6G at 50mM, $t= 2.23$, df= 94, $P< 0.01$ (2 days), for 3C at 100mM, $t= 2.34$, df= 94, $P=0.02$ (2 days) for 6G at 100mM NaCl, $t= 2.14$, df= 94, $P< 0.01$ (2 days) for 3C at 150mM, $t= 2.01$, df= 94, $P< 0.01$ (2 days) for 6G at 150mM, Figure 14: $t= 2.43$, df= 94, $P< 0.01$ (4 days) for 3C at 0mM, $t= 3.12$, df= 94, $P< 0.01$ (4 days) for 6G at 0mM, $t= 4.394$, $P< 0.01$ (4 days) for 3C at 50mM, $t= 4.253$, df= 94, $P< 0.01$ (4 days) for 6G at 50mM, $t= 3.77$, df= 94, $P< 0.01$ (4 days) for 3C at 100mM, $t= 5.606$, df= 94, $P< 0.01$ (4 days) for 6G at 100mM NaCl). However, lateral root number and lateral root density of wild-type Arabidopsis was drastically decreased in 150mM NaCl containing plates.

ANOVA analysis for lateral root count at two days after transfer shows that lateral root number is not significantly greater in wild-type compared to OEX lines (F-calculated= 3.784, $P= 0.074$, where, F-critical is 4.757). However, lateral root count at
four days after transfer shows lateral root number in wild-type is significantly greater than OEX lines (F= 10.66, P< 0.01, where, F-critical is 4.757). Therefore, the null hypothesis that means are all equal (i.e., there is no difference in number of laterals between OEX lines and wild-type) is rejected and genotype has an effect on the difference between lateral root number seen in OEX and wild-type lines.

ANOVA analysis of lateral root count data obtained at both two days and four days after transfer reveals that NaCl has significant impact on the difference of lateral root number between OEX lines and wild-type (where F-calculated=9.58, df= 2, 3, P< 0.01 for two days, F-calculated= 24.29, df= 2, 3, P< 0.01 for four days).
Figure 5: Primary root growth of *Arabidopsis* plants in response to 0mM NaCl. Four days post germination seedlings were transferred to media supplemented with 0mM NaCl and grown for four additional days. Primary root length was measured respectively at two and four days following transfer. The reported values are averages of 64 plants (+SEM) for each line. The primary roots of OEX lines at four days post transfer were significantly longer than wild-type.
Figure 6: Primary root growth of *Arabidopsis* plants in response to 50mM NaCl. Four days post germination seedlings were transferred to media supplemented with 50mM NaCl and grown for four additional days. Primary root length was measured at two and four days following transfer. The reported values are averages of 64 plants (+SEM) for each line. The primary roots of OEX lines at four days post transfer were significantly longer than wild-type.
Figure 7: Primary root growth of *Arabidopsis* plants in response to 100mM NaCl. Four days post germination seedlings were transferred to media supplemented with 100mM NaCl and grown for four additional days. Primary root length was measured respectively at two and four days following transfer. The reported values are averages of 64 plants (+SEM) for each line. The primary roots of OEX lines at four days post transfer were significantly longer than wild-type.
Figure 8: Primary root growth of Arabidopsis plants in response to 150mM NaCl. Four days post germination seedlings were transferred to media supplemented with 150mM NaCl and grown for four additional days. Primary root length was measured respectively at two and four days following transfer. The reported values are averages of 64 plants (+SEM) for each line. The primary roots of OEX lines at four days post transfer were significantly longer than wild-type.
Figure 9: Primary root growth of OEX lines and wild-type Arabidopsis plants per day in response to NaCl. Four days post germination seedlings were transferred to media supplemented with NaCl concentrations indicated above and grown for four additional days. Primary root length was measured when seedlings were two and four days old following transfer. The reported values are averages of 64 plants (+SEM) for each line. The primary roots of OEX lines were significantly longer than wild-type.
Figure 10: Primary root length of *Arabidopsis* plants in response to NaCl. Four days post germination seedlings were transferred to media supplemented with A (0 mM), B (50 mM), C (100 mM) and D (150 mM) NaCl concentrations and grown for four additional days. Primary root length was measured when seedlings were two and four days old following transfer. The reported values are averages of 64 plants (+SEM).
Figure 11: Average lateral root number in *Arabidopsis* plants (2 days) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with 0mM (control), 50mM, 100mM and 150mM NaCl and grown for four additional days. Lateral roots that emerged during the first two days following transfer were counted and marked to allow roots emerging following this period to be identified. The reported values are averages of 48 plants (+SEM) for each line. Lateral root number of OEX lines at each salt concentration, were compared with the wild-type at two days post transfer by student t-test and 2-way ANOVA.
Figure 12: Lateral root density in *Arabidopsis* plants (2 days) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with 0mM (control), 50mM, 100mM and 150mM NaCl and grown for four additional days. Lateral roots that emerged during the first two days following transfer were counted and marked to allow roots emerging following this period to be identified. The reported values are averages of 48 plants (+SEM) for each line. Student’s t-test (P<0.05) were significant between OEX lines and wild-type for all treatments.
Figure 13: Average lateral root number in *Arabidopsis* plants (4 days) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with 0mM (control), 50mM, 100mM and 150mM NaCl and grown for four additional days. Lateral roots that emerged during the last two days following transfer were counted and marked to allow roots emerging following this period to be identified. The reported values are averages of 48 plants (+SEM) for each line. Lateral root number of OEX lines at each salt concentration, were compared with the wild-type at four days post transfer by student’s t-test and 2-way ANOVA. The lateral root numbers of wild-type were significantly higher than OEX lines.
Figure 14: Lateral root density in *Arabidopsis* plants (4 days) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with 0mM (control), 50mM, 100mM and 150mM NaCl and grown for four additional days. Lateral roots that emerged during the last two days following transfer were counted and marked to allow roots emerging following this period to be identified. The reported values are averages of 48 plants (+SEM) for each line. Student’s t-test (P<0.05) were significant between OEX lines and wild-type for all treatments.
**Histochemical detection of expression pattern of p2.3LHA2:: GUS**

The expression pattern of *LHA2::GUS* changed during the course of exposure to different NaCl concentrations. In the primary roots of untreated (control) *Arabidopsis* plants, *LHA2::GUS* expression was highest in columella cells and the remainder of the root cap in the elongation zone of the root vascular cylinder. However, with increasing concentrations of NaCl, *LHA2::GUS* expression became more condensed in columella cells of root cap and also decreased in the elongation zone of the root vasculature tissue at 100mM and 150mM NaCl (Figure 25, 26, 27, 28 and 29).

In leaf, the expression of *LHA2::GUS* decreased with increasing of NaCl concentration. The highest expression was seen in control plants (0 mM NaCl) throughout the leaf and very little expression was observed in highest concentration of NaCl (150 mM NaCl). Expression decreased in the mesophyll and gradually became limited to the leaf vasculature system with increasing amount NaCl (Figure 22, 23 and 24).

In lateral roots, the expression of *LHA2::GUS* increased with increasing amounts of NaCl. *LHA2::GUS* expression was low in the root cap and vasculature tissue of young lateral roots at 0mM and 50mM NaCl and high in root cap and vasculature tissue of young lateral roots at 100mM and 150mM NaCl (Figure 16, 17 and 18). In addition, LHA2::GUS expression increased with increasing NaCl concentration in mature lateral roots (Figure 19, 20 and 21) and also it appears to be expressed more highly in mature laterals than young laterals at 50mM, 100mM and 150mM concentrations of NaCl.
Figure 15: *Arabidopsis* (*LHA2::GUS* line) root with key regions and tissues identified.
Figure 16: The expression pattern of LHA2::GUS in young laterals of Arabidopsis seedlings (2 days, 24 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM (control), (B) 50mM, (C) 100mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained for twenty four hours at 37°C.
Figure 17: The expression pattern of \textit{LHA2::GUS} in young laterals of \textit{Arabidopsis} seedlings (2 days, 2 hrs of staining) in response to NaCl. Four days post germination; seedlings were transferred to media supplemented with (A) 0mM (control), (B) 50mM, (C) 100mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained for two hours at 37\(^\circ\) C.
Figure 18: The expression pattern of *LHA2::*GUS in newly emerged lateral roots of *Arabidopsis* seedlings in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained for sixteen hours at 37° C.
Figure 19: The expression pattern of LHA2::GUS in mature laterals of Arabidopsis seedlings (2 days, 24 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained for twenty four hours at 37°C.
Figure 20: The expression pattern of \textit{LHA2::GUS} in mature laterals of \textit{Arabidopsis} seedlings (4 days, 16 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about sixteen hours at 37\degree C.
Figure 21: The expression pattern of LHA2::GUS in mature laterals of Arabidopsis seedlings (4 days, 24 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about twenty four hours at 37°C.
Figure 22: The expression pattern of \textit{LHA2::GUS} in mature leaves of \textit{Arabidopsis} seedlings (2 days, 2 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained about two hours at 37° C.
Figure 23: The expression pattern of \textit{LHA2::GUS} in mature leaves of \textit{Arabidopsis} seedlings (2 days, 16 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained about sixteen hours at 37$^\circ$C.
Figure 24: The expression pattern of $LHA2::GUS$ in mature leaves of $Arabidopsis$ seedlings (4 days, 24 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about twenty four hours at $37^\circ$ C.
Figure 25: The expression pattern of *LHA2::GUS* in primary root tips of *Arabidopsis* seedlings (4 days, 2 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about two hours at 37° C.
Figure 26: The expression pattern of LHA2::GUS in primary root tips of Arabidopsis seedlings (4 days, 16 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about sixteen hours at 37°C.
Figure 27: The expression pattern of *LHA2::GUS* in primary root tips of *Arabidopsis* seedlings (4 days, 24 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were four days old following transfer and stained about twenty four hours at 37° C.
Figure 28: The expression pattern of \textit{LHA2::GUS} in primary root tips of \textit{Arabidopsis} seedlings (2 days, 2 hrs of staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained about two hours at 37\(^\circ\) C.
Figure 29: The expression pattern of LHA2::GUS in primary root tips of Arabidopsis seedlings (2 days, 16 hrs staining) in response to NaCl. Four days post germination seedlings were transferred to media supplemented with (A) 0mM NaCl (control), (B) 50mM NaCl, (C) 100mM NaCl and (D) 150mM NaCl and grown for four additional days. Histochemical analysis was done when plants were two days old following transfer and stained about sixteen hours at 37\(^0\) C.
Beta-glucuronidase (GUS) activity assay

The level of expression of \textit{LHA2::GUS} was determined in activity assays of \textit{Arabidopsis} roots by measuring fluorescence produced in assays containing root extracts and GUS assay buffer carried out for thirty and sixty minutes at 37\degree C. GUS activity assays demonstrated that the expression level was significantly decreased with increasing concentration of NaCl (Figure 30: t-test; \(t=5.943, \text{df}=8, P<0.01\) for 50mM NaCl, \(t=7.709, \text{df}=8, P<0.01\) for 100mM NaCl and \(t=4.675, \text{df}=8, P<0.01\) for 150mM NaCl).
Figure 30: Relative LHA2::GUS activity of Arabidopsis roots. Four days post germination seedlings were transferred to media supplemented with NaCl and grown for four additional days. Values in the figure represent the means of two independent replicates and one pseudo replicate (+SEM). Each experiment carried in triplicates. GUS activity at 50mM, 100mM and 150mM NaCl concentrations were compared with 0mM NaCl at four days post transfer by Student’s t-test. Student’s t-test results were significant (p < 0.05) for each treatment compared to 0 mM NaCl.
DISCUSSION

In this study, in order to test the role of the plasma membrane H\(^+\) pump in salt tolerance, *Arabidopsis* lines which had previously been shown to be overexpressing the pump and wild-type *Arabidopsis* plants were analyzed for differences in primary root growth and lateral root initiation in response to salt (NaCl). In addition, a *LHA2::GUS* containing transgenic *Arabidopsis* line was used to analyze the expression of a GUS reporter gene driven by the LHA2 promoter to examine the regulation of *LHA2* in response to salt. In order to test the response of the LHA2 promoter, seedlings were treated with NaCl and subjected to histochemical GUS detection to determine the expression pattern of the LHA2::GUS gene and subjected to fluorometric assays to determine the level of GUS expression.

**Primary root growth and p2.3LHA2::GUS expression in primary root tips in response to increasing NaCl**

The results of this study reveal that with increasing concentrations of salt (50mM, 100mM and 150mM) root lengths of both overexpressing and wild-type lines decreased. However, across all concentrations primary root growth in overexpressing (OEX) *Arabidopsis* lines was significantly greater than in wild-type *Arabidopsis* lines.

Histochemical detection of *LHA2::GUS* expression in *Arabidopsis* GUS lines revealed expression of *LHA2::GUS* in the elongation zone of the primary root, a site in which cell expansion occurs at a high rate. *LHA2::GUS* expression decreased in the entire root cap area and elongation zone of the wild-type *Arabidopsis* with increasing NaCl concentrations. These findings suggest that there is a direct relation between the
activity of LHA2 and cell elongation. The increased primary root growth in overexpressing lines compared to wild-type lines also suggests that the activity of the proton pump (H^+ pump) is directly responsible for cell expansion and overexpression of the proton pump enhanced salinity tolerance in Arabidopsis. In a similar study, Bose et al. also observed that increased proton pump activity correlates with salt tolerance in Arabidopsis. The researchers used Arabidopsis lines that were overexpressing haem oxygenase (HO) and observed an increase in membrane potential and net H^+ efflux from the elongation root zone upon salinity (NaCl) treatment when compared with the wild-type and the HO knockout mutant. Furthermore, treatment with the P-type H^+-ATPase inhibitor vanadate abolished the NaCl-induced H^+ efflux in this HO overexpressing transgenic line which suggests that the observed increased H^+ efflux was mediated by a P-type H^+-ATPase (Bose et al., 2013).

While Bose et al. (2013) showed that activation of the H^+ pump contributes to salt tolerance, in our study LHA2::GUS expression decreases in salt treated roots. This suggests that LHA2 is not up-regulated at the transcriptional level in response to salt, however, it may be up-regulated downstream of transcription or, possibly, another isoform is regulated in response to salt. Moreover, the induction at mRNA level of isoform LHA8 in response to NaCl has been shown in a study conducted by Kalampanayil and Wimmers (2001) and increased level of LHA8 message accumulation has been seen in the expanded leaves, unexpanded leaves and roots. While LHA2 may not be up-regulated to provide salt tolerance, increased primary root growth of
overexpressing lines demonstrates that, at least by this measure, increased H\(^+\)-ATPase activity can provide increased performance under salt stress.

Furthermore, the results of our study support the Acid Growth Hypothesis. According to the Acid Growth Hypothesis, protons (H\(^+\)) secreted by the activated proton pump decrease the apoplastic pH (i.e. increase acidification of the cell wall) (Cleland and Rayle, 1978, Hager, 2003). The acidification of cell wall then causes cell wall loosening (Serrano, 1989), allowing turgor driven cell expansion (Rayle and Cleland 1977, Rayle and Cleland 1992, Hager, 2003). If the proton pump functions in this way, then roots of overexpressing plants should elongate more rapidly than wild-type lines, as was observed in this study.

**Effects of NaCl on lateral root growth in wild-type compared to the overexpressing Arabidopsis lines**

Lateral root initiation (LRI) is an essential process in the formation of the root system, which is crucial for the nutrient uptake and water-use efficiency in the plant. This developmental process occurs throughout the life-span of a plant and is a key determinant of root system architecture that contributes to the ability of the plant’s to adapt to changing soil environments. In *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), the first emerged primary root does not contain lateral root primordia. The cells within the xylem-adjacent pericycle layer of the mature root are activated by various signals to begin to divide in initiating a lateral root primordium which is the primary event in the formation of a new lateral root. The newly formed lateral roots then elongate and undergo further branching to allow the plants to utilize the soil resources and provide underground support for the plant (Dubrovsky *et al.*, 2009).
Initiation of a new lateral root is regulated by a plethora of external and endogenous signals (Malamy, 2005). Soil conditions, including availability of nutrients, are external factors that are perceived by the plant and contribute to the control of lateral root initiation (LRI). Also, in response to these environmental signals auxin and other plant hormones control LRI process (Malamy, 2005, Dubrovsky et al., 2009).

In this study, wild-type Arabidopsis (control) plants and Arabidopsis plants overexpressing the tomato proton pump were transferred to medium with a range of concentrations of NaCl to test the effects of increasing salt concentration on lateral root development in overexpressing lines compared to wild-type. In wild-type Arabidopsis, lateral root number (LRn) and lateral root density (LRd) are higher than in OEX lines except at the highest concentration of NaCl (Figure 11-14). If activation of the H\(^+\) pump contributes to lateral root initiation then an increased number of lateral roots would have been expected to be observed across all concentrations of NaCl--which was not the case here. In fact, fewer lateral roots were observed at 0, 50 and 100 mM NaCl while there was no significant difference at 150 mM (P = 0.0491). The reduction in the number of laterals could have resulted from a change in hormone transport as a result of overexpression of the H\(^+\) pump or, possibly, some other effect such as changes in expression of other isoforms.

In Arabidopsis, at least eleven isoforms of the plasma membrane proton pump have been identified and more than one isoform is present in every tissue or organ type. H\(^+\)-ATPase isoforms differ in their regulatory and biochemical properties, for example the isoforms AHA1, AHA2 and AHA3 have been shown to differ in their affinity for
ATP and in the degree of activation by lysophosphatidylcholine (Palmgren and Christensen, 1994, Ewing and Bennett, 1994). We studied phenotypic effects caused by overexpressing the tomato proton pump LHA2 by treating with salt. We can’t rule out the possibility of the regulation of other isoforms that can be either down-regulated or up-regulated in a feedback response to the constitutive overexpression of the proton pump. It is possible that the concomitant regulation of the other isoforms in response to the overexpression of the H\(^+\) pump in *Arabidopsis* causes the lateral root growth difference between wild-type and overexpressing lines in response to salt.

**Expression of p2.3LHA2::GUS in lateral roots in response to NaCl**

Histochemical analysis demonstrates that the expression of *LHA2::GUS* is significantly increased in lateral roots with increasing salt concentrations. In 0mM and 50mM salt LHA2 promoter activity is detected in the pericycle founder cells of the lateral root primordia with little expression in adjacent layer of cells in young lateral roots. *LHA2* activity gradually increases with increasing concentrations of NaCl to 100mM and 150mM and is detected as highly expressed in columella cells of lateral root tip and the entire vascular cylinder of the elongation zone of emerged lateral roots. Since expression of *LHA2::GUS* begins very early in lateral root initiation we hypothesize that *LHA2* activity may play a role in lateral root initiation despite the fact that fewer lateral roots are observed in overexpressing lines compared to wild-type lines. Furthermore, *LHA2* activity gradually increases with increasing NaCl concentrations in mature lateral roots which suggests a role for *LHA2* in responding to
external environmental signals, in this case salt, across different developmental stages of the \textit{Arabidopsis} lateral root.

\textbf{Expression level of p2.3 LHA2::GUS in different NaCl concentrations}

The \textit{Arabidopsis} line containing \textit{LHA2::GUS} was treated with increasing concentrations of NaCl and the expression level of GUS was determined utilizing the GUS activity assays. GUS activity assays reveal that, overall in the root, the expression level of GUS driven by LHA2 promoter decreases with increasing NaCl concentrations. This result suggests that in the presence of high NaCl, LHA2 promoter activity decreases. Furthermore, the decreased activity of GUS correlates with the observation of decreased \textit{LHA2::GUS} expression in the elongation zone and root cap of the \textit{Arabidopsis} GUS line following histochemical detection. This result also correlates with the fact that overexpression of the proton pump causes increased primary root growth in overexpressing lines of \textit{Arabidopsis} relative to wild-type in response to salt.

Although in many studies salt stress or salinity has been reported to cause the decrease or inhibition of the PM H$^+$-ATPase, the activity of plasma membrane proton pump is often increased by the NaCl stress, for example NaCl stress increases the activity of proton pumps in cultured tomato cells (Kerkeb \textit{et al.}, 2001, Pons \textit{et al.}, 2011) and decreases the activity of membrane proton pumps in maize leaves (Zorb \textit{et al.}, 2005, Pons \textit{et al.}, 2011). These types of variations are related to the tissue type, developmental stages, experimental conditions as well as plant species (Pons \textit{et al.}, 2011).
In a recent study, overexpression of haem oxygenase (HO) was shown to increase the expression levels of PM H⁺-ATPase (*AHA1/2/3*) and modifies salinity tolerance in *Arabidopsis*. The combination of pharmacological experiments and gene expression analysis demonstrated that HO enhanced salinity tolerance via regulation of PM H⁺-ATPase and Na⁺/H⁺ antiporter (SOS1) (Bose *et al.*, 2013). In the wild-type *Arabidopsis* background *LHA2::GUS* is down regulated with increasing salt which demonstrates that *LHA2* is responsive to salt levels. It is possible that another isoform is up-regulated or that *LHA2* is activated downstream of transcription. It is also possible that since wild-type *Arabidopsis* does not tolerate salt well, no isoform is up-regulated and that up-regulation of the H⁺ pump can improve salt tolerance--as is suggested by the improved primary root length of the overexpressing lines relative to wild-type lines.
CONCLUSIONS

The maintenance of chemiosmotic circuits through the membrane of cellular compartments requires coordinated regulation of membrane transport proteins that contribute to the ability of plant cells to adapt to an altering environment and to grow. The purpose of this study was to determine the role of the predominant membrane protein, the PM H⁺-ATPase in the plant’s response to a major environmental factor, salt. Further, we tested whether overexpressing of the proton pump would improve the plant’s growth and ability to tolerate salt.

We have successfully used transgenic lines consisting of a non-native constitutively overexpressing proton pump to explore of the plant’s phenotypic changes during development in response to NaCl. In this study, continuously overexpressing the pump caused increased primary root growth with increasing NaCl compared to wild-type (control) plants. Primary root growth is considered to be a predominant phenotypic characteristic of plants growth and survivability. Therefore, our results suggest that H⁺ pumps play a critical role in the ability of plants to tolerate salt.

In recent work in Dr. Ewing lab, Robert Boyce used four independent transgenic Arabidopsis lines overexpressing the proton pump, including the lines used in this study, and analyzed root growth and lateral root initiation as was done in this study. He observed increased primary root growth in overexpressing lines compared to wild-type while grown on normal MS agar plates.
Furthermore, he observed increased lateral root numbers in overexpressing lines compared to the wild-type line when grown on simple MS agar plates. He also demonstrated increased pump activity since overexpressing lines caused a significant decrease in the pH of medium when grown overnight in liquid culture. From his work he concluded that cytosolic pH changes resulting from the activated proton pump may trigger lateral root initiation. Increased primary root lengths for all lines compared to wild-type supports the hypothesis that activation of H⁺ pump causes cell wall loosening allowing turgor driven expansion of the cell as described in the Acid Growth Theory.

In our study, a similar trend was observed for primary root length data, with overexpressing lines having increased root length compared to wild-type when grown on normal MS media and having greater primary root length than wild-type in the presence of various concentration of NaCl. However, we observed more lateral roots in wild-type Arabidopsis than in the OEX lines at all concentrations of NaCl including the 0 mM (control). If our results support the hypothesis that the H⁺ pump contributes to lateral root initiation then there should be more lateral roots in OEX lines than in wild-type. The difference in lateral root number in wild-type and OEX lines between our study and the previous studies may be due to a difference in media conditions or environmental conditions. It is also possible that changes in auxin transport resulting from overexpression could be a factor since auxin plays a critical role in lateral root initiation process and its transport is coupled to the H⁺ gradient.
Although our resulting data of the lateral root number in wild-type and overexpressing lines contradicts the findings of the previous study, the idea that the H$^+$ pump plays a role in lateral root initiation process is supported in part by the results of our study that show a difference in lateral root number across the range of NaCl concentration between wild-type and overexpressing lines (Figure 31). From our study the number of newly emerged lateral roots in OEX lines is fairly constant across the range of NaCl concentrations, in contrast, the number of newly emerged lateral roots in wild-type decreases significantly at 150 mM NaCl.

In addition, the analysis of the pattern of expression of LHA2::GUS in the primary root cap and the elongation zone shows decreased GUS activity in the tip of primary roots but GUS expression appears to increase in lateral roots. The observation that the expression pattern of LHA2::GUS changes in the primary root cap and other areas of the primary root as well as in lateral roots and leaves in response to salt supports the hypothesis that LHA2 is regulated by NaCl. Furthermore, these results shows that the activity level of LHA2::GUS is also changed with increasing NaCl which provides further support for the hypothesis that LHA2 is modulated by NaCl.

However, this study only provides insight into a portion of the big question of how the proton pump works in response to salt. Our study characterized the pump activity through the basic phenotypic analysis of the plant.
Figure 31: Average lateral root number in OEX line and wild-type Arabidopsis in response to 0mM (control), 50mM, 100mM and 150mM NaCl. Four days post germination seedlings were transferred to media supplemented with NaCl and grown for four additional days. Lateral roots that emerged during the first two days following transfer were counted and marked to allow roots emerging following this period to be identified. The number of lateral roots in OEX lines is fairly constant across the range of NaCl concentrations. The reported values are averages of 48 plants (+SEM).
Future study that includes biochemical analysis such as measurement of net H\(^+\) efflux and membrane potential will further characterize the H\(^+\)-ATPase activity in response to salt. Gene expression analysis such as qRT-PCR and RNA sequencing needs to be done to determine the differential patterns of expression and post-transcriptional regulation of each isoform of H\(^+\)-ATPase involved in salinity tolerance.

Moreover, histochemical analysis and GUS activity assay provides support for the hypothesis that \textit{LHA2} is regulated by salt and we conclude that it is regulated at the transcriptional level. The analysis of mRNA that includes qRT-PCR assay will provide additional insight into the regulation of individual isoforms and their roles in conferring salt tolerance.
Literature Cited


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