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8-2018

Identification and Characterization of Salinity Tolerance Genes by Activation Tagging in Arabidopsis

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Identification and Characterization of Salinity Tolerance Genes
by Activation Tagging in Arabidopsis

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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August 2018
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Abstract

Salinity often affects irrigated areas in arid and semi-arid regions of the world. The existence and accumulation of soluble salts in the soil layers limit the growth of crops essential for our food. Salt stress dramatically affects plant growth, plant development, as well as crop yield. *Arabidopsis thaliana* is the plant model that provides a comprehensive knowledge of plant development, genetics and physiology, and response to abiotic stresses such as salinity. The redundancy of genes due to duplication, even in the simple model genome of Arabidopsis, limits the value of knockout (KO) mutagenesis to provide complete information on gene function. 'Gain-of-function' mutants are an alternative genetic tool to identify gene functions for redundant genes, and those with small effect or that respond to an environmental condition. Transposon-mediated 'activation tagging' is an efficient genetic tool that can randomly generate 'gain-of-function' mutants for a large number of genes. In the method used here, the transposable element Enhancer-Inhibitor (En-I/dSpm) system of maize was modified to develop an activation tag (AT) mutant library in Arabidopsis. The mobile I-AT transposon contains a transcriptional enhancer, from the cauliflower mosaic virus (CaMV) 35S promoter, located close to the right border of the transposon. This I-AT element was mobilized to randomly insert into the plant genome by transposition from the T-DNA, and can give rise to mutants differing in the level of overexpression of the adjacent genes. Consequently, the gain-of-function dominant phenotypes generated are displayed by the I-AT plants due to enhanced expression of the gene(s) adjacent to the 35S enhancer. In this study, the I-AT library was used to screen for salt tolerance, identified by enhanced growth or biomass of the tagged mutants compared to the wild-type grown in saline conditions. A number of tagged salt tolerance candidate genes were identified flanking the I-AT insertion, and their tagged genes characterized for their role in salt tolerance.

Acknowledgements

First of all, I would like sincerely to thank my God "Allah" for his mercies and blessings and for making my dreams a reality. I would like to express my sincere gratitude to my advisor Dr. Andy Pereira for the continuous support of my PhD research over the past five years, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this dissertation. Besides my advisor, I would like to thank the rest of my advisory committee: Dr. Kenneth L. Korth, Dr. Daniel J. Lessner and Dr. Ainong Shi, for their wonderful contribution and support to the success of my study.

In particular, I would like to extend my deep thanks, gratitude and appreciation to my mom for always loving me, supporting my dreams, and being there for me when I needed her. I would like to express my appreciation to my husband and my kids for supporting me spiritually throughout my research.

A special thank you goes to Dr. Julie Thomas, for her patience and assistance in completing experiments in an appropriate manner. I would like to extend my deep thanks to my lab members: Ramegowda Venkategowda, Chirag Gupta, Anuj Kumar, Sara Yingling, Yheni Dwiningsih, and Bhuvan Pathak for their encouragement and support. I would like also to thank my sponsor King Saud University for providing the scholarship to obtain my degree. I would like to extend my gratitude to Cell and Molecular Biology program and University of Arkansas for giving me an opportunity to complete my PhD.

Dedication

This dissertation is lovingly dedicated to my mother Norah for her support, sincerity, encouragement, patience, prayers and mellifluous affections that inspired me to achieve my goal and pursue my dreams.

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Chapter 1

General Introduction

Introduction

Arabidopsis as a plant genetic system

The fact that plants are sessile make them important organisms to investigate the effect of the environment, due to their necessity to respond to environmental conditions by changes in their physiology. Some environmental conditions, such as temperature, nutrients and salinity have a substantial impact on plant growth and development. *Arabidopsis thaliana* is a dicotyledonous species that belong to Brassicaceae family. Arabidopsis is widely used as a model system to study the genetics of plant biological systems, as the species has advantages such as a short generation time, small size, a wide range of genetic and trait variations, and a high number of offspring, all of which make it unique for genetic studies (Shindo et al., 2007). The small genome of Arabidopsis makes it simpler to identify mutations and evaluate genomic responses to different experimental treatments. Moreover, determining the function of Arabidopsis's genes facilitates scientists to extrapolate the functions of many important genes in diverse plant species as well as crops. The Arabidopsis Information Resource (TAIR) database, developed at the Carnegie Institution of Washington in Stanford, California, stores and provides information for Arabidopsis research. Arabidopsis Biological Resource Center (ABRC) stores the germplasm and makes it available for research use (Garcia-Hernandez et al., 2002).

Abiotic Stress in Plants

Like all other kinds of living organisms, plants experience various environmental stress factors that affect their growth and survival (Rahnama et al., 2010; Quados, 2011). 'Abiotic stress' refers to any environmental condition that affects the ability of plants to develop, grow, and produce

below optimal levels (Rahnama et al., 2010; Quados, 2011). Abiotic stress, by itself, has the potential of reducing crop yields by as much as 69% which makes it an important challenge to plant growth (Bray, 2001). The major abiotic stress factors that affect plants include drought/desiccation, extreme temperatures, and high salinity of soils (Chinnusamy et al., 2004; Wood, 2005; Wahid et al., 2007; Mantri et al., 2012).

Water is essential for the growth and development of plants, and water deficiency in areas where plants grow typically result in the inhibition of plant growth and development (Boyer, 1982). Apart from water deficit, extreme temperatures are another abiotic stress that can affect the plants' ability to grow and reproduce. The most significant effect of high temperatures can be seen on plants still at the reproductive stage of development, by disrupting the pollination process that may sometimes lead to plant sterility or inability to reproduce (Hatfield and Prueger, 2015). In crops yielding grains, such as corn, high temperatures can lead to 80-90% yield reduction (Hatfield and Prueger, 2015). The main reason for this is that high temperatures shorten the duration for grain filling and formation among grain-yielding crops. Aside from extreme high temperature, extreme low temperatures can also have detrimental effect on productivity by hampering reproductive development. For example, extreme low temperatures can cause abortion of the formed grains and sterility in grain-yielding crops such as wheat (*Triticum aestivum*) (Uemura et al., 2006; Hatfield & Prueger, 2015).

High Salinity Stress in Plants

Stress related to high salinity levels in soil is recognized as one of the most severe abiotic stress experienced by plants. Stress caused by high salinity levels in soils is estimated to affect 20% of

overall cultivated lands and 33% of overall irrigated agricultural lands worldwide (Ghassemi et al., 1995; Gupta & Huang, 2014; Shrivastava and Kumar, 2015). This can be translated to as much as one (1) billion hectares of land being affected by high salinity levels in soil (FAO, 2008; Tanji, 2002; Metternicht and Zinck, 2003;). It is estimated that every year lands affected by high salinity levels in soil increase by 10%, and by the year 2050 it is expected that as much as 50% of overall arable lands worldwide will suffer from high salinity levels that would make agriculture more difficult (Shrivastava and Kumar, 2015).

Stress associated with elevated levels of salinity in the soil affects plants from all developmental stages: germination, vegetative growth and reproductive development. During the reproductive stage, salinity adversely affects plant development by inhibiting micro-sporogenesis and elongation of stamen filament, enhancing programmed cell death in some tissues of the plant, abortion of ovules, and senescence of fertilized embryos (Rahnama et al., 2010; Quados, 2011). Despite this, plants are generally more susceptible to high salinity stress during the seedling stage, after transplanting, and when exposed to other forms of stressors such as disease, insect infestation, and nutrient imbalance (Kotuby-Amacher et al., 2000). Once affected by high salinity stress, plants become stunted and their leaves turn dark green which may appear to be thicker and more succulent than what is normal for their type. In plants belonging to woody species, high salinity stress is associated with leaf burn and defoliation (Kotuby-Amacher et al., 2000). For example, alfalfa plants subjected to high salinity stress show reduced productivity and increased leaf-to-stem ratio which proves that high salinity has an influence on plants' forage quality (Kotuby-Amacher et al., 2000). Another example is grass, which appears stunted with a darker green color and leaf burn symptoms (Kotuby-Amacher et al., 2000).

Soil salinity is generally referred to as the total quantity of soluble salt that can be found in a certain soil, from a land mass or land area (Kotuby-Amacher et al., 2000; Provin and Pitt, 2001; Munns and Tester, 2008). The severity of soil salinity is measured according to salinity or "the salt concentration in soil solution" and sodicity or "concentration of sodium on the exchange complex of the soil" (Munns and Tester, 2008; Yan et al., 2015). The United States Department of Agriculture (USDA) has devised a system that classifies saline soils into three (3) categories: (1) saline, (2) sodic, and (3) saline-sodic (Yan et al., 2015).

Saline soils have a pH level lower than 8.5 and are sometimes referred to as "white alkali" and characteristically form white salt crust on their surface as they dry (Chapman, 1995; Provin and Pitt, 2001). Sodic soils, on the other hand, are soils that have characteristically high levels of sodium (Chapman, 1995; Provin and Pitt, 2001). Contrasting saline soils, sodic soils are referred to as "black alkali" due to the absence of white crusts that form on the surface as they dry (Chapman, 1995; Provin and Pitt, 2001). The high sodium levels in sodic soils are typically coupled with low levels of calcium and magnesium which result in the dispersion of clay particles that lead to the formation of structure-less soil with low water content and air permeability (Harivandi, 1984). The third kind of saline soil, the saline-sodic soil, is characterized by unstable pH levels that sometimes reach higher than 8.5 (Harivandi, 1984). Saline-sodic soils assume the properties of either saline or sodic soils depending on two conditions: (1) if the existing soluble salts remain while the levels of exchangeable sodium in the soil profile remains constant, the soil assumes the properties of saline soil but (2) if the existing soluble salts are leached downward while the levels of exchangeable sodium in the profile of soil remain constant, the soil assumes the properties of sodic soils (Harivandi, 1984).

Soil salinity can be measured by the "electrical conductivity extracted from a water-

saturated soil paste" (Kotuby-Amacher et al., 2000; Tanji, 2002). In saline soils, electrical conductivity (EC) is greater than 4 deciSiemens per meter (dS.m^{-1}) and the exchangeable sodium percentage (ESP) is lower than 15 (Harivandi, 1984; Chapman, 1995; Provin and Pitt, 2001; Munns, 2005). In sodic soils, on the other hand, the EC is less than 4 dS.m^{-1} and the ESP is higher than 15 (Harivandi, 1984; Chapman, 1995; Provin and Pitt, 2001). In cases of saline-sodic soils, the EC is greater than 4 dS.m^{-1} and the ESP is greater than 15 - a combination of traits found in saline and sodic soil types (Harivandi, 1984 Chapman, 1995; Provin and Pitt, 2001).

High levels of salts in the soil can accumulate when there is not enough water to leach the salt ions from the soil, which may happen in cases where there is insufficient precipitation and irrigation (Shrivastava and Kumar, 2015). In the hot and dry regions of the world such as India, the problem of high salinity levels in the soil is more pronounced (Shrivastava and Kumar, 2015). The lack of sufficient precipitation to compensate for the hot and dry climate, as well as the common practice of inadequate irrigation, all lead to secondary salinization of soils which significantly increase salinity levels (Shrivastava and Kumar, 2015). As explained by one source, a dry and hot climate leads to dried soil which has more concentrated salt content that further exacerbates salinity stress in plants (Kotuby-Amacher et al., 2000). This issue affects as much as 20% of overall arable lands worldwide (Ghassemi et al., 1995). In general, the effects of toxicity caused by high salinity levels in plants may be divided into two mechanisms: (1) disturbance in osmotic regulation, and (2) ionic toxicity.

Figure 1 shows the effect of salinity stress on the regulation of osmosis and ions in plants. During the osmotic disturbance caused by salinity stress, dehydration occurs which leads to the inhibition of water uptake, cell elongation, and development of leaves (Gupta and Huang, 2014). During ionic stress, the second phase of salinity toxicity in plants, potassium ions significantly

decrease while sodium ions rise excessively. This imbalance leads to toxicity which results in the rapid aging and dying of leaves, and impairment of photosynthetic ability, protein synthesis and enzyme activity (Greenway et al, 1972; Munns and Tester, 2008; Gupta and Huang, 2014). Signal transduction may take place during the early stage of osmotic and ionic stress which may either establish osmotic adjustment and ion homeostasis or cell death (Greenway et al, 1972; Munns and Tester, 2008; Gupta and Huang, 2014). In the event that the signal transduction successfully initiates osmotic adjustment and ion homeostasis, recovery or adaptation may be expected. However, if cell death occurs, then the chances that the plant recovers from the salinity-induced toxicity become significantly low. These two phases of plant's response to salinity stress is described in detail below.

Salinity-Induced Osmotic Disturbance

The mechanism by which high salinity levels affect plant growth is initiated by the occurrence of osmotic stress, as the first phase of response. The plant cells begin to strain in maintaining balanced osmotic adjustment after prolonged exposure to an environment that has exceeding levels of ions coming from high levels of salts (Munns and Tester, 2008; Gupta & Huang, 2014). Impaired osmotic regulation in plants is most likely caused by the abnormal levels of the phytohormone Abscisic Acid (ABA) which mainly functions in signaling stress among plants (Davies et al, 2005; Waidyarathne, 2015). ABA specifically functions by inducing stomatal closure as a way to regulate ions in order to release water from the plant's guard cell through osmosis (Waidyarathne, 2015). ABA may then be considered as a regulator osmotic activity in plant cells during exposure to abiotic stresses like salinity (Davies et al, 2005; Waidyarathne, 2015).

During the initial stages of high salinity stress, various physiological changes can be observed in plants which include interruption of membranes, nutrient imbalance, impaired ability to detoxify reactive oxygen species (ROS), alteration of antioxidant enzymes, reduced and altered photosynthetic activity, and decreased stomatal aperture (Sharma and Dubey, 2005; Tanou et al, 2009; Gupta and Huang, 2014). Interruption of cell membranes is largely attributed to excessive accumulation of sodium in the cell walls which leads to osmotic stress and even cell death (Shrivastava and Kumar, 2015). Nutrient imbalance, on the other hand, takes place since salts in the soil serve as important sources of nutrients for the plants (Shrivastava and Kumar, 2015). An upset in their balance, such as in the case of high salinity levels, the nutrients available for plants to absorb also become imbalanced (Shrivastava and Kumar, 2015). Impaired photosynthetic ability associated with high salinity stress is due to the decrease in leaf area, chlorophyll content and stomatal conductance, and reduced photosystem II efficiency caused by high salt levels (Sharma et al., 2012).

The main mechanism used by plants to recover from or adapt to the osmotic stress induced by high salinity levels is through the osmolytes and osmoprotectants (Hasegawa et al. 2000; Munns and Tester, 2008). Sugars, cyclic and acyclic polyols, amino acids and derivatives of amino acids, fructans, quaternary amino and sulfonium compounds are some of the known organic solutes capable of accumulating in the cells of bacteria, alga, and plants to regulate osmosis during stress (Hare and Xu, 1998; Munns and Tester, 2008). The compounds mentioned above are collectively known as compatible solutes or osmolytes due to their ability to accumulate in excessive amounts without impairing cellular functions (Cushman, 2001). Compatible osmolytes form massive units of compounds that function in restoring the osmotic potential of cytoplasm to facilitate water uptake and maintain turgor in the cell (Cushman, 2001).

Apart from such functions, compatible osmolytes are also capable of replacing the water surrounding proteins and stabilizing protein complexes and membranes (Cushman, 2001).

Salinity-Induced Ion Toxicity

The second phase of plant toxicity is characterized by the accumulation of ions and is known as the ionic phase which occurs due to the impairment of osmotic adjustment caused by the initial stage of toxicity (Munns and Tester, 2008; Gupta and Huang, 2014; Roy et al., 2014). The occurrence of ion toxicity shows that salinity stress is also a form of hyperionic stress (Gupta and Huang, 2014). Ion toxicity due to high salinity stress occurs when high concentrations of NaCl in the soil result in the excessive accumulation of Na^+ and Cl^- ions in the plant tissues exposed to the soil. The accumulation of Na^+ and Cl^- ions causes severe ion imbalance and physiological disorders (Munns and Tester, 2008; Gupta and Huang, 2014). Elevated concentrations of Na^+ ions can lead to inhibited uptake of K^+ ions which is necessary for plant growth and development (Gupta and Huang, 2014). Apart from sodium, chloride and potassium ions, high salinity stress can also cause imbalance of other ions in plants such as boron and calcium (Shrivastava and Kumar, 2015). Plants typically battle the surge of Na^+ ions in saline environments by adjusting and maintaining ion homeostasis (Sun et al., 2009).

Ion homeostasis during salt stress is enhanced through a variety of mechanisms such as the reduction of Na^+ and Cl^- ion levels and increasing concentrations of nutrition elements such as K^+ (Sun et al., 2009). Specifically, ion homeostasis is described as the restriction of sodium ion accumulation, hence, the Na^+ and Cl^- ions are generally reduced in plants exhibiting adaptive ion homeostasis activity (Ji et al., 2013). Ion homeostasis in plants is supported by various

signaling pathways, and in case of salinity stress the most common signaling pathway is the so-called *Salt Overly Sensitive* (SOS) signaling pathway (described below) that is responsible for exclusion of sodium ions from the roots as the first line of defense against toxicity and cell death (Zhu, 2000; Ji et al., 2013). However, prolonged exposure to high salinity levels may disrupt this pathway, eventually leading to the accumulation of sodium in the shoot (Zhu, 2000; Ji et al., 2013).

In the SOS pathway mechanism, salinity stress triggers significant increases in various ions (Gupta and Huang, 2014). Ca^+ ions, are among the ions significantly increased during salinity stress and the increase in their concentration mediates their penetration into the cytoplasmic region of the cell wherein they attach to the SOS3 protein which specifically binds with Ca^+ ions (Zhu, 2000; Quan et al., 2007; Huang et al., 2012; Gupta and Huang, 2014). Once Ca^+ ions bind to the SOS3 proteins, the SOS2 protein kinases are activated which leads to phosphorylation and triggers the activation of SOS1, a protein that is primarily located in the plasma membrane and functions in exchanging Na^+ and H^+ ions (Zhu, 2000; Quan et al., 2007; Huang et al., 2012; Gupta and Huang, 2014). This mechanism leads to the maintenance of ion or ion homeostasis. Overexpression of the Na^+/H^+ antiporter *SOS1* in plasma membrane in Arabidopsis caused improving in plant growth, number of seeds, chlorophyll content, as well as reduction in Na^+ content compared to control plants in salt stress condition (Shi et al, 2002). The mechanism of the SOS pathway is primarily activated once the salinity toxicity affects the regulation of ions in the cells.

Secondary Effects of Salinity Stress

Apart from the osmotic disturbance and ionic toxicity, plants exposed to saline stress can also experience secondary salinity toxicity, which affects different plant mechanisms such as the regulation of K^+ and the accumulation of ROS. Potassium ions play an important role in inducing salinity tolerance traits in plants. K^+ has the ability to control and maintain cell turgor and osmotic regulation during salinity stress (Wang et al., 2013; Chakraborty et al., 2016; Huang et al., 2017). However, during exposure to highly saline environments, K^+ in plants may significantly be reduced resulting in accumulation of ROS and cellular damage (Wang et al., 2013). Specifically, a low amount of K^+ in the plant cell cytosol may lead to the activation of caspase-like proteases which are responsible for triggering programmed cell death (Wang et al., 2013).

Apart from the disturbance of K^+ acquisition, salt-induced stress may result in the accumulation of ROS which is also partly triggered by the reduction in K^+ ions (Wang et al., 2013). ROS include the "singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals" (Tripathy and Oelmüller, 2012). There are various factors that contribute to the generation and accumulation of ROS in plant cells and among these are the abiotic stress factors including salinity stress. ROS are normally controlled and regulated by various enzymatic and non-enzymatic processes (Tripathy and Oelmüller, 2012). However, in cases wherein the ROS levels exceed normal, the mechanisms that regulate them are impaired and various cell damaging consequences may occur (Sharma and Dubey, 2005; Tanou et al, 2009; Tripathy and Oelmüller, 2012). High levels of ROS in the cell may lead to photo-oxidative damage to DNA, proteins, and lipids which eventually result in cell death (Tripathy and Oelmüller, 2012). Both, the reduction of K^+ ions and accumulation of ROS in plant cells, are secondary effects of salinity stress.

Improving Salt Tolerance in Plants

While alteration or remediation of the soil conditions is an agronomic solution of reducing salinity stress for plants, modern science is now looking into the ability of plants to tolerate highly saline conditions on their own. Since the 1930s, salt-tolerant plants have been known to exist and since this time, scientists have studied the inherent mechanisms of salt-tolerant plants in order to help salt-sensitive plants evolve or be developed to increase their survivability in saline conditions (Flowers, 2004). To date, there are three known ways by which a plant is able to tolerate saline environment: (1) osmotic tolerance, (2) ion exclusion, and (3) tissue tolerance (Munns & Tester, 2008; Roy et al., 2014).

Plants conduct osmotic tolerance by regulating long distance signals that limit and reduce shoot growth just before shoots accumulate Na^+ (Munns & Tester, 2008; Roy et al., 2014). Specifically, osmotic tolerance involves rapid, long-distance signaling and experts assume that such signaling happens through processes that include ROS waves, Ca^{2+} waves, and/or long distance electrical signaling (Roy et al., 2014). Osmotic tolerance capacity differs from plant to plant and experts assume that such variance is due to different long-distance signaling involved or different initial perception of the salt or different responses associated with the signals (Roy et al., 2014).

The ion exclusion mechanism that responds to the ionic phase of salt toxicity is more understood compared to osmotic tolerance. As mentioned earlier, plants respond to this salt toxicity phase by reducing toxic ions that have accumulated in their leaf blades through various signaling pathways such as the SOS pathway (Zhu, 2000: Ji et al., 2013). Apart from the said mechanism, salt-tolerant plants also manifest an ability to increase tolerance of salts that remain

in the shoot by compartmentation into the vacuoles (Li et al. 2006; Roy et al., 2014). Both mechanisms used by some plants to tolerate the ionic phase of salt toxicity involve transporters and their respective controllers at both cell membrane and tonoplast (Roy et al., 2014).

The third category of mechanism used by plants to tolerate saline environment is the so-called 'tissue tolerance'. Tissue tolerance mechanisms are generally carried out by removing Na^+ from the cytosol, and storing it by compartmentation in the vacuoles before the ions elicit an adverse reaction from the plant cells (Roy et al., 2014). Tissue tolerance mechanisms require "the synthesis of compatible solutes and higher level controls to coordinate transport and biochemical processes", resulting in osmoprotection and osmotic adjustment (Roy et al., 2014). Among the compatible solutes synthesized to enable tissue tolerance of salt ions are mannitol, ononitol, proline, glycinebetaine, trehalose, ectoine, and fructan all of which function in increasing hyperosmotic tolerance in plants (Nakayama et al., 2005).

The discovery of these three mechanisms involved in the plant cells' ability to tolerate high salinity levels in the soil is considered to be an important breakthrough in plant science. However, the need for more sophisticated methods to increase salt tolerance in plants is still increasing, since the mechanisms tackled above are still confined to salt-tolerant plants and most crops nowadays remain to be salt-sensitive, necessitating efforts to either alter salinity levels of their environment or modify their physiological functions in order to allow tolerance for highly saline environments.

Literature showing the ubiquity of the high salinity problem in large areas of arable land worldwide, presents the fact that the continuing efforts to change saline environments are most likely ineffective. Changing salinity levels in the soil through irrigation methods is highly

ineffective due to its high cost that makes it unsustainable, providing only a temporary solution to the problem. This leads experts to focus more on the plants' ability to tolerate saline environments and deliberately altering plants' physiological ability to tolerate high salinity levels in soil to improve their growth and production ability. Among the many methods being largely evaluated nowadays to improve salt tolerance among plants are by breeding for salt stress or genetic manipulation (Cushman et al., 2001).

Salt Stress Screening Methods

Screening methods for salt-tolerance traits and genetic resources are crucial in fighting the effects of soil salinity stress. Screening methods are necessary to identify specific genetic lines that are associated with salt tolerance traits compared to sensitive non-salt-tolerant lines (Arzani, 2008; Bhute et al., 2012). Effective screening methods can ensure the success of breeding programs as well as the speedy development of salt-tolerant genotypes of plants. Faster screening methods are particularly useful in determining potential parents for breeding salt-tolerant progeny of plants. Salt stress screening methods may be based on growth or yield, damage or tolerance to high salinity levels, and/or physiological mechanisms (Munns and James, 2003).

The screening methods based on growth or yield are focused on measuring root elongation, leaf elongation, biomass, and yield (Munns and James, 2003). Screens based on plant damage or tolerance to high salinity levels are focused on measuring leakage from leaf discs, chlorophyll content, and chlorophyll fluorescence (Munns and James, 2003). Screening methods focused on specific traits study measure Na^+ exclusion, K^+/Na^+ discrimination, and Cl^- exclusion

(Munns and James, 2003). Apart from these basal properties, screening for salt-tolerant crops may also be influenced by the types of environment within which the plants grow.

For plants selected at the germination or seedling stage, the traits determined by specific screening methods include rate of germination, seedling vigor, and dry matter (Arzani, 2008). Screening methods in the greenhouse conditions are focused on chlorophyll content, photosynthesis, stomatal conductance, leakage from leaf discs, leaf Na^+ concentration, leaf K^+/Na^+ ratio, leaf Cl^- concentration, biomass, yield, harvest index, and use of molecular markers for QTL identification (Arzani, 2008). Screening methods for plants grown *in vitro* are focused on traits that include somaclonal variants, induced mutations, and development of screening tools (Arzani, 2008).

Breeding programs and screening methods both heavily rely on the genetic analysis of plants and present technologies specifically target the genetic background of plants to develop novel salt-tolerance traits. Given this, it is important to examine the role of genetic variation and strategies in improving plant salt tolerance traits.

Genetic Strategies for Identifying Salt Tolerance Genes

Genetic strategies are one of the most favored techniques for improving salt and drought tolerance among plants (Winicov, 1998). Salinity tolerance is considered to be a quantitative trait at the genetic level and quantitative traits have profound influence on plant productivity (Winicov, 1998). Given this, methods used to genetically improve salinity tolerance traits of plants also affect their maximum yield potential. Oftentimes, strategies that genetically improve the salinity tolerance trait of plants have the consequence of lowering the plants' yield even

under normal conditions (Winicov, 1998). This complexity makes genetic strategies still largely ineffective in terms of directly improving salinity tolerance traits in crop plants. Despite this, genetic strategies are still considered useful in improving salinity tolerance traits in plants by functioning as detectors of salt-tolerance trait genes. Among the most common genetic strategies used for detecting salt-tolerant genes are the forward and reverse genetics.

Forward and Reverse Genetics

Forward and reverse genetics are two different strategies that enable thorough analyses of certain traits and functions in an organism. In forward genetics, organisms are treated to mutagens to induce random lesions in their DNA and general modifications in their genome to alter gene function (Ahringer, 2006; Lawson and Wolfe, 2011). The resulting mutant genotypes that may either display a targeted (based on screen) or random detectable phenotype are analyzed through standard molecular genetic techniques to locate the underlying gene mutation (Ahringer, 2006; Lawson and Wolfe, 2011). Forward genetics may therefore be described as a genetic strategy that works from the phenotypic level to the genotype, making it possible to identify the function of a gene sequence on the phenotype that they influence (Ahringer, 2006; Lawson and Wolfe, 2011).

The forward genetics approach always starts with the induction of heritable mutations in a population of the organism which is then expected to be passed onto their progeny for observation of detectable phenotypes that are linked to effect of the mutagenesis treatment (Lawson and Wolfe, 2011). N-ethyl-N-nitrosourea (ENU) is the mutagen of choice in most forward genetics procedures, although in some cases, radiation and insertional mutagens such as

retroviruses and transposons are more preferred (Lawson and Wolfe, 2011). After mutagenesis, genetic strategies are employed to monitor production and selection of progeny with the target phenotype of interest that can be attributed to the mutation induced on the parent line (Lawson and Wolfe, 2011). The forward genetics strategies depend on many experimental factors such as the phenotypes to be assayed and practical considerations such as space available, personnel, and total cost (Lawson and Wolfe, 2011). After phenotyping is performed to identify mutations associated traits of interest, molecular genetic analysis is performed to identify the gene sequence altered that is responsible for the mutation under study (Tierney and Lamour 2005). Apart from forward genetics, reverse genetics is also widely employed in determining particular traits in organisms.

In contrast, reverse genetics aims to determine the phenotype that arises from specific alterations in the genetic sequence (Tierney and Lamour 2005; Sessions et al., 2002). Reverse genetics makes it possible to systematically determine the functions of gene sequences and how alterations in them can affect the development and behavior of an organism (Ahringer, 2006; Sessions et al., 2002). Furthermore, reverse genetics makes it possible to investigate the function of an entire gene family as well as the function of a certain gene involved in a particular biological process (Tierney and Lamour 2005). Reverse genetics has been useful in determining genetic sequences and their corresponding functions in various model organisms such as Arabidopsis (Sessions et al., 2002).

A popular reverse genetics approach is the use of insertional mutagens, particularly the transposons a kind of transposable element (TE).

Transposable Elements

Transposable elements (TEs) are generally defined as DNA sequences that have the ability to move within the genome through a process called transposition, and may have an effect on the genome's function by changing gene expression (Muñoz-López and García-Pérez, 2010). TEs were first discovered by Barbara McClintock in 1940s during a study that analyzed the genome of maize (Fedoroff, 2012; Pray, 2008). Although TE are ubiquitously present in almost all life-forms, in plants like maize, TEs account for more than 80% of the genomic composition (Muñoz-López and García-Pérez, 2010).

Classes of Transposable Elements

TEs that occur in eukaryotic organisms are categorized into two classes depending on the transposition intermediate involved (Wessler, 2006). Class I involves TEs whose transposition intermediate is RNA and Class II involves TEs with DNA as their transposition intermediate (Wessler, 2006). Class I TEs are further categorized into two groups according to transposition mechanism and structure (Carnell & Goodman, 2003; Wessler, 2006). These two groups are the LTR retrotransposons and non-LTR retrotransposons (Ostertag and Kazazian, 2001; Eickbush and Malik, 2002). LTR stands for long terminal repeats and LTR retrotransposons are known to have these elements while non-LTR retrotranposons are remarkable for lacking them (Wessler, 2006). LTR retrotransposons function by being transcribed into RNA which then undergoes reverse transcription mechanism to produce a DNA copy that recombines with DNA in the genome (Ostertag and Kazazian, 2001; Eickbush and Malik, 2002). LTR retrotransposons are responsible for encoding of proteins necessary for the retrotransposition of Class I TEs (Ostertag and Kazazian, 2001; Eickbush and Malik, 2002).

Non-LTR retrotransposons, on the other hand, are responsible for encoding the reverse transcriptase and endonuclease proteins which are necessary for the mobilization of Class I TEs and other non-autonomous elements (Ostertag and Kazazian, 2001; Eickbush and Malik, 2002). Non-LTR retrotransposons are further divided into two superfamilies called the autonomous long interspersed elements (LINEs) and non-autonomous short interspersed elements (SINEs) (Ostertag and Kazazian, 2001; Weiner, 2002). LINEs are known for having an "internal promoter for RNA polymerase II, a 5' untranslated region (UTR), two open reading frames (ORFs), and a 3' terminal polyadenylation site" in their structures (Loeb et al., 1986). The two (2) ORFs in LINEs have different functions. ORF 1 is a protein known for binding to RNA proteins while ORF2 is known for encoding both the reverse transcriptase and DNA endonuclease (Loeb et al., 1986). SINEs, on the other hand, have an "internal promoter for RNA polymerase III and a 3' A-rich tract" in its structure (Ostertag and Kazazian, 2001; Weiner, 2002). SINEs measure from 80-400 bp in length and necessitate activities supported and encoded by autonomous retrotransposons and/or their mobility host (Ostertag and Kazazian, 2001; Weiner, 2002).

Class II TEs, on the other hand, have DNA as their DNA intermediate and function through mechanisms that are largely different from those that fuel Class I TEs (Muñoz-López and García-Pérez, 2010; Wessler, 2006). DNA transposons contain a transposase gene that is flanked by two (2) terminal inverted repeats (TIRs) (Muñoz-López and García-Pérez, 2010). The transposase gene relies on the TIRs to accomplish excision or movement of the transposable unit as it gets inserted into a new location within the genome (Muñoz-López and García-Pérez, 2010). During insertion, a few nucleotides of DNA as low as 3 at the target site are duplicated, leading to the formation of target site duplications (TSDs) which are a remarkable feature among DNA

transposons (Muñoz-López and García-Pérez, 2010). Like RNA retrotranposons, DNA transposons are also categorized into classes called families (Muñoz-López & García-Pérez, 2010).

The differences in DNA transposons families are based on the different sequences that determine the different kinds of DNA transposons (Muñoz-López & García-Pérez, 2010). Class I and II TEs have different modes of mechanism and motion within the genome which are influenced by the transposition intermediates involved in the structure (Fedoroff, 2012; Muñoz-López & García-Pérez, 2010). Class I TEs, with RNA as transposition intermediates, are generally termed as RNA retrotansposons and are known to move within the genome through a copy-and-paste mechanism (Ostertag and Kazazian 2001; Hacket et al., 2013). Class I TEs generate "double-stranded DNA intermediate from their RNA template that is then integrated into chromosomes by a mechanism similar to that used by DNA-mediated mobile elements (transposons)" (Ostertag and Kazazian 2001; Eickbush and Malik, 2002). Class II TEs, on the other hand, are generally known as DNA transposons and are described to move within the genome through a cut-and-paste mechanism (Smit and Rigg, 1996).

DNA transposons have various functions and one of these is their ability to inactivate or modify gene expression via insertion within introns, exons or regulatory regions (Muñoz-López & García-Pérez, 2010).

Applications of Transposable Elements

Since the discovery of their mobilization mechanism within the genome, TEs have become widely utilized in the field of biotechnology and medicine (Poćwierz-Kotus and Wenne, 2010).

For instance, transposons have an important role to play in the emergence of antibiotic resistance trait in microorganisms (Van Opijnen and Camili, 2013). This finding enabled experts to learn more about antibiotic resistance and how such can be remedied in cases of humans. But aside from biotechnology and medicine, TEs are remarkably useful in the field of genetics (De Lima Fàvaro et al., 2005; Poćwierz-Kotus and Wenne, 2010).

TEs are typically employed as natural tools used in genetic engineering (Vizvàryovà and Valkovà, 2004). Transposons act as mutagens producing mutations in the form of insertions (thus termed insertion elements), deletions, inversions, and translocations during their transposition, especially if they occur in more than one copy (De Lima Fàvaro et al., 2005; Vizvàryovà and Valkovà, 2004). Mutations generated by transposons alter the phenotypes since transcription of the original gene sequence is blocked and/or the transcription pattern is modified (De Lima Fàvaro et al., 2005). Given this, TEs are useful tools in deliberately inducing genotypic alterations in order to study gene sequences and the phenotypes associated with them (Kumar and Narayanan, 1998; Aarts et al., 1993). TEs may also be used in gene identification and cloning, and as genetic markers for specific genotypes originating from common genetic ancestors (Kumar and Narayanan, 1998; Aarts et al., 1993; De Lima Fàvaro et al., 2005).

Another important function of TEs is their ability to detect stress-related genes. Evidently, TEs often show increased activity under stress conditions (Capy et al., 2000; Grandbastien, 1998; Wessler, 1996), and may remain dormant otherwise, perhaps indicating a genome's adaptive response to stress. Given the role played by TEs in determining stress response in organisms exposed to abiotic stress, such as plants, they are a significant part of this study as will be shown in succeeding discussions.

Insertional Mutagenesis

Since their discovery, transposons have been widely utilized as insertional mutagens (Van and Camili, 2013). Insertional mutagenesis, as the name implies, is the process by which "insertional mutations" are induced into the genome through the use of viruses or transposons (Hackett et al., 2013). Mostly, the insertions that are located in promoter or coding regions of the gene cause mutant phenotypes. Considering the scope of this study, the discussion will be focused on insertional mutagenesis mediated by transposons.

Insertional mutagenesis with well-described transposable elements as tags was first used in Drosophila melanogaster (Bingham et al., 1981). The same approach was employed in plants after characterization of transposable elements of maize and snapdragon (Walbot, 1992). There are two kinds of insertion elements in plants that are commonly used: one is the T-DNA from Agrobacterium tumefaciens, and the other one is transposons. Both endogenous transposon and heterologous transposon have been employed effectively for tagging genes (Lightner and Caspar, 1998; Martienssen, 1998; Pereira, A., 2000). There are different types of inserts that function in Arabidopsis, such as T-DNA from Agrobacterium tumefaciens and retrotransposon Tnt1 element from tobacco (Feldmann et al., 1991; Grandbastien et al., 1992; Lucas et al., 1995).

A large number of genes have been characterized from populations of insertional mutants that were generated by T-DNA or transposons (Parinov and Sundaresan, 2000; Pereira, A., 2000; Sussman et al., 2000). However, there are different pros and cons of T-DNA or transposons. For example, the biggest advantage of using T-DNA is the stability, which does not exist when using transposons as a tag. T-DNA always acts as stable insertions when they are inserted in the plant genome, but it is unlikely that they can result in complex integration patterns or chromosomal rearrangements in the genomic DNA.

Alternatively, using transposons is more appropriate to implement targeted tagging (Speulman et al., 1999, 2000). Generally, transposon tagging is defined as a technique wherein the transposon is used to generate a DNA "tag" with a known sequence (Speulman et al., 1999, 2000). The transposon sequence is usually employed to detect DNA sequences near the TE (Speulman et al., 1999, 2000). TEs, particularly the transposons, can only be useful in functioning as DNA tags if the target sequence is known (Speulman et al., 1999, 2000). The known gene sequence is normally used to detect clones that contain mutant alleles that have their own transposons (Weigel and Glazebrook, 2002). The sequence adjacent to the insertion can be identified using different techniques based on the type of the insert that was used in the generation of mutants. Effective techniques for identifying sequences adjacent to the insertion can be performed in short time comparing to gene mapping such as inverse PCR, thermal asymmetric interlaced or TAIL-PCR (Deng et al., 1992; Pereira and Aarts, 1998; Liu and Whittier, 1995; Tsugeki et al., 1996). The identification of an unknown sequence adjacent to the insertion allows the identification of the position of the insert in the genome.

Knockout Insertional Mutagenesis Using T-DNA

Knockout insertional mutagenesis is one of the strategies commonly used to identify mutants by loss-of-function mutations, but is not able to reveal the redundant genes that are complemented by other genes or possess additional roles (Tani et al., 2004). In the process of knockout mutagenesis tagging, transfer DNA or T-DNA is widely employed. T-DNA is defined as the DNA transferred specifically from *Agrobacterium* species to plant genomes (Martineau et al., 1994). The transfer of T-DNA from bacterium to plant genome induces insertional mutagenesis,

and through this the T-DNA has become a useful tool that provides detailed analysis of the plant genomes by insertional mutagenesis (Kuromori et al., 2009; Martineau et al., 1994).

Aside from its ability to provide systematic analysis of the plant genomes, T-DNA is also popularly employed because it is easily produced or generated in large numbers (Kuromori et al., 2009). However, T-DNA mostly causes recessive phenotype and homozygous plants are necessary to detect if a mutant phenotype is caused by the insert or to another mutation arose through transformation (Marsch-Martinez et al., 2002). In addition, T-DNA insertions are considered to be too complex and the chromosomal rearrangements of T-DNA could lead to reversed configurations, such as multiple inverted or tandem copies or a truncated T-DNA insert, which might be problematic in analysis of adjacent genomic sequences to the insertion (Marsch-Martinez et al., 2002). Moreover, the frequency of T-DNA dominant morphological mutants is very low, typically appearing in 1 out of 1000 mutated plants (Tissier et al., 1999).

Because of these challenges with the use of T-DNA in activation tagging methods, our group has developed a method that incorporates transposable elements for transposon based activation tagging (Marsch-Martinez et al., 2002).

Activation Tagging Using Transposons

Apart from knockout tagging using T-DNA, activation tagging is another procedure commonly employed to study plant genomes. Activation tagging in plants is a novel gene isolation approach that was first proposed by Walden et al., 1994 and has been successfully implemented using T-DNA inserts (Kakimoto, 1996; Kardailsky et al., 1999; Borevitz et al., 2000; Weigel et al., 2000; Zhao et al., 2001; Marsch-Martinez et al., 2002) and applying Ac-Ds transposon system (Wilson

et al., 1996). The activation tagging method is used to observe genes whose functions cannot be identified by knockout insertional mutagenesis due to gene redundancy or for phenotype of genes whose expression is restricted to only specific conditions (An et al., 2005).

Activation tagging in Arabidopsis using transposons was initially demonstrated to be a novel gene identification method (Aarts and Pereira, 2000; Marsch-Martinez et al., 2002). Activation tagging methods using transposons is an effective vehicle that introduces transcriptional enhancer sequences and creates a powerful system that generates gain-of-function mutants (Marsch-Martinez et al., 2002; Robinson et al., 2009). In Arabidopsis, populations possessing a tetramer of cauliflower mosaic virus (CaMV) 35S enhancer are commonly used to screen mutants (Aarts and Pereira, 2000; Marsch-Martinez et al., 2002). The CaMV 35S enhancer's main function is to induce overexpression of closely present tagged genes, to reveal dominant gain-of-function mutant phenotypes (Marsch-Martinez et al., 2002; An et al., 2005; Robinson et al., 2009).

The maize *Ac/Ds,* and *Spm-dSpm* or *En-I* are the most frequently used transposable element systems for insertional mutagenesis in plants (Tissier et al., 1999; Marsch-Martinez et al., 2002). Initially, the transposon tagging Ac-Ds system was employed in Arabidopsis as a heterologous transposon system. However, the transposition frequency of introducing transposon-tagging Ac-Ds the first time was found to be very low, around 0.2-0.5% (Dean et al., 1992; Schmidt et al., 1995; Aarts and Pereira, 2000). Therefore, transposon-tagging Ac was not an appropriate method for gene isolation. On the other hand, the use of transposon EN-I activation tagging methods had a high success frequency rate which was about 10 in 1000 lines (Marsch-Martinez et al., 2002).

The *En-I (Spm-dSpm)* transposons based system of maize is the most common tool for transposon-based activation tagging, which was first used in tobacco and subsequently developed in Arabidopsis (Pereira and Saedler, 1989; Marsch-Martinez et al., 2002). The ability of the *En-I (Spm-dSpm)* system to transpose to unlinked locations and the high transcription frequency of its independent transpositions was observed when it was used with Arabidopsis, which was not seen in tobacco or when using the Ac transposon in Arabidopsis (Pereira and Saedler, 1989; Marsch-Martinez et al., 2002).

The *En-I (Spm-dSpm)* system exploits the *BAR* marker that confers plant resistance to the herbicide Basta, as well as the *SU1* marker, which converts the pro-herbicide R7402 into sulfonylurea which inhibits or reduces the growth of plants that contain it (Marsch-Martinez et al., 2002; Harb and Pereira, 2013). The first Arabidopsis genes that were isolated using En/Spm system were CER1 and MS2 (Aarts et al., 1993; 1995). Moreover, there are a number of genes that have been tagged and analyzed using the system, including the Arabidopsis HARDY gene (Karaba et al., 2007) for drought tolerance and water use efficiency, and the SHINE gene for regulation of wax biosynthesis (Aharoni et al., 2004) and lignocellulose regulation (Ambavaram et al., 2011).

Advantages of using activation tagging over knockout tagging

Activation tagging using the *En-I (Spm-dSpm)* system is said to be better than knockout tagging using T-DNA because the generation of gain-of-functions gene in activation tagging makes its mutagenesis more dominant, allowing the analysis of the function of duplicated genes (Weigel et al., 2000; Marsch-Martinez et al., 2002; An et al., 2005; Harb and Pereira, 2013). In addition, the

mutant spectrum of activation tagging systems have the capacity to generate novel and beneficial traits that could improve crops compared to the loss-of-function mutants used in knockout tagging (Weigel et al., 2000; Marsch-Martinez et al., 2002; An et al., 2005; Harb and Pereira, 2013). Another advantage of activation tagging using transposon over knockout tagging T-DNA is overcoming the redundancy problem and the high frequency of transpositions (Weigel et al., 2000; Marsch-Martinez et al., 2002; An et al., 2005; Harb and Pereira, 2013).

Given the pros of activation tagging using transposons, this will be the main procedure that will be employed in this study.

Scope of the dissertation

The main objective of this study is to identify novel salt stress tolerant genes by screening a number of Activation tagging lines from the model plant Arabidopsis using the *En-I* transposon system. In this study, we have identified genes from Arabidopsis conferring salt tolerance in Arabidopsis.

Objectives

1- Identification of Arabidopsis Activation tagged mutant lines for salt stress tolerance.

2- Characterization of genes for salinity tolerance identified from Arabidopsis activation tagged mutant lines.

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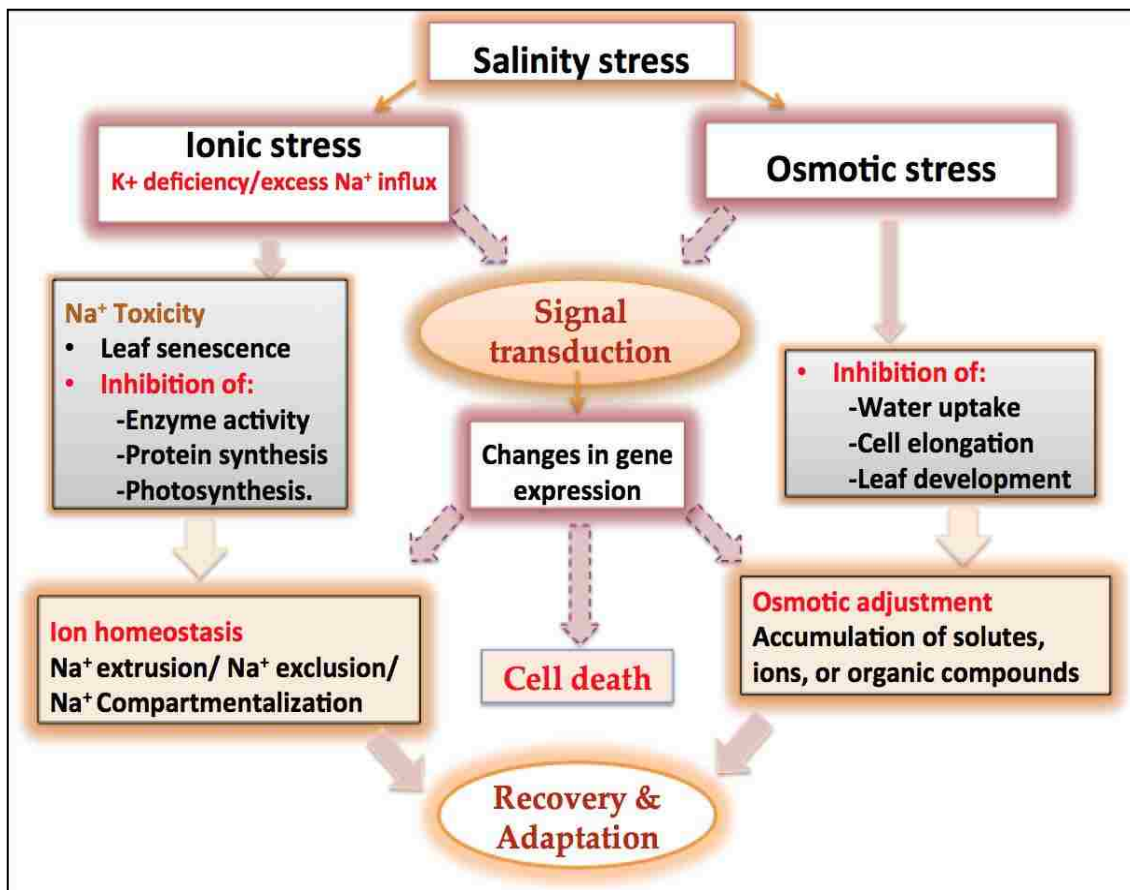
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Figure 1: Diagrammatic representation of the recovery/adaptation mechanism exhibited by most plants when exposed to salinity stress (Horie et al., 2012).

Chapter 2

Identification of Arabidopsis Gain-of-Function Mutants for Salt Stress Tolerance

Abstract

Agricultural production faces restrictions by abiotic stress factors such as high salt concentration in the soil, drought, heat and cold. Naturally occurring genomic and genetic variation undergoes selection by abiotic stress factors leading to evolution of stress tolerance mechanisms by selection in specific environments. *Arabidopsis thaliana* is a weed adapted to grow throughout the different climatic conditions of the world, and has enormous genetic diversity. To tap the latent diversity in Arabidopsis, independent lines of maize derived *En-I* transposon activation tagged (ATag) population of Arabidopsis plants were screened in a quantitative assay for salt tolerance. A salt stress treatment of 150 mM NaCl was applied for a week to 21 days old plants of 300 independent *En-I* ATag Arabidopsis lines grown in replications. This gain-of-function activation tagging approach enabled the identification of 15 lines with altered response to salt treatment, based on the evaluation of salt tolerance physiological traits. Two tolerant lines were systematically characterized at the genetic and molecular level for identification and characterization of putative tagged candidate genes involved in the altered salt tolerance response. These mutant lines can help identify new genes and mechanisms for salt tolerance that are likely naturally occurring as expression allele genotypes of genes in different natural populations, and help develop salt tolerant crops.

1.0 Introduction

There are a multitude of abiotic stresses, the most prominent being high salinity, drought, cold, and heat encountered by plants during their growth and development. Salt accumulation in arid regions, coastal flooding, poor irrigation and improper drainage facilities induce soil salinity stress, affecting around 21% of the world's irrigated land area (Ghassemi et al., 1995). The significant factors for high salinity are increased evapotranspiration and improper leaching leading to abnormal accumulation of soluble salts (the most soluble being sodium chloride) in the soil (Munns and Tester, 2008). A concentration of 40 mM NaCl, equivalent to the electrical conductivity of 4dS/m, is considered the ideal concentration for fertile soil (Munns, 2005).

Under high salinity conditions, ion imbalance takes place by disturbing the osmotic homeostasis in salt sensitive plants, which can be sensed rapidly. As a result, these plants are not able to manage an optimal ion transport ratio, which should be high potassium ions (100-200 mM) and low sodium ions (10-20 mM) for normal growth (Munns and Tester, 2008). Primarily, roots are affected by osmotic imbalances or water deficit created by high salt concentration which restricts nutrients entrance (Munns, 2002). Prolonged high salt soil exposure then leads to leaf necrosis, chlorosis, senescence and enzymatic degradation resulting in the loss of seed germination (Munns and Tester, 2008). This ultimately inhibits plant growth and causes losses in seed germination, plant height, fresh and dry weight during the growth stages, as well as reduction in crop yield, as documented for *Vicia faba* (L.) (Rahnama et al., 2010; Quados, 2011).

Plants have developed an innate ability to respond to stresses through responses that can be permanent through evolutionary adaptations, depending on the type and duration of stress. Tissue and cell specific responses during development involve signal transduction, hormonal

release, and others that confers structural, morphological, physiological, biological and molecular tolerance (Ahmad and Prasad, 2012). The basis of physiological adaptations induced by plants under stress can be found in molecular mechanisms such as osmotic adjustment during abiotic stress response following early signal transduction, diverse response pathways and their genetic regulation (Pereira, 2016). This offers tolerance through re-programming developmental, physiological and metabolic pathways in plants (Asensi-Fabado et al., 2017).

The high salinity environments can be combated by growing salt tolerant plants developed to have various tolerance mechanisms, such as by the exclusion of excess sodium ions from the cytoplasm, or their accumulation in vacuoles by overexpressing Na^+/H^+ antiporters (NHX1) (Munns and Tester, 2008). Genes such as the vacuolar H^+ translocating pyrophosphatase (AVP1) in Arabidopsis, have been found to pump excess sodium ions to vacuoles enhancing salt tolerance (Pasapula et al, 2011). Another inherent mechanism plants employ is to reduce dehydration losses, oxygen scavenging, and offering chaperone like activities by retaining water inside the cell through accumulation of osmoprotectants like sugars, organic acids, amino acids and amines (Valliyodan and Nguyen, 2006). The accumulation of osmotic solutes and activation of the antioxidant system are the first phase of defense employed by the plant for salt tolerance as explained in the previous chapter (Tang et al., 2015). The calcium binding proteins like calmodulins, calcium dependent protein kinases (CDPKs), calcineurin B-like proteins and calmodulin-like proteins are reported to be involved in ABA dependent and independent signaling during stress responses (Kader and Lindberg, 2010; Zhang et al., 2005).

Transcription factor families such as NAC, bZip, AP2/ERF, WRKY, and Trihelix have been well documented for their association with abiotic stresses in plants, including salt tolerance

in Arabidopsis and other crops such as rice, soybean, pea and maize, (Yamaguchi-Shinozaki and Shinozaki, 2006; Liu et al., 2007; Ambavaram et al., 2014)).

Salt tolerance features are exhibited by plants in the field, but can also be evaluated in the salt sensitive genotypes to identify the response to stress by genes that might be induced to confer stress tolerance. Such natural defense responses employed by the plants against salt stress can be either screened directly in the field or in the greenhouse by treating plants in hydroponics under optimal and high salt concentrations during different growth phases. There are many physiological factors that can be considered for salt screening: ion selectivity, ion accumulation, osmotic adjustment, organic solutes, and water use efficiency are commonly evaluated (Shannon, 1993). Ion selectivity is the ability of plants to maintain mineral nutrient ion balance and limit toxic ions, which is measured in salt tolerance screening whereas the accumulation capacity of sodium ions is termed ion accumulation. Osmotic adjustment is a measure of the increase in solutes, decreasing water and osmotic potential. Organic solute measurements indicate the accumulation of organic salts that maintain turgor pressure and render tolerance. High water use efficiency indicates the slowing down of salt accumulation in roots, offering salt stress tolerance. Physiological trait-based field screening studies have been performed in rice and maize by primarily analyzing the multiple phenotypic features for selection of salt tolerant mutants, or the analysis of natural variation for genome wide association studies (GWAS) (Pereira, 2016).

In the current study, quantitative assays were performed in screening for phenotypic changes such as growth and biomass accumulation in response to salt stress in Arabidopsis activation tagging lines using a forward genetic screen strategy.

Transposons are mobile pieces of DNA, first identified genetically in plants, that can move around the genome and can modify the regulation of genes, a principle referred to by Barbara McClintock as 'Controlling Elements' as agents that could modify gene activity or regulation (Fedoroff, 2012; Pray, 2008). Transposons can help in identifying genes in the host genome with the aid of forward and reverse genetics for genes that display a phenotype with altered expression. Transposons have been identified in drought tolerant maize as controlling 20% of the abiotic stress responsive genes (Makarevitch et al., 2015), suggesting their natural role in altering gene expression under stress (Pereira, 2016).

There have been many approaches described for the identification of genes for tolerance to abiotic stresses and this information has been used to improve the resilience of plants to stresses such as salinity. A widely popular classic genetic approach includes induction of loss-of-function mutations, which are important to describe genes required for expression of the trait or function. However, they are not able to unveil the contribution of redundantly working genes that are either complemented by other genes or regulatory circuits or possess additional roles (Tani et al., 2004; Krishnan et al., 2017). Activation tagging as a gene isolation approach, proposed first by Walden et al in 1994, circumvents these limitations aiding plants in unveiling their genomic potential through analysis of gain-of-function phenotypes, of redundant genes or those with minor effects on the trait, which is useful for the identification of stress tolerant gene candidates for use through transgenics (Kondou et al., 2010). Activation Tagging is a method of identifying a gene with an insertion, such as *En-I* (*Spm/dSpm*) transposon system that is inserted by transformation into heterologous plants such as Arabidopsis. Activation Tagging is used for identification of the function of the tagged gene on the basis of its enhanced expression that provides a dominant gain-of-function phenotype (Tissier et al., 1999; Marsch-Martinez et al.,

2002). There has been a shift from T-DNA insert application to transposon systems using the *En-I (Spm-dspm)* from maize was used initially by Marsch-Martinez et al. in 2002.

Targeted transposon based tagging systems overcome the shortcomings of T-DNA based system that show complexity in integration patterns and rearrangements in chromosomes (Marsch-Martinez et al., 2002). The *En-I* (*Spm-dSpm*) heterologous transposon from maize (*Zea mays*) was used in this present study, which was found efficient in generating independent transpositions and with transposing ability to unlinked locations (Marsch-Martinez et al., 2002). The tagging construct (Figure 1) was created by using two selectable markers, *BAR* (resistance to BASTA herbicide) and *SU1* (converts R7402 into herbicide sulfonylurea that restricts plant growth); a non-autonomous element (*I/dSpm*), an immobile transposase (*En/Spm* element minus terminal repeats) and a multiple copy 35S enhancer (Harb and Pereira, 2013). The marker BAR gene is within the *I/dSpm* element and the SU1 in the T-DNA insert so that the application of both herbicides renders the selection of plants with stable insertion elements. The strong enhancer within the *I/dSpm* enables activation tagging on a large scale and this entire unit along with BAR is designated as the activating I element (AIE) (Marsch-Martinez et al., 2002). The fact that there has been an advancement of technology is evident from the fact that the mutant selection through T-DNA activation tagging is maximum 1 per 1000 mutant lines whereas with *En-I* is 10 in 1000 lines (Marsch-Martinez et al, 2002).

The Arabidopsis genome has been explored widely by many research groups for identification of novel stress related candidate genes via T-DNA activation tagging. Zhao et al in 2001 have identified roles of the flavin mono-oxygenase family members in auxin biosynthesis. *Thread* was another gain-in-function mutant belonging to the same mono-oxygenase family that was identified via the En-I activation tagging system (Marsch-Martinez et al., 2002). A gain-of-

function mutation in Arabidopsis using the En-I ATag identified the *Hardy* gene i.e. AP2/ERF like transcription factor that renders Arabidopsis salt and drought tolerant, and also enhance biomass, photosynthesis and water use efficiency on transformation into rice (Karaba et al., 2007). Another such mutant *Shine* identified from the En-I ATag system renders drought tolerance by leaf structure modification with reduced stomatal density and significant increase in cuticular wax (Aharoni et al., 2004). Ahmad et al in 2015 have identified a salt tolerant line stc-1 (salt tolerant callus 1) that overexpressed the gene AT4G39800 (expressing myo-inositol-1-P-synthase-1 protein) by employing a T-DNA based activation tagging system on genome wide screening. *ORCA3* encoding a DNA binding domain in AP2/EREBP transcription factor that is involved in TIA pathway in *Catharanthus roseus* was identified using activation tagging and is categorized to act commonly during stress response actions (Tani et al., 2004). The drought tolerant rice line AH01486 was identified through T-DNA activation tag screening and was found to activate two glutamate receptor-like genes (Lu et al., 2014). It also offers tolerance to Arabidopsis plants against drought. Another rice mutant BPT-5204 was also identified via gain-of-function mutagenesis by over-expressing the transcription factor nuclear factor Y (NF-YC13) that offered salt stress tolerance (Manimaran et al., 2017). The presence of an mPing MITEs (miniature-Ping) transposon insertion at the 5'gene region up-regulated the nearby genes NAC gene of maize (ZmNAC111) that conferred drought stress tolerance (Pereira, 2016; Mao et al., 2015). The gene polygalacturonase involved in expansion2 or the *PGX2* gene is one of the genes in plants that was identified and characterized through activation tagging via transposons (Xiao et al., 2016).

An insertional activation tagging strategy has been employed in this research to screen the genetic variation among a population of activation tagged (ATag) mutant lines of

Arabidopsis to identify salt tolerant/sensitive lines in a quantitative assay in comparison to the salt sensitive wild type.

2.0 Objectives

The objective of this research is to identify novel genes for salt tolerance using a forward genetics strategy of activation tagging in *Arabidopsis thaliana*, a plant model for molecular genetics studies. Tolerance to abiotic stresses such as salinity is a quantitative trait, the phenotype being measurable and needs to be conducted on the basis of the phenotype of multiple plants, quantified by the difference of the tagged mutant being statistically different from the wild type.

3.0 Materials and Methods

3.1 Arabidopsis Transposon Activation Tagged Mutant lines

In previous studies the population of I-transposon ATag mutant lines were generated in Columbia (Col) ecotype plants that were transformed with the *Agrobacterium tumefaciens* T-DNA activation-tag construct (Marsch-Martinez et al., 2002). The transposable elements used were derived from the maize Enhancer (En)- Inhibitor (I) transposon system cloned and sequenced from maize (Pereira et al., 1985, 1986), and shown to transpose in heterologous plants (Pereira & Saedler, 1989). The activation-tag I-transposn (I-ATag) construct (Marsch-Martinez et al., 2002) has two greenhouse selectable markers: a 'positive' selection marker BAR (for Basta Resistance) on the mobile I-transposon that confers resistance to the herbicide Basta/glufosinate; and a 'negative' marker SU1 (O'Keefe et al., 1994), that converts the pro-herbicide R7402 to its active form (N-dealkylation), which reduces plant growth and can be identified by spraying R7402 (Dupont). Thus used for segregating out the active En-transposase on the T-DNA. This Arabidopsis population of stable transposed activation tag (I-ATag) mutant lines (Marsch-Martinez et al., 2002) generated from ecotype Columbia (Col) were obtained as T3 generation seed from the PhD research work of Dr. Amal Harb at Virginia Tech (Blacksburg, VA) and described previously in publications (Harb & Pereira, 2011; 2013). These lines had been selected for stable (non-transposing) I-ATag elements bearing the BAR gene for Basta resistance, that had transposed from the main construct bearing the En-transposase by selecting progeny that had segregated away the T-DNA bearing the En-transposase with the negative selection marker of the SU1 gene (Figure 1). These stable I-ATag lines therefore contain a stable I-transposon bearing 4 copies of the 35S promoter, that can effectively activate inserts as far as 10kb away (Marsch-Martinez et al., 2002)

In the present study, the Arabidopsis ecotype Columbia (Col) with the reference genome was used in all experiments as the wild type. The ATag genotypes were cataloged with their original names/numbering and additional numbers added from 1-300. For synchronous Arabidopsis seed germination, the seed of 300 Activation tagged lines were moist stratified in the cold at 4 °C in the dark for approximately 3 days. Next, 10 seeds for each line were sown in small pots filled with moist soil (professional growing mix) from Sungro Horticulture Company, and all trays of the pots were kept in the growth chamber under light (150 -200 μmole m^{-2} s^{-1}) at 22°C growth conditions (12 h of light and 12 h of dark). The trays were covered with clear plastic domes for 5 days, and the plastic covers were subsequently removed. Plants were fertilized once a week using the water-soluble fertilizer MiracleGro® All Purpose Fertilizer (24N-8P-16K).

3.2 Selection of BASTA Resistant Arabidopsis Activation tagged lines

After one week of germination, 10 seedlings for each pot were sprayed twice a week for 2 weeks with 0.7 mL/L Finale (Basta herbicide contains 150 g/L glufosinate ammonium). After five to seven days of the last spray, sensitive ATag lines could be identified and individual seedlings of the resistant seedlings were cautiously transferred into new pots with new soil. The genotypes of 106 ATag Basta resistant lines were divided into 6 batches systematically according to their number for salinity screening in batches.

3.4 Salt Stress Screen at the Vegetative Stage

Arabidopsis T3 generation seeds of the Col Activation tag genotypes and WT Col were stratified, grown, and treated with Basta herbicide for selection of the ATag insert as described above, and selfed seed was used for further analysis. For salt treatment, 21-day old seedlings at the vegetative stage were separated into two sets, one for salt treatment and the other a non-treated control set. The set of salt-treated plants were maintained in 150 mM NaCl solution for 7 days, while the set of the control plants were grown with normal watering. The plants of both sets were fertilized twice, prior to salt treatment for providing essential nutrients required for optimal growth. After 7 days of salt stress treatment, photographs of each genotype/plant were taken, and the individual plants were harvested and kept in the oven at 70 °C for complete drying. Next, the dry biomass of each plant sample was measured using a sensitive scale and recorded. The relative reduction in biomass (RB) was calculated using the following equation [(Biomass under control condition) – (Biomass under stress condition) / (Biomass under control condition)], and used as a measure of growth.

3.5 Phenotypic Screen of Arabidopsis Tagged lines for Salt Tolerance

Two groups of 14 AIE lines (440-B4-7, 440-F2-20, 440-G3-25, 440-B3-34, 440-F2-64, 440-G3-68, 440-H1-70, 440-A1-73, 441-E2-83, 441-E4-85, 441-G3-89, 440-G4-90, 440-H2-47, 440-D4-60) as well as the wild type Col0 with three replicates were used for the phenotypic screen for salt tolerance. The first group (at the vegetative stage): was treated with continuous salt application of 150 mM NaCl starting at day 21 at the vegetative stage. At day 21 after applying salt treatment, growth parameter measurements and data collection was initiated. The number of

leaves and diameter of rosette surface area was measured with a ruler every week until day 49. Every alternate day from day 21 until day 62, plants were scored to determine the percentage of bolting and flowering (Chan et al., 2013). The second group (at the flowering stage) was treated continuously with 150 mM NaCl starting at day 30 at the flowering stage. Measurements were made for plant height and number of stems (recorded at day 62). The samples were then harvested and dried in the oven at 70 °C to a constant weight to calculate the dry weight (DW), and kept further for ion analysis. At the end of the experiment, the dried plants samples were sent for ion content analysis (Dr. John Hatten, Laboratory ALTH 313, University of Arkansas).

3.6 Measurements of chlorophyll content

The chlorophyll content was measured in sample leaf tissue of 10 replicates of each genotype and wild type under control (H_2O) and salinity (150 NaCl) conditions after 10 days of treatment using SPAD-502 Plus Chlorophyll Meter (Konica Minolta; Tokyo, Japan).

3.7 Statistical Analysis

The data collected in this study were analyzed by the t-Test (Two-Sample Assuming Equal Variances). A 99% level and a 95% level of confidence were used in the study to determine the significance of differences between treatment and control at two levels of p-value (≤ 0.01 and ≤ 0.05). Analysis of Variance (ANOVA) using JMP version 12 was also performed to determine whether the different Arabidopsis genotypes had significant differences in their morphological phenotypic levels.

4.0 Results

The screening of the independent Arabidopsis activation tagged lines to salt stress treatment (150mM NaCl) during their early growth phase provided differences in genotypes to be identified. As a quantitative measure for plant growth and tolerance to salt, the reduction in biomass was adopted as a means to identify significant tolerant and sensitive lines. Based on the genetic analysis that will be shown in the next chapter, 14 significant mutant phenotypes with 7 tolerant and 7 sensitive Arabidopsis AIE genotypes were selected for physiological analysis of the following parameters number of leaves, rosette diameter, chlorophyll content, plant height and number of stems, in response to salt stress, at several time intervals ranging from three to seven weeks.

4.1 Selection of BASTA Resistant Arabidopsis Transformants

The results of seed germination of 300 Activation tagging T2 lines showed that after one week of growth 262 genotypes germinated and grew well to yield mature plants. T3 seeds were produced from all of the 262 T2 Activation tag lines. These plants were generated to harbor an activation-tag construct (Figure 1) as described by Marsch-Martinez et al. (2002). The activation-tag construct has two kinds of greenhouse selectable markers: positive marker BAR, that confers resistance to the Basta/glufosinate herbicide and the negative selectable marker SU1, that converts the pro-herbicide R7402 to an active form.

The Basta herbicide treatments on progeny seedlings of the 262 T2 A-Tag lines from multiple original transformants, as well as the wild type (Col-0), showed that the wild type seedlings and progeny from 59 of A-Tag lines were completely dead, while seedling progeny of

57 activation tagging lines segregated for resistance, and all progeny seedling of 106 A-Tag lines survived. The A-Tag lines that were dead after Basta treatment were untransformed lines (or undergone silencing or mutation). The lines that survived were transformants with transposed A-Tag inserts. Since all of the seedlings must have been BASTA resistant (dominant), these lines were selected for salt stress screening.

4.2 Screening of Arabidopsis Genotypes for Salt Tolerance at Vegetative Stage

The differences in biomass production under salt treatment and control conditions for an extended time period indicated genotypic differences that can be referred to the level of salt tolerance (Munns and James, 2003), and the current experiments are aimed at determining the same. The 106 Arabidopsis Activation Tag (ATag) lines were grown for a week and thereafter i.e. at 21 days after germination treated with moderately high 150mM NaCl concentration (Figure 2). The vegetative phase was chosen for the high salt stress application as the other stages, such as germination, are not documented to be tolerant enough. Subjecting the Arabidopsis genotypes to this salt concentration assured the selection of salt tolerant lines, while at the same time the sensitive lines would not be able to survive a prolonged exposure.

4.3 Arabidopsis Activation Tagged Lines- Biomass in Response to Salinity

Plant biomass is defined as an organic matter of green plants converting sunlight into plant material during the photosynthetic process (McKendry, 2002). Thus, plant biomass is one of the important factors as a basis for analyzing plant growth rate and for calculating net primary

production (Golzarian et al., 2011). In this study, the measurements were made based on the relative reduction in biomass by weighing all above-ground dry matter for both control and stressed plants at the vegetative stage using a quantitative analysis of biomass accumulation as an estimator of growth. For ease of analysis, the 106 Arabidopsis A-Tag lines were grown in six batches with control Col plants grown alongside. The plant dry biomass was measured for plants under control and under stress, then used to estimate the relative reduction of plant biomass (Figure 4 and 5). Based on the analysis on variance, there were significant differences found between wild type Col0 and the individual genotypes among the six batches of the 106 A-Tag lines in terms of relative reduction of plant biomass.

The results of the analysis of the relative reduction in biomass yielded 25 genotypes that were categorized as tolerant genotypes with lower relative reduction in plant biomass compared to the wild type Col-0. Subsequently, 15 genotypes were classified as sensitive with higher relative reduction in plant biomass as compared to the wild type Col-0, while the remaining ten of genotypes exhibited no significant difference between the A-Tag lines and the wild type.

It is evident from the analysis of relative reduction in biomass of all the batches that certain mutant lines were highly tolerant or sensitive while others were only moderately tolerant/sensitive compared to the wild type. The genotypes that were found to exhibit marked differences compared to the wild type being moderately tolerant were 440-C2-37, 440-H1-46, and 440-D1-57. However, most tolerant genotypes were found to exhibit high tolerance i.e. 440-B4-7, 440-C4-11, 440-E1-15, 440-G3-25, 440-B3-34, 440-B4-35, 440-H4-48, 440-G3-68, 440-H1-70, 441-E4-85, 441-G4-90, 441-H4-94, 441-D1-97, 441-A1-98, 441-A3-99, 441-B4-100, 441-D1-102, 441-H3-109, 441-H4-110, 442-C2-114, 412-D1-116, and 411-E1-172. Similarly most of the sensitive genotypes were highly sensitive towards high salt conditions and included

the genotypes 440-F2-20, 440-F2-64, 441-E2-83, 441-G3-89, 442-C1-113, 442-E3-129, 442-G2-131, 442-H4-138, 412-A3-189, 412-B2-192, 440-A2-2, 440-H4-72, 441-E3-84, and 412-C4-198 which displayed a significant reduction in the biomass. Only one sensitive genotype, 440-B4-52, was in the moderate sensitivity range.

4.4 Phenotypic Screen of Arabidopsis Activation Tagged Mutants for Salt Tolerance

The analysis of the salt tolerance parameter of relative reduction in biomass identified 14 ATag mutant lines from the primary phenotypic screen and physiological analysis (Figure 2 and 3). Several physiological factors that are significantly affected by salinity and are phenotypically evident in plant growth. The parameters were: the number of leaves and rosette diameters, bolting and flowering, plant height, and number of stems. The 14 ATag mutant lines identified in the phenotypic screen with altered response to salt are 440-B4-7, 440-F2-20, 440-G3-25, 440-B3-34, 440-F2-64, 440-G3-68, 440-H1-70, 440-A1-73, 441-E2-83, 441-E4-85, 441-G3-89, 440-G4-90, 440-H2-47, 440-D4-60. These include the sensitive Atag 440-F2-20, 440-F2-64, 441-E2-83, and 441-G3-89 along with ATag lines 440-H2-47 and 440-D4-60. Their biomasses were not significantly changed in response to salt and they were mostly similar to the wild type. The tolerant ATag genotypes identified are 440-B4-7, 440-G3-25, 440-B3-34, 440-G3-68, 440-H1-70, 440-A1-73, 441-E4-85, and 440-G4-90. The phenotypic analysis was carried out for the wild type and the 14 mutant lines by initially analyzing the plant growth parameters using three replications each for control and salt stress conditions during the vegetative stage, which enabled their characterization either as tolerant (resistant) or sensitive.

The phenotypic screen results show that in the absence of salt stress treatment, all plants were healthy during their life cycle with slight differences in some physiological parameters. Some AIE lines displayed varying phenotypes under normal growth conditions. The ATag lines 440-B4-7, 440-B3-34, 440-H1-70, 441-E4-85, 440-A1-73, and 440-G4-90 showed faster early growth, and these lines reached 6 true leaves (6TL) at around 14 days after sowing compared to the 4-5TL for the wild type Col0 and other mutant lines. This already indicates a difference in growth rate among the ATag lines, which indicates their superiority to the wild type parent. On the other hand, out of the four selected sensitive variants, two ATag lines, 440-F2-20 and 440-F2-64, had smaller plants than the wild type, with round small leaves and pale green color under normal conditions visible in the photographs of the plants. However, the sensitive genotypes 441-E2-83 and 441-G3-89 had healthy and tall plants under normal conditions similar to the wild type. Other lines had normally growing plants with appearance similar to the wild type under normal conditions and a very prominent reduction in growth parameters upon salt treatment.

4.4.1 Number of Leaves and Rosette Diameters

The wild type Col and ATag mutant lines exhibited significant visibly distinguishable changes in the number of leaves and rosette diameters under salt stress treatment compared to control non-treated conditions. Beginning with day 21 of the salt treatment the number of leaves and rosette diameters were the same for both control and stress treated plants in all of the selected mutant lines. Under normal control growth conditions, all the AIE lines and wild type Col0 showed a normal significant increase in the number of leaves and rosette diameters, from about four weeks until seven weeks of growth. However, the plants revealed marked alterations in response to

long-term exposure to salt stress through this same phase from Day 21 to Day 49. The differences begin to be evident from the third week of treatment and are most noticeable in the fourth and fifth weeks of treatment. The results of relative reduction on the number of leaves of ATag lines showed that there was a significant tolerance to salt stress in tagged lines 440-B4-7, 440-F2-20, 440-G3-25, 440-B3-34, 440-G3-68, 440-H1-70, 441-E4-85, and 440-G4-90. The ATag lines 440-G3-68 and 441-E4-85 440 were found to have the maximum number of leaves under high salt conditions. There was not a significant changed in the leaf number of the sensitive lines and their response to the salt stress as the wild type (Figures 6).

Some ATag lines also displayed salt tolerance with significant increase on their rosette diameter as being higher relative to the wild type. The high tolerance ability to salt stress treatment, by maintaining leaf growth for survival was shown by the Atags 440-B4-7, 440-G3-25, 440-B3-34, 440-G3-68, 440-H1-70, 441-E2-83, 441-E4-85, and 440-G4-90. The ATag 440-H1-70 showed the most tolerance, having the highest rosette diameter amongst all the genotypes under analysis. In sensitive ATag lines 440-F2-20, 440-F2-64, and 441-G3-89 the rosette diameter was dramatically smaller with salt treatment especially after two weeks of treatment compared to that of the wild type. The lines 440-H4-47 and 440-D4-60 had rosette diameters most similar to the wild type under normal and salt stress conditions (Figures 7).

4.4.2 Bolting and Flowering

The bolting and flowering percentage of the wild type were measured every other day from day 21 till day 62, and the figure were taken at day 42 (Table 1 and Figure 3). The results of the screening of three replicates of each AIE lines indicated that under control conditions the 3

replicates of ATag lines (440-B4-7, 440-B3-34, 440-H1-70, 440-A1-73, 441-E4-85 and 440-G4-90) bolted 100% at Day 28, and flowered 100% at day 35 which is about one week prior to the WT (Col-0). The other mutants (440-G3-25, 440-G3-68, 441-E2-83, 441-G3-89, 440-H2-47, and 440-D4-60) bolted and flowered 100% at day 35 which is mostly around the same time as that of the WT Col-0. However, the sensitive genotypes (440-F2-20 and 441-F2-64) exhibited an extremely later flowering phenotype than the WT, bolting around day 42 and flowering around day 49. Under continuous salinity stress, the results revealed that two replicates of ATag line 441-E4-85 bolted 100% at day 28 and flowered 100% at day 35 which is around the same time of this line under non-treated condition, and one week earlier than the other mutant lines in response to salt stress. The ATag lines (440-B4-7, 440-G3-25, 440-B3-34, 440-G3-68, 440-H1-70, 440-A1-73, 441-E2-83, and 440-G4-90) displayed moderate delay in bolting and flowering, one replicate of each line bolted 100% at day35 and flowered at day42. The wild type Col0 and ATag lines 440-F2-20, 441-F2-64, 441-G3-89, 440-H2-47, and 440-D4-60 were the most sensitive in terms of flowering in the continuous salt stress condition. These lines were not bolted or flowered at all and dead after two weeks of treatment with 150 NaCl.

4.4.3 Plant Height, and Number of Stems at the Flowering Stage

The height of the plants was further found to be significantly different in non-treated condition and salt stress condition for the wild type and ATag mutant lines (Figure 8). Noticeably, the relative reduction on plant height in most of the tolerant mutant lines under salt stress conditions were significantly higher than the wild type Col0. Characteristically, the tolerant genotypes 440-B4-7, 440-G3-25, 440-B3-34, 440-H1-70, 441-E4-85, 440-G4-90, 440-G3-25, and 440-B3-34

have the lowest relative reduction in plant height. The results also indicated that in comparing ATag lines to wild type, there was a significant decrease in plant height in the sensitive ATag line 440-F2-20 compared to the wild type Col0. For the seven remaining ATag lines, no significant change was observed in plant height compared to the wild type under salt stress condition and these lines maintained minimal reduction (Figure 8).

The number of stems in tolerant lines was characteristically more than the wild type in salt stress conditions. The stems decreased in number with continuous salt treatment (Figure 8). The plants with greater height borne relatively more stems, and the shorter ones had lesser stems. The effect of salt stress on the stems was even more prominent as there was a huge reduction in their number. However, there was a significant increase in number of stems in response to salt treatment compared to the wild type in ATag lines 440-B4-7, 440-G3-25, 440-B3-34, 440-G3-68, 440-H1-70, and 440-G4-90, and surprisingly also the sensitive genotypes 441-E2-83 and 441-G3-89 showed more stems number than the wild type. The sensitive ATag line 440-F2-20 showed a significant relative reduction in stems number compared to the wild type (Figure 9).

4.4.4 Chlorophyll content

The chlorophyll content of individual plants was measured using a SPAD meter (Figure 10). Under normal conditions, all the plants including the wild type, tolerant and sensitive mutant lines had chlorophyll content in the average range of 33 to 40 μmol/ m^2 of leaf area. After elongated and continuous exposure to moderate salt concentration (150 mM NaCl) during the flowering stage, there was a remarkable reduction in the chlorophyll content. The relative reduction on chlorophyll content was reduced by ~0.2 - 0.3 μmol/m^2 of leaf area in most of the

tolerant genotypes including 440-B4-7, 440-G3-25, 440-B3-34, 440-H1-70, 440-A1-73, 441-E2-83, 441-E4-85, and 440-G4-90. The wild type plant under salt stress had highly relative reduction on the chlorophyll content by around 0.4 μmol/m^2 of leaf area with the genotypes 440-H2-47 and 440-D4-60 sharing almost similar patterns as the wild type. The sensitive genotypes 440-F2-20, 440-F2-64 and 441-G3-89 had a higher relative reduction of ~0.6 μmol/m^2, which was more than the wild type. Noticeably, one sensitive line, 441-E2-83, did not exhibit a higher reduction as did other sensitive genotypes but the relative reduction in chlorophyll content was similar to the tolerant genotypes.

4.4.5 Mineral content analysis

Two highly tolerant ATag genotypes of Arabidopsis, 440-B4-7 and 441-H1-70 were chosen for mineral composition analyses. The mineral elements that were focused for analysis were potassium (K), phosphorous (P), calcium (Ca), magnesium (Mg), sulphur (S), sodium (Na), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu) and boron (B). The two ATag mutants and the wild type were analyzed for their mineral composition and the data was tabulated for comparative analysis (Tables 1 and 2, Figure 11). The elements that showed a decrease in wild type and increase in tolerant genotypes under stress condition were zinc (whose concentration was less in mutants under normal conditions compared to the wild type but increased under stress in contrast to the wild type), iron (whose content was increased under stress in mutants whereas it was lesser than wild type under normal conditions), and calcium (which remained same in wild type under normal and stressed conditions but its content raised slightly in both mutant genotypes).

The minerals that increased in the wild type and decreased in ATag mutant lines include magnesium, which slightly increased in wild type under stress but decreased in genotype 440-B4-7 and increased in 441-H1-70. Some had a similar pattern in wild type as well as both the mutant genotypes. Sodium content was most noticeably highly raised under stress treatments in all three plant types contrary to the fact that it was minimal under normal conditions. Boron content decreased in all under salt stress. Copper content also reduced under stress (was less in mutants than wild type under normal conditions) and manganese content also decreased in all the mutants. The sulphur content did not very much in the three plant types and also not much after subjected to normal and stressed situations. Sodium, potassium and calcium are mainly involved in cellular mechanisms and regulation of cellular homeostasis, therefore these three will be discussed further

5.0 Discussion

Arabidopsis is naturally a salt sensitive species and its growth is restricted as soon as the threshold concentration of the salt in soil exceeds its natural tolerance value, that has been identified in literature as ~150mM NaCl (Sanders, 2000; Sun and Hauser, 2001; Xiong and Zhu, 2002).

The present study was successfully conducted to study a wide range of gain-of-function genotypic genotypes of Arabidopsis in the greenhouse under controlled conditions, primarily to identify and select activation tagged Arabidopsis lines for salt tolerance. Activation tagging using the maize En-I transposon system (Marsch-Martinez et al., 2002), with the mobile I transposon bearing a 35S-enhancer tetramer in Arabidopsis has been shown to generate 10 times as many gain-of-function mutants compared to T-DNA activation tagging (Weigel et al., 2000) that tend to be methylated due to multiple T-DNA copy insertions (Chalfun-Junior et al., 2003). The ATag mutants induce a gain-of-function mutation by altering the level of gene expression (i.e. transcriptional activation) by bringing it under the control of the adjacent strong enhancer of the cauliflower mosaic virus active promoter 35S (Weigel et al., 2000). This way the activated genes (at locus up to 10kb up- or down- stream of genes) in the genome are overexpressed to enhance the phenotype in a quantitative way with an over-dominant gene action, and provide phenotypes for genes with small effect that are easily distinguished as mutants in a way that is not possible using gene knockout strategies. Apart from the current application in selection of abiotic (salt stress) tolerant mutants, such approaches have been previously used by researchers to generate characterized mutations such as developmental (Marsch-Martinez et al., 2002), phenotypic, flowering abilities, several biochemical mutations, parthenocarpy and many more (Marsch-Martinez & Pereira 2011).

The 300 Arabidopsis En-I transposon Activation Tagged (ATag) mutants were grown and screened in the controlled growth chambers, out of which 106 genotypes were found suitable for analysis. The variant salt tolerant mutant lines were grouped into six batches to give 25 tolerant and 15 sensitive lines relative to the wild-type control, out of which 14 mutant lines were further selected for analysis. This included 8 tolerant and 4 sensitive lines for salt tolerance that were selected to identify the tagged genes. These 14 lines showed significant p-values compared to the wild type for salt tolerance scores using relative reduction in biomass, which was complemented by analysis of variance (Figure 4, A-F). The phenotypic analysis included several visibly distinguishable tolerance parameters whereby the tolerant genotypes mostly had significant p-values in comparison to wild type plants under salt stress. The sensitive lines also showed significant difference to the wild type, indicating the presence of salt stress mechanisms also in the wild type. Two sensitive genotypes, 440-H2-47 and 440-D4-60, were chosen to be similar to the wild type and they exhibited many similar patterns for each criteria corresponding to the wild type under normal as well as stressed environment.

5.1 Selection of Basta resistant ATag lines, and salt screening

The screening protocol for selection of Basta resistant ATag lines is described by Marsch-Martinez in 2002. The Bar gene in the construct was used for selection of Basta herbicide resistance in Arabidopsis transgenic AIE lines. The application of glufosinate herbicide to the growing array of ATag lines in the greenhouse was essential to check the presence of the BAR gene which confers Basta resistance. This gives an effective greenhouse based selection strategy yielding the transposed Basta resistant ATag containing lines.

The screening for salt tolerance was set at a selection level based on growth at 150 mM NaCl concentration. This concentration of sodium chloride was selected as LD_{50} NaCl (lethal dose for 50% plant population) for Arabidopsis and many other moderately salt tolerant species like wheat cultivars has been identified to be 150mM (Orsini et al., 2010; Munns et al., 1995). Arabidopsis is highly sensitive toward high salinity during the seed germination and seedling stages, to the extent that callose deposition and abnormal alterations in embryo lead to seed death (Xiong and Zhu, 2002). Studying the salt tolerant physiological traits that are prior to the characterization of the candidate genes was not possible during the seedling stage, therefore high salt treatment was imposed during the vegetative phase. If the salt treatment were to be applied during the seedling stage, only the resistant ATag transposants would have been able to survive and no sensitive line would be obtained for comparative analyses.

Plant biomass production is under genetic control by multiple factors. Genes, such as the putative vacuolar $Na^+(K^+)/H^+$ antiporter gene from *Panicum virgatum* L. (switchgrass), are known for increasing the biomass by enhancing several physiological factors like height, longer leaves and large stem, which impart salt stress tolerance, indicating that biomass decrease is a symptom of salt stress effects (Huang et al., 2017). Hence, primarily the relative reduction in biomass was analyzed for all the surviving genotypes to select the highly potential tolerant and sensitive lines for further analysis. The treatment of the entire population with moderately high salt treatment at its vegetative growth phase, and subsequently measuring the relative biomass reduction, explains the abundance of tolerant lines (about 30) obtained after screening 146 genotypes in the greenhouse as compared to the few sensitive lines (about 11) identified. It is understandable that the naturally sensitive lines must have been selected out at the initial stage. A similar approach was employed by Harb and Pereira (2013) whereby 10 mutant lines were tested

for the relative reduction in biomass analysis under salt treatment that yielded one sensitive line, C421, and 5 tolerant lines, C65, C394, C420, C437 and C490. This was a useful result as we aimed to study the gain-in-function mutations. Finally, 8 tolerant and 4 sensitive genotypes were chosen for further analysis in the present study.

5.2 Phenotypic Characteristics under Salt Stress condition

5.2.1 Number of leaves and Rosette diameter

Some mutant lines, especially the sensitive genotypes 440-F2-20 and 440-F2-64 plants were short in size while some tolerant genotypes showed much healthier and faster growth, indicating an effect in the ATag mutant under normal conditions. In the early research of Marsch-Martinez et al. (2002) the maize *En-I* transposon based ATag insertional strategy identified both dominant and recessive mutants. Two characteristic mutants identified with altered phenotypes were the recessive *fiddlehead* mutant with variegated leaves and a dominant mutant *thread* that was sterile and late flowering with long curved leaves and siliques without seeds.

The other tolerant lines and two more sensitive mutants had indistinguishable physical appearance from the wild type. It is interesting here to note that the tolerant ATag lines like 440-B4-7, 440-B3-34 and 441-E4-85 showed early vigorous growth. In addition, these lines showed more tolerance to salt stress effects on leaf number with respective means of 0.41, 0.46, and 0.35, compared to a wild type mean of 0.61, and showed more tolerance to salt stress effects on their rosette diameter with respective means of 0.37, 0.41, and 0.29 compared to a wild type mean of 0.544.

There could be some physical mechanism in response to salt stress preventing any hampering of leaf growth and development.

5.2.2 Plant Height and Stalk numbers

On exposure to high salt concentration, plant height and number of stalks/stems were reduced in each plant, but the relative reduction in tolerant ATag lines was lesser than the wild type, indicating that the tolerance phenotype could be attributed to the gain function by transposon activation tagged genes, which could involve some changes in the cell wall components such as cellulose, pectin and lignin during plant growth development stages. Therefore, a plant can overcome the harmful effects posed by salt stress, enabling the plant to survive under saline soil condition. Such a strategy has been used before as shown by a T-DNA activation tagged Arabidopsis mutant PGX[AT] over expressing a polygalacturonase enzyme and exhibiting the phenotype of cell expansion and the regulation of pectin (Xiao et al., 2016). PGX[AT] mutants possess long hypocotyls, larger rosettes and early flowering, but reduced stem thickness because the increased polygalacturonase enzyme degrades pectin and promote cell expansion and separation (Xiao et al., 2016).

Along with pectin, which is a cell adhesive component, the mechanical support contributor lignin has also been shown to be involved in enhancing plant height through hypocotyl elongation. The anatomical analysis of Arabidopsis plant parts like roots, hypocotyl and leaves have revealed higher content of lignin in salt tolerant ATag lines compared to their wild-type counterparts. This indicates that a firm anatomical infrastructure is required for plant sturdiness and height maintenance, which also confers tolerance by contributing to the continuity

of plant growth under salt stress (Sessions et al., 2002). The enzyme Cu/Zn superoxide dismutase has been found to be associated with increasing lignin content and being involved in salt stress tolerance (Gill et al., 2010). Hence, it will be fruitful to undertake anatomical evaluations of the tolerant lines to identify any changes in the cell wall for pectin and lignin composition, and validate the discussed predictions.

5.2.3 Bolting and Flowering

In screening for their flowering behavior, the mutants displayed an expected pattern whereby most of the tolerant ATag lines showed early bolting and flowering, even prior to the wild type. However, a few others corresponded with that of the wild type and the two most sensitive lines had delayed flowering under normal conditions. The sensitive ATag lines which did not flower in response to saline condition indicating the restriction of growth factors under salt stress. These observations are in support with earlier documentations where wild type Arabidopsis Col ATag lines have been recorded with reduced vegetative growth, less flowering, no or delayed bolting, and flowering along with chlorosis and necrosis on exposure to high salinity conditions (Chan et al., 2013). Overexpression of miRNA *gma-mir172a* has been found to promote early flowering in *Arabidopsis*, maize, rice and soybeans, as it up-regulates LFY, AP1 and FT whereas *mir156* promotes late flowering (Wang et al., 2016). A molecular level evaluation of the tolerant mutant lines from the current screen showing early flowering, by testing the response of *mir172a* expression, would question its role in up-regulation through activation tagging.

5.2.4 Chlorophyll content

The salt stress treatment reduced the chlorophyll in leaves, which is the basic source for carrying out the essential process of photosynthesis. The tolerant plants were able to limit this reduction to a large extent but sensitive lines and wild type were greatly affected. In saline conditions, the tolerant plants tend to reduce leaf expansion compared to the controlled conditions, which increases the chlorophyll density per unit leaf area but may slow down the photosynthetic process under salt stress (Munns and Tester, 2008). Saline conditions affect the photosynthetic rates by initially decreasing the stomatal aperture that has been observed in sensitive durum wheat mutants (James et al., 2002). This reduction slows down the stomatal conductance that disrupts the ionic balance in cells affecting the photosystem II, which leads to degradation of chlorophyll in the sensitive genotypes (Negrao et al, 2017). These factors negatively regulate the Rubisco enzyme that leads to the slowing down of photosynthetic rate and hence supports the results observed, wherein sensitive lines had significantly reduced chlorophyll content and tolerant lines were able to relatively retain the amount. The salt stress tolerance response mechanism here could be that the stomatal closure is more effective in mutants than the wild type, which saves water from transpiring, along with maintaining the conductance that leads to retaining chlorophyll to a great extent (Sessions et al., 2002).

5.2.5 Mineral Nutrient Analysis (K, Na, Ca and Mg)

Nutrients such as basic mineral elements are required by plants in micro or macro quantities to carry out the cellular processes and biological mechanisms. Potassium, calcium, magnesium and sodium have been long documented to have a significant role during salt stress, being

participants of signal transduction, as messengers, activators/repressors, co-factors and more. Their concentration was important to find out, as these four nutrients show varying concentrations in both Activation tagged lines 440-B4-7 and 441-H1-70 as compared to the wild type. Potassium was found to slightly decrease under stress in ATag lines 440-B4-7, 441-H1-70 and Col0 under salt condition, whereas sodium significantly increased in ATag lines and Col0. K^+ and Na^+ ions are involved in the maintenance of cellular homeostasis and ionic balance which is essential for optimal growth and development (Reguera et al., 2014). Hence, it was thought that an unbalance amongst them, such as the highly raised sodium content in all of these plant types, must work in causing a salt related sensitivity as it causes leaf necrosis, interference with ion channels, growth hampering, and disrupts enzymes and injures the plasma membrane (Parwaiz and Satyawati, 2008). But, in concordance with the obtained results, sodium has been found previously to also increase in content under salt stress without hampering the tolerance mechanism and has been listed as a factor of shoot ion independent tolerance (Chan et al., 2013; Munns and Tester, 2008). Similarly, in certain salt tolerant Arabidopsis transgenics the sodium ions increased under salt stress but were less in the wild type, and potassium ions decreased but transgenics accumulated more of it than the wild type (Huang et al., 2017). However, sodium ions are not required for plant growth but interfere in potassium uptake and ion binding sites. Their excess is then referred to as sodium toxicity (Quan et al., 2007).

$Na^+(K^+)/H^+$ antiporter proteins have been shown to be involved in improving cellular homeostasis, mainly through potassium ion accumulation that reportedly combat salt stress effects (Huang et al., 2017). This has been documented experimentally in several plant species like tobacco, mungbean, cowpea and alfalfa for inducing salt stress tolerance (Zang et al., 2015; Sahoo et al., 2016). However, certain species have shown reverse results with increased K^+ under

salt stress (Huang et al., 2017). Another contrary data has been obtained from peanut cultivars where Na^+ exclusion and external K^+ administration enhanced salt tress tolerance (Chakraborty et al., 2016). These facts suggest that there might be a swift internal mechanism (like osmoprotectants) working in maintenance of the ion balance in cells, such that this high rise of Na^+ and slight reduction of K^+ does not adversely affect the cells when under salt stress. An alternative explanation could be that the plant responds to high salt concentration by growth reduction due to the disruption of balance in osmolarity of external surroundings, and not due to internal concentration in growing tissues (Munns, 2002).

Calcium displayed this reverse trend and increased in both mutant genotypes under stressed conditions, whereas in the controls its content remained almost the same in normal and stress situations, suggesting its role in offering tolerance against salt stress. It has been studied that calcium ion accumulation curbs the high sodium ion induced ill effects and has been supplemented in salinity experiments to study tolerance (Cramer, 2002; Negrao, 2017). Calcium ions are involved in the salt overly sensitive (SOS) pathway that has recently been studied at the tissue level, and is conserved in several crop species including rice (Zhu, 2000; Martinez-Atienza et al., 2006). The SOS3 pathway in roots and SOS calcium associated binding protein 8 (SCABP8) in shoots senses raised calcium levels under salt stress, and works in a cascade to combat the salt stress effects (Quan et al., 2007). Interestingly, SOS mutants have been found to be deficient in the maintenance of K^+/Na^+ homeostasis. The SOS pathway has several components including SOS1 which functions as a sodium/proton antiporter in the plasma membrane, which is vital for proper exchange of Na+ and H+ ions. SOS2, a Ser/Thr protein kinase is another component of the cascade that is pivotal for interactions with SOS3 and SCABP8. All of these factors work together in conferring salt tolerance in Arabidopsis and

reducing sodium ion toxicity in roots and shoot respectively.

Magnesium was found to increase in only one tolerant mutant, 441-H1-70, and not in other tolerant mutants, suggesting its role in salt stress tolerance that might be responsible in some ATag genotypes. It is essentially the most abundant divalent cation in plant cells, and silencing of its transporter magnesium transporter 6 (MGT6) through RNAi results in growth retardation as magnesium levels drop (Mao et al., 2014). This highlights the importance of Mg^{+2} in plants during growth processes including photosynthesis, and recently the role it plays in chloroplast-nucleus signaling has also been reported, as it is needed for protoporphyrin IX concentration which directs chlorophyll biosynthesis (Pontier et al., 2006). Hence, the increasing concentration in a tolerant line under salt stress must be a reflection of rising chlorophyll content as a salt stress response.

These phenotypic and physiological analyses are the primary experiments toward the unraveling of the salt tolerance response mechanisms at the cellular and genetic levels. The mutant lines that displayed salt stress tolerance through various characteristic observations were then analyzed for each factor, such as chlorophyll density increase, calcium ions increase, and plant height changes. These processes were then followed for their connections at the cellular level with factors like enzymes, proteins, co-factors and signal transduction pathways, which were then studied for their association with the corresponding genes complemented with genetic analyses tools and techniques. Ultimately this will lead to the identification of candidate genes that can then be utilized for crop improvement and protection from salt stress scenarios.

In summary, Activation tagging has been identified as a productive approach for the random generation of gain-in-function mutants of otherwise redundant or lowly expressing genes

that are not possible to identify using conventional knockout mutant approaches. The high efficacy and ease of getting transformants and their progeny in a reduced time span are the advantages that upbeat its usefulness. It is evident from the present study conducted that the gain of function mutant analysis yielded results that were unpredictable earlier. It suggested that the role of calcium in offering tolerance is more significant than that of sodium, which showed no change in content or trend in wild type and mutant lines under normal or stressed situations. A forward genetics approach that involves use of the En-I transposon Activation tagging system based on the I-ATag activating insert was proposed to reveal the hidden mysteries of the complex plant genome. The loss of function strategy is at times unable to identify a mutant phenotype or one which may not be evident after screening knockouts of a single target gene, which may be attributable to several factors such as the presence of a closely related or duplicate gene. These problems do not arise with activation tagging involving a transposon ATag, as its specific and definite gene targets are overexpressed. Application of such a fruitful approach in a convenient ecotype like Columbia (with its completely sequenced genome) will aid in the identification of stress related responses and factors working alongside, which can also be applied to commercially important crop species such as rice mutants for improvement.

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List of Tables

Table 1: Bolting and flowering percentage during plant growth Day 21-Day 49 of 3 biological replicates of 14 Columbia activation tagging lines (ColATag) comparing to the wild type Col0 (Control 0 mM NaCl and Stress 150 mM NaCl). Salt treatments were initiated at Day 21 after sowing.

Genotypes	3 Plants- Control 0 mM NaCl		3 Plants- Stress 150 NaCl	
	100 % Bolting	**100 % Flowering**	**100 % Bolting**	**100 % Flowering**
Col0	3/3 Day 35	3/3 at Day42	0/3	0/3
440-B4-7	3/3 at Day 28	3/3 at Day35	1/3 at Day 35	1/3 at Day42
440-F2-20	3/3 at Day 42	3/3 at Day49	0/3	0/3
440-G3-25	3/3 at Day 35	3/3 at Day42	1/3 at Day 35	1/3 at Day42
440-B3-34	3/3 at Day 28	3/3 at Day35	1/3 at Day 35	1/3 at Day42
440-F2-64	3/3 at Day 42	3/3 at Day49	0/3	0/3
440-G3-68	3/3 at Day 35	3/3 at Day42	2/3 at Day35	2/3 100 at Day42
440-H1-70	3/3 at Day 28	3/3 at Day35	1/3 at Day 35	1/3 at Day42
440-A1-73	3/3 at Day 28	3/3 at Day35	1/3 at Day 35	1/3 at Day42
441-E2-83	3/3 at Day 35	3/3 at Day42	0/3	0/3
441-E4-85	3/3 at Day 28	3/3 at Day35	2/3 at Day 28	2/3 at Day35
441-G3-89	3/3 at Day 35	3/3 at Day42	0/3	0/3
440-G4-90	3/3 at Day 28	3/3 at Day35	1/3 at Day 35	1/3 at Day42
440-H2-47	3/3 at Day 35	3/3 at Day42	0/3	0/3
440-D4-60	3/3 at Day 35	3/3 at Day42	0/3	0/3

Table 2: Composition analysis for common elements measured on basis of dry weight of wild type Col0 and activation tagged line (A) 440-B4-7, (B) 441-H1-70 for control 0 mM NaCl and stress 150 mM NaCl treatment. % Indicating plant macronutrients (percentage in dry weight), Ppm indicating plant micronutrients (parts per million-mg/kg Dw), Sig= significance, NS= no significance. The data are average of three replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05 using t-test.

(A) Analysis of ATag line 440-B4-7

Nutrient	Symbol	Unit	Col0 Control	Col0 Stress	440-B4-7 Control		440-B4-7 Stress	
			Mean	Mean	Mean	Sig.	Mean	Sig
Phosphorus	P	%	0.7632	0.66	0.7675	NS	0.6131	NS
Potassium	K	%	3.21	2.495	3.416	**	2.374	NS
Calcium	Ca	%	2.484	2.634	3.3736	**	3.714	**
Magnesium	Mg	%	0.475	0.5178	0.635	**	0.5487	**
Sulfur	S	%	1.346	1.326	1.2043	NS	1.241	NS
Sodium	Na	Ppm	2417.5	26595.8	3284.5	**	26354.0	NS
Iron	Fe	Ppm	137.4	126.3	91.85	**	112.5	NS
Mg	Mn	Ppm	29.67	24.77	24.19	NS	19.87	**
Zinc	Zn	Ppm	99.29	93.41	72.38	**	88.31	NS
Copper	Cu	Ppm	5.222	3.561	4.166	**	3.559	NS
Boron	B	Ppm	32.455	24.76	30.19	*	18.79	**

(B) Analysis of mutant line 440-B4-7

Nutrient	Symbol	Unit	Col0 Control	Col0 Stress	441-H1-70 Control		441-H1-70 Stress	
			Mean	Mean	Mean	Sig	Mean	Sig
Phosphorus	P	%	0.7632	0.66	0.7604	NS	0.6134	NS
Potassium	K	%	3.21	2.495	3.459	NS	2.1303	**
Calcium	Ca	%	2.484	2.634	3.075	**	3.5106	*
Magnesium	Mg	%	0.475	0.5178	0.498	*	0.649	**
Sulfur	S	%	1.346	1.326	1.061	*	1.253	NS
Sodium	Na	ppm	2417.5	26595.8	2200.4	*	24834.8	NS
Iron	Fe	ppm	137.4	126.3	82.62	**	107.9	NS
Manganese	Mn	ppm	29.67	24.77	27.14	NS	16.71	*
Zinc	Zn	ppm	99.29	93.41	71.09	**	88.44	NS
Copper	Cu	ppm	5.222	3.561	4.115	**	2.508	**
Boron	B	ppm	32.455	24.76	27.29	*	18.88	*
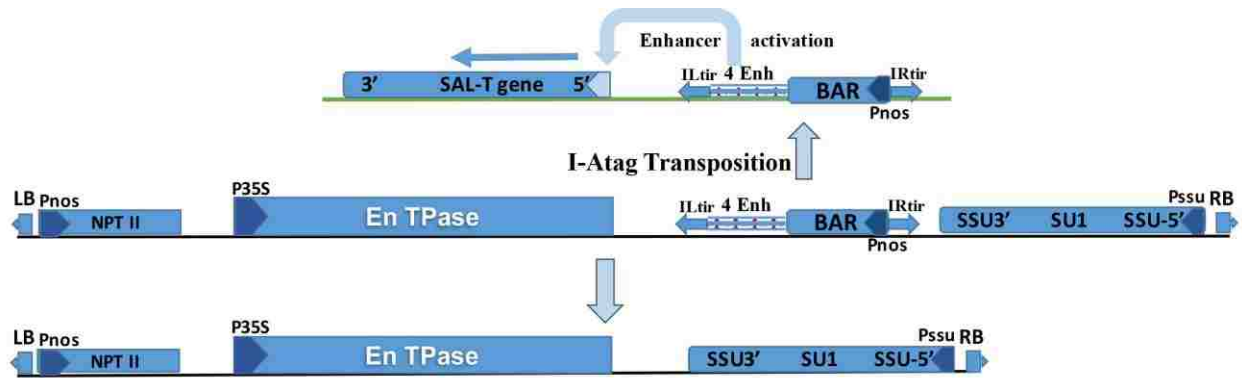
Figure 1: Schematic representation of the Activation-Tag lines using the En-I transposon system for generation of Salinity Tolerant (SAL-T) mutants by gain-of-function, adapted from Marsch-Martinez et al., (2002). The elements of the construct are as follows: T-DNA LB (Left border) and RB (right border); P35S CaMV35S promoter; *En*TPase, *En* immobile transposase; *I*-element left (ILtir) and right (IRtir) terminal-inverted repeat; 4-Enh (tetramer of the CaMV 35S enhancer). Selectable marker: positive selectable marker BAR (glufosinate/Basta resistance) and negative selectable marker SU1 (Pro-herbicide R740).

Figure 2: Morphological phenotypes during vegetative stage of 3 biological replicates of 14 Columbia activation tagged lines (ColATag) comparing to the wild type Col0 (Control 0 mM NaCl and Stress 150 mM NaCl). Salt treatments were initiated at Day 21 after sowing.

Figure 2 (Cont.)

Figure 2: Morphological phenotypes during vegetative stage of 3 biological replicates of 14 Columbia activation tagged lines (ColATag) comparing to the wild type Col0 (Control 0 mM NaCl and Stress 150 mM NaCl). Salt treatments were initiated at Day 21 after sowing.

Figure 3: Morphological phenotype at flowering stage of 14 Columbia activation tag lines (ColATag) comparing to the wild type Col0, with Control 0 mM NaCl (left) and Stress 150 mM NaCl (right). Salt treatments were initiated at Day 21 after sowing.

A
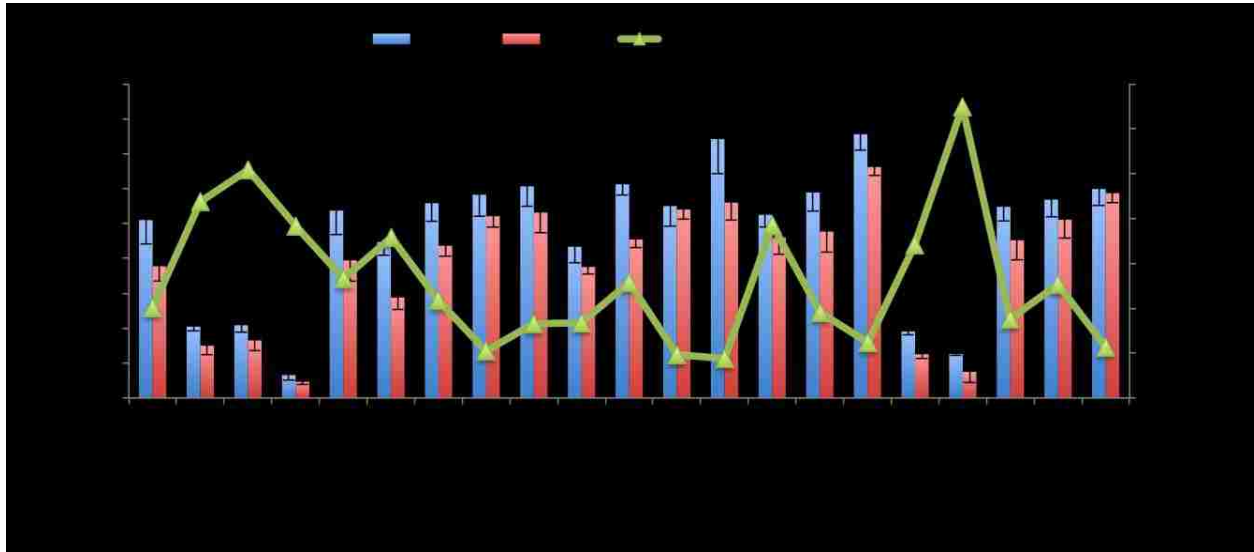
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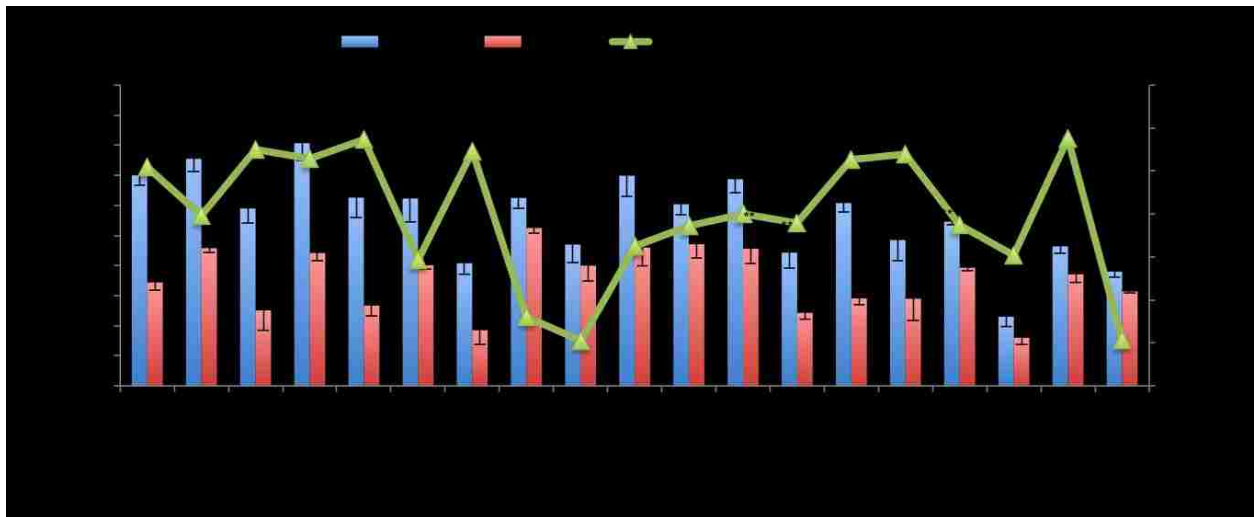
Figure 4: Comparative analysis of the response of salt stress on plant biomass of ATag lines analyzed in batches A-F compared to WT Col0. All the data are average of ten replicates; the error bars show the 99% and 95% confidence interval of the t-test. The green line shows the relative reduction in biomass. The data are average of ten replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. (A) Batch 1, (B) Batch 2, (C) Batch 3, (D) Batch 4, (E) Batch 5, (F) Batch 6.

Figure 4: (Cont.)

C
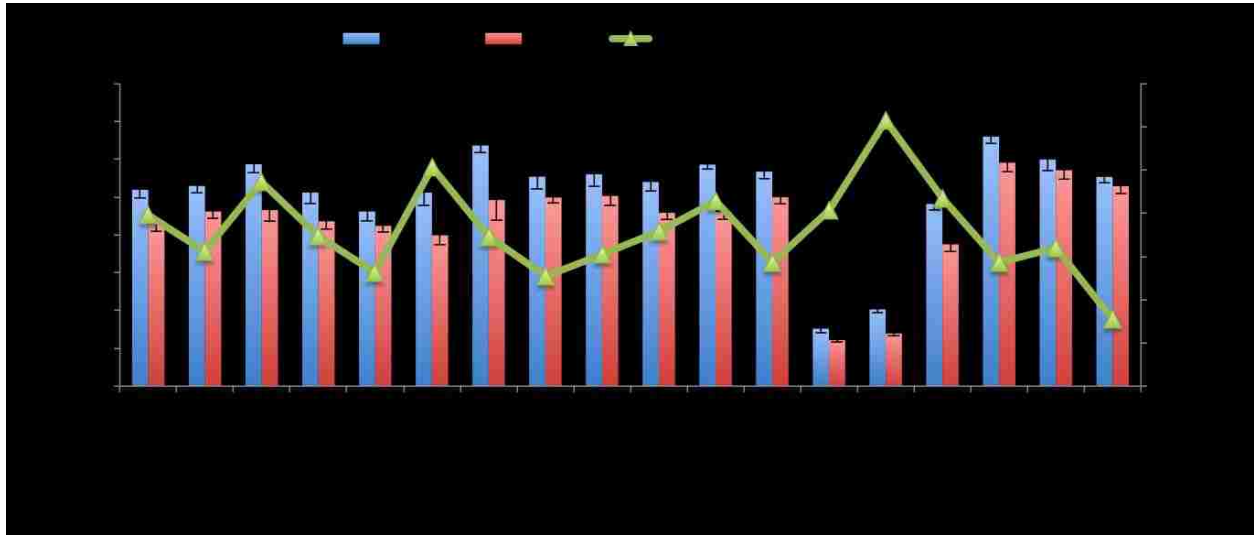
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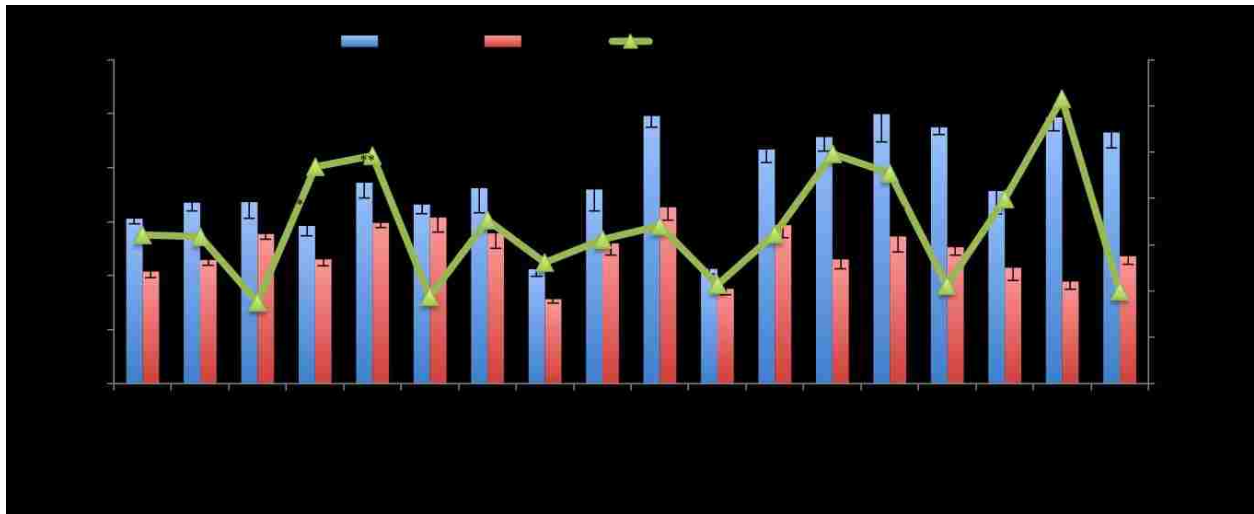
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Figure 4: (Cont.)

E
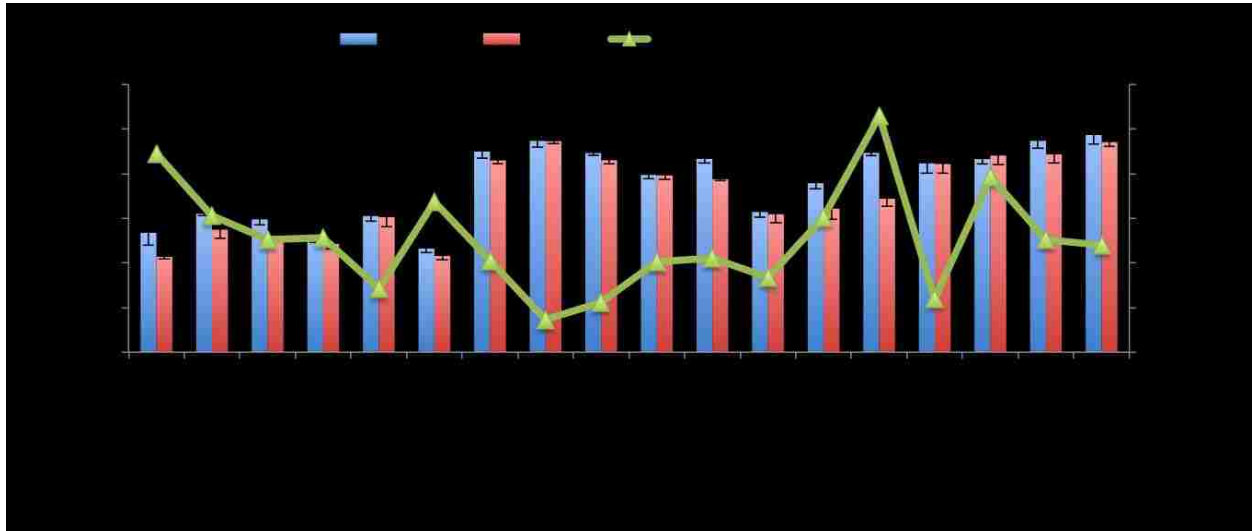
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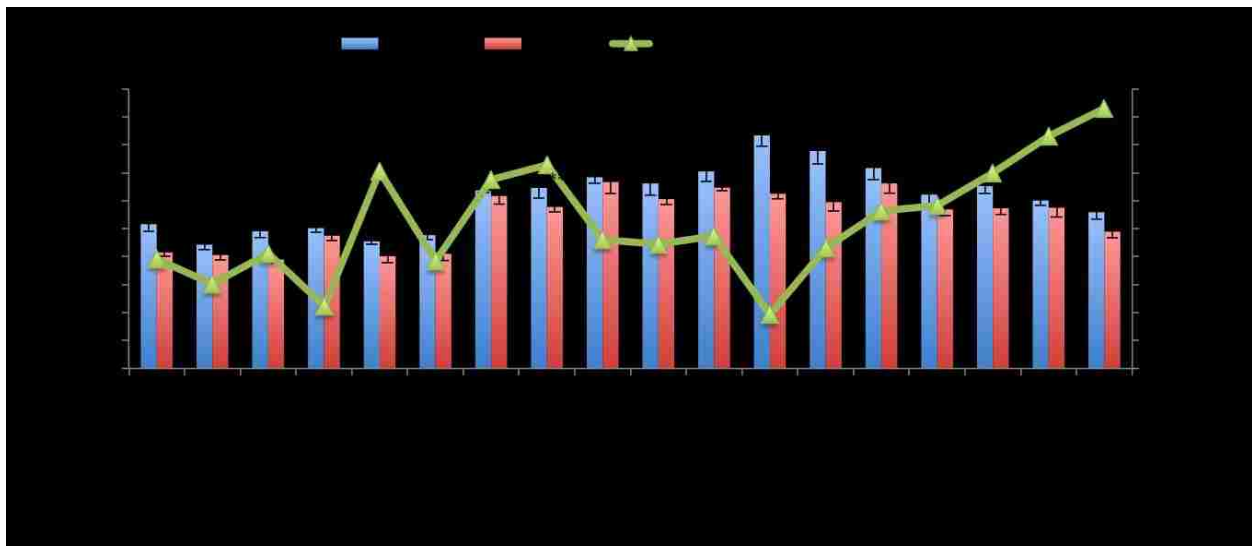
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A
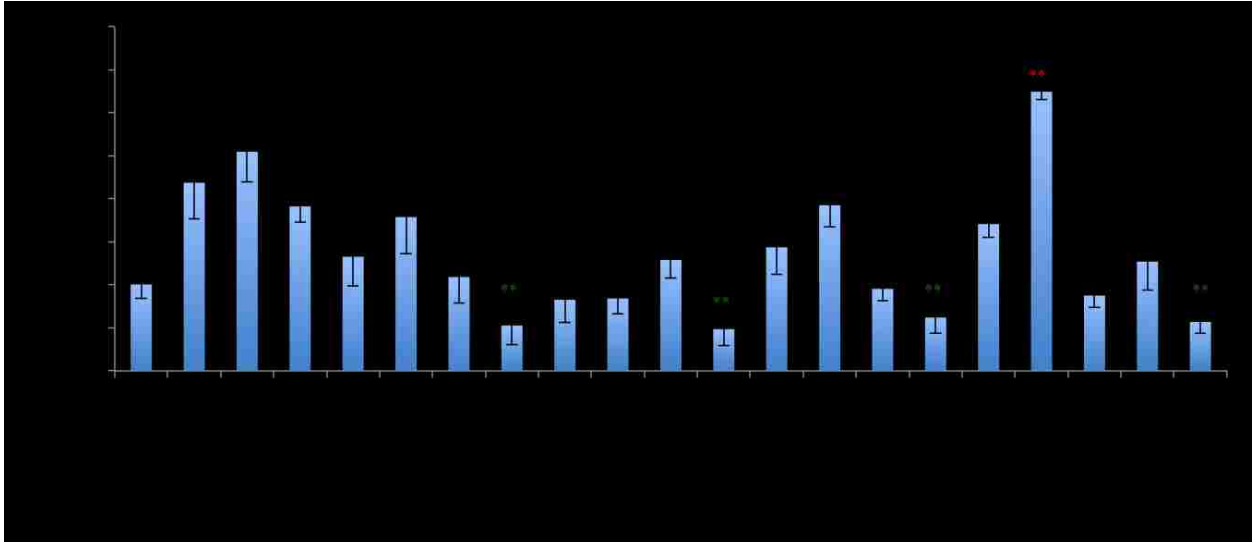
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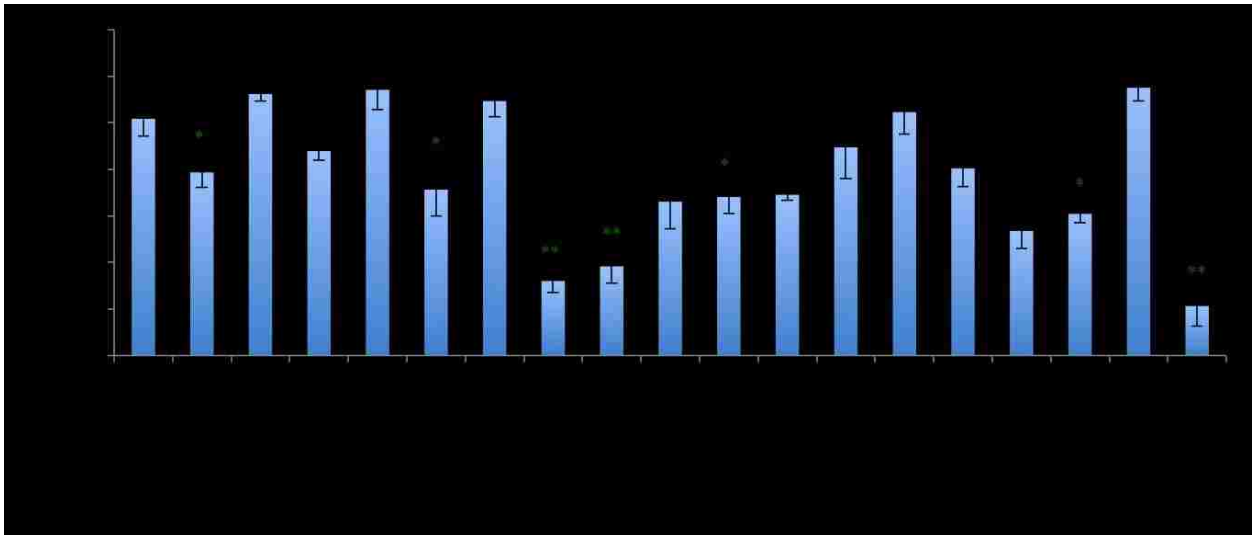
Figure 5: Relative reduction in biomass (RB) of salt screen in batches (A-F) of ATag lines compared to WT Col0. Bars represent ± SE, N= 10. The data are average of ten replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicates sensitive lines, green * indicates tolerant lines. (A) Batch 1, (B) Batch 2, (C) Batch 3, (D) Batch 4, (E) Batch 5, (F) Batch 6.

Figure 5: (Cont.)

C
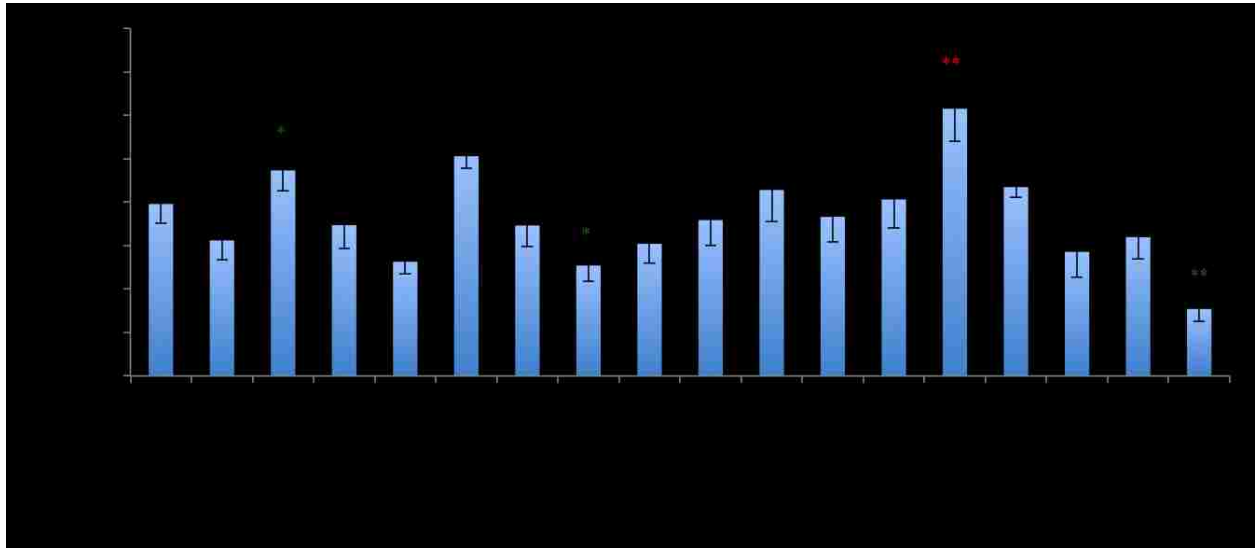
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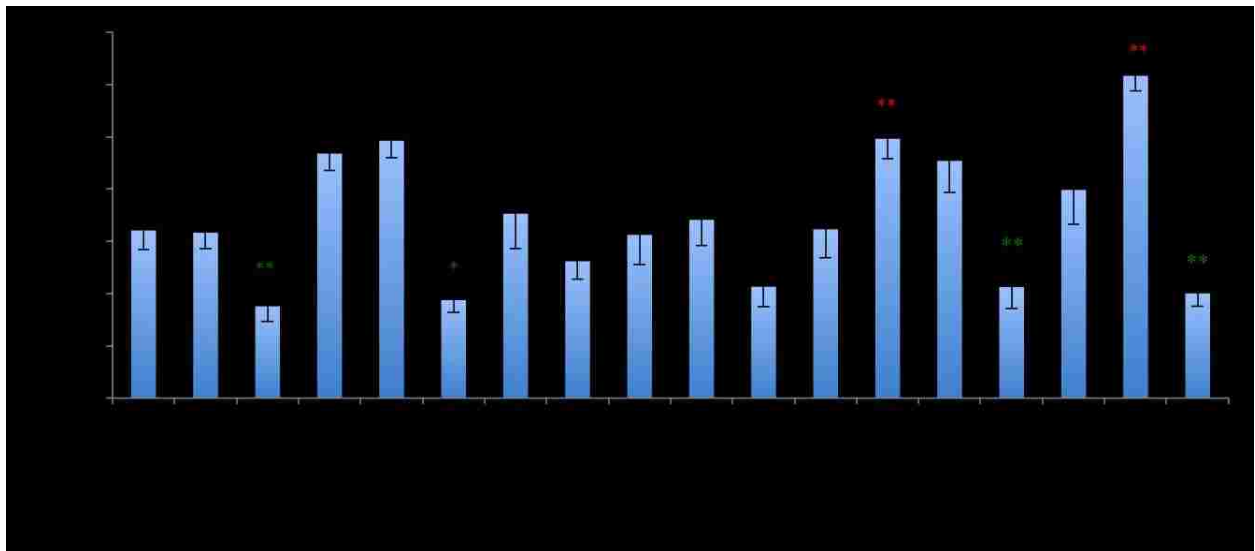
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Figure 5: (Cont.)

E
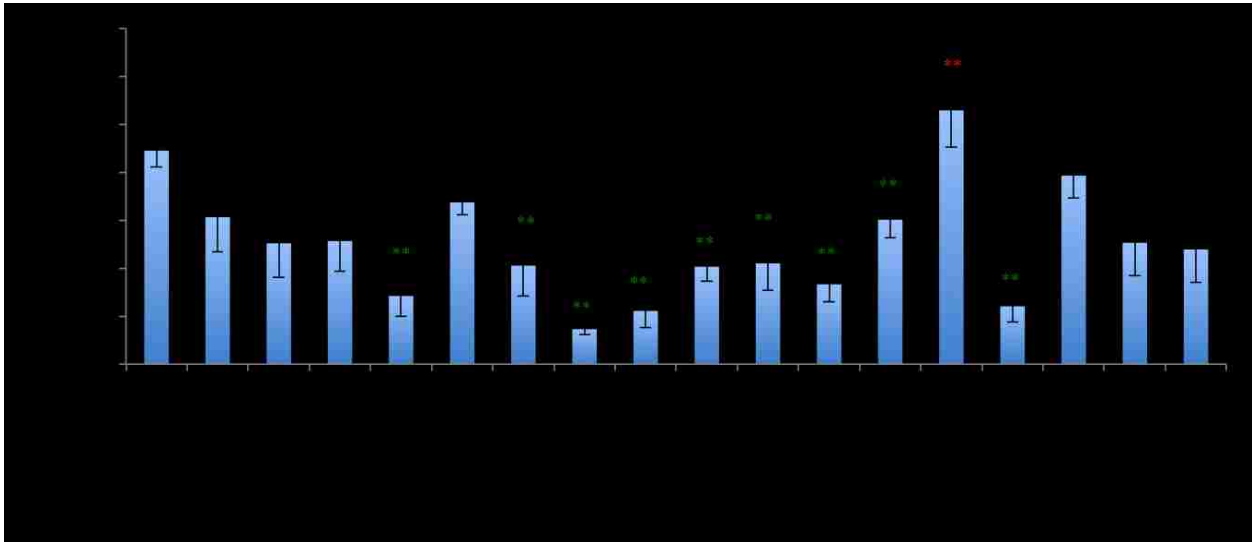
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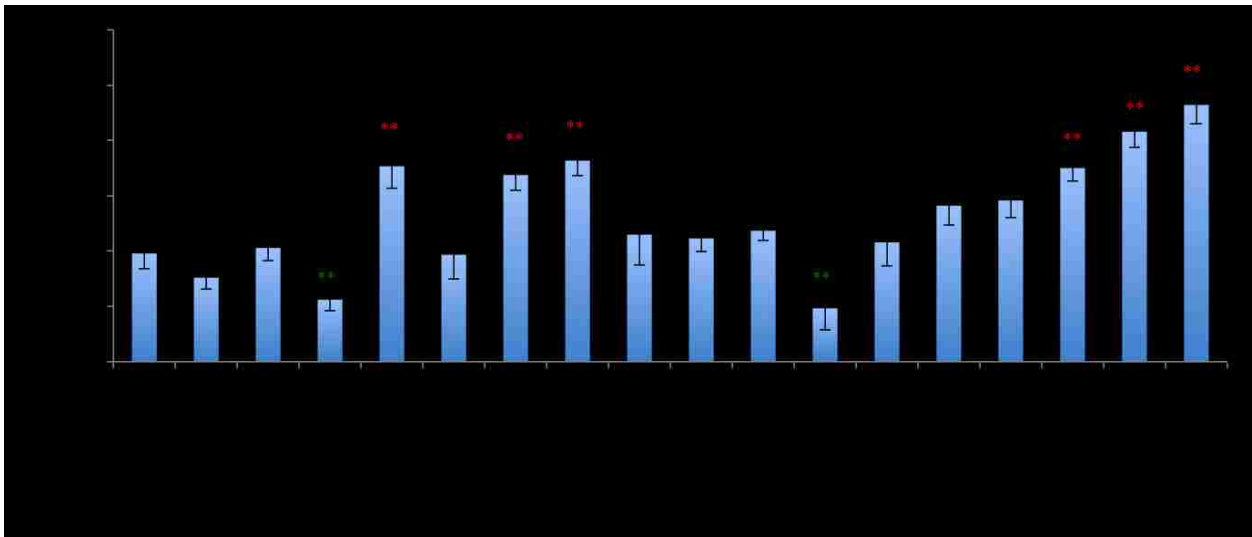
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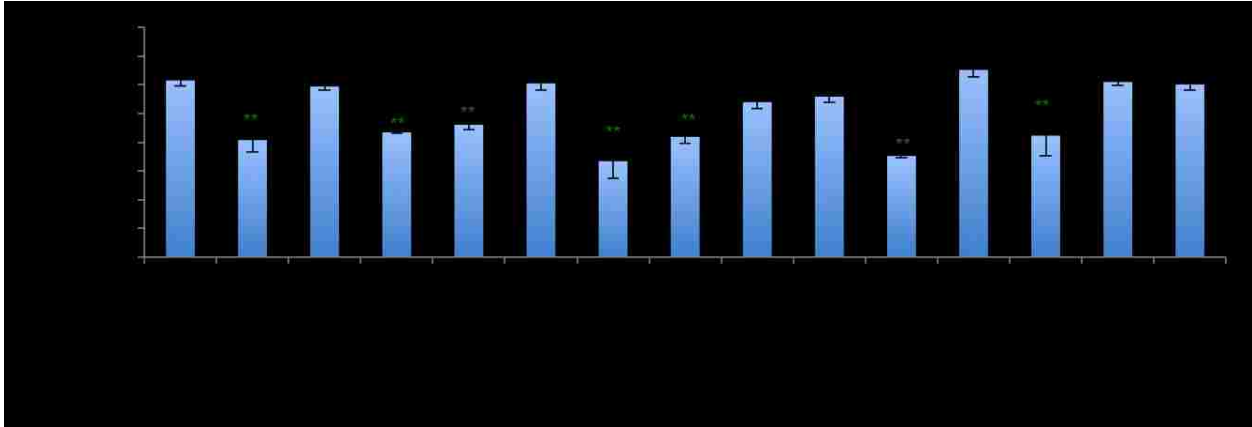
Figure 6: Bar plot showing response of continuous salt stress among flowering stage on relative reduction in plant number of leaves of 14 selected ATag lines compared to WT Col0. Bars represent ± SE, N= 3. The data are average of 3 replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicating sensitive lines, green * indicating tolerant lines.
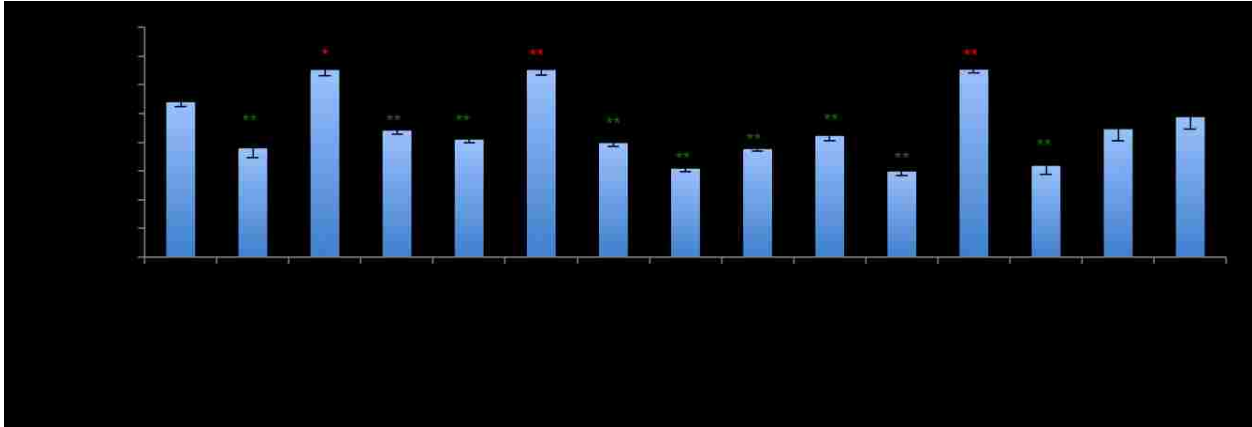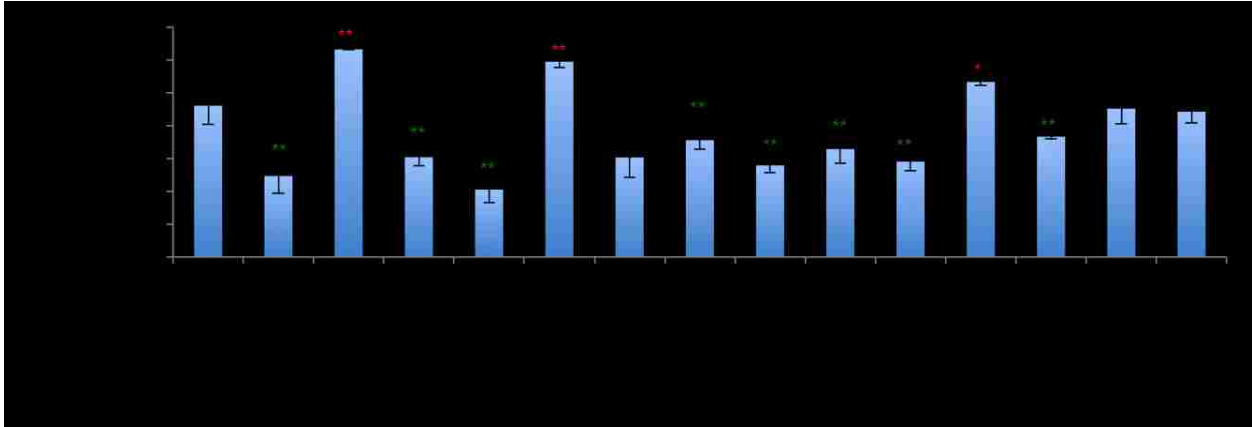
Figure 7: Bar plot showing response of continuous salt stress among flowering stage on relative reduction in rosette diameter on day 49 of 14 ATag lines compared to WT Col0. Bars represent ± SE, N= 3. The data are average of 3 replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicating sensitive lines, green * indicating tolerant lines.
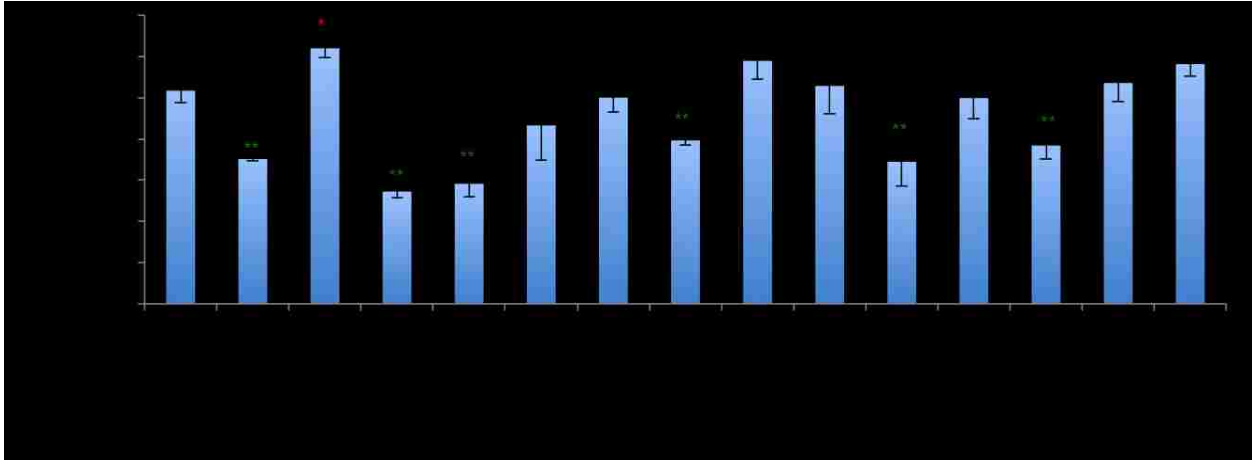
Figure 8: Bar plot showing response of continuous salt stress among flowering stage on Relative reduction in plant height on day 62 of 14 ATag lines compared to WT Col0. Bars represent ± SE, N= 3. The data are average of 3 replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicating sensitive lines, green * indicating tolerant lines.
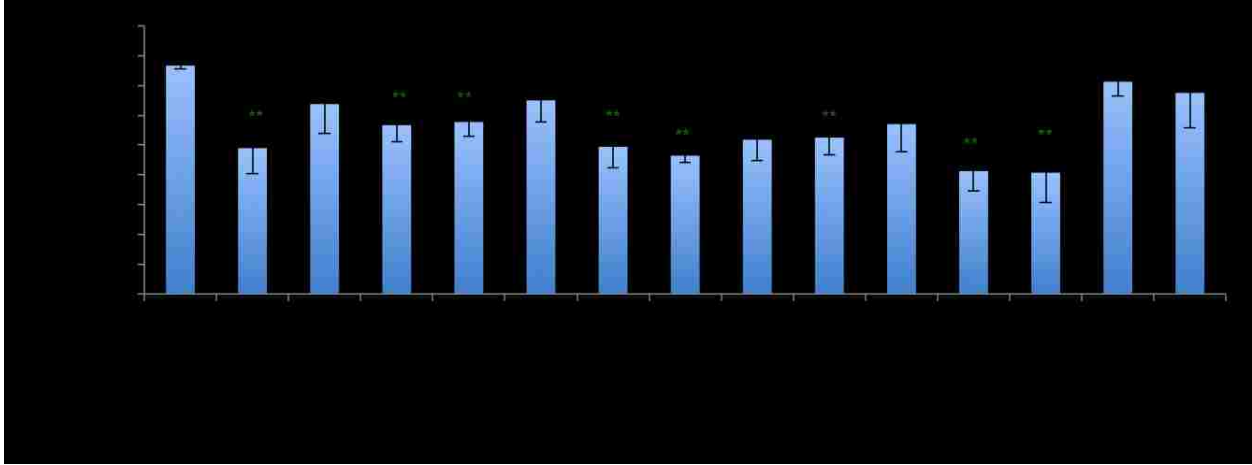
Figure 9: Bar plot showing response of continuous salt stress among flowering stage on Relative reduction in number of stems on day 62 of 14 ATag lines compared to WT Col0. Bars represent ± SE, N= 3. The data are average of 3 replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicating sensitive lines, green * indicating tolerant lines.

Figure 10: Bar plot showing response of continuous salt stress among flowering stage on Relative reduction in Chlorophyll of 14 ATag lines compared to WT Col0. Bars represent ± SE, N= 10. The data are average of 10 replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. Red * indicating sensitive lines, green * indicating tolerant lines.
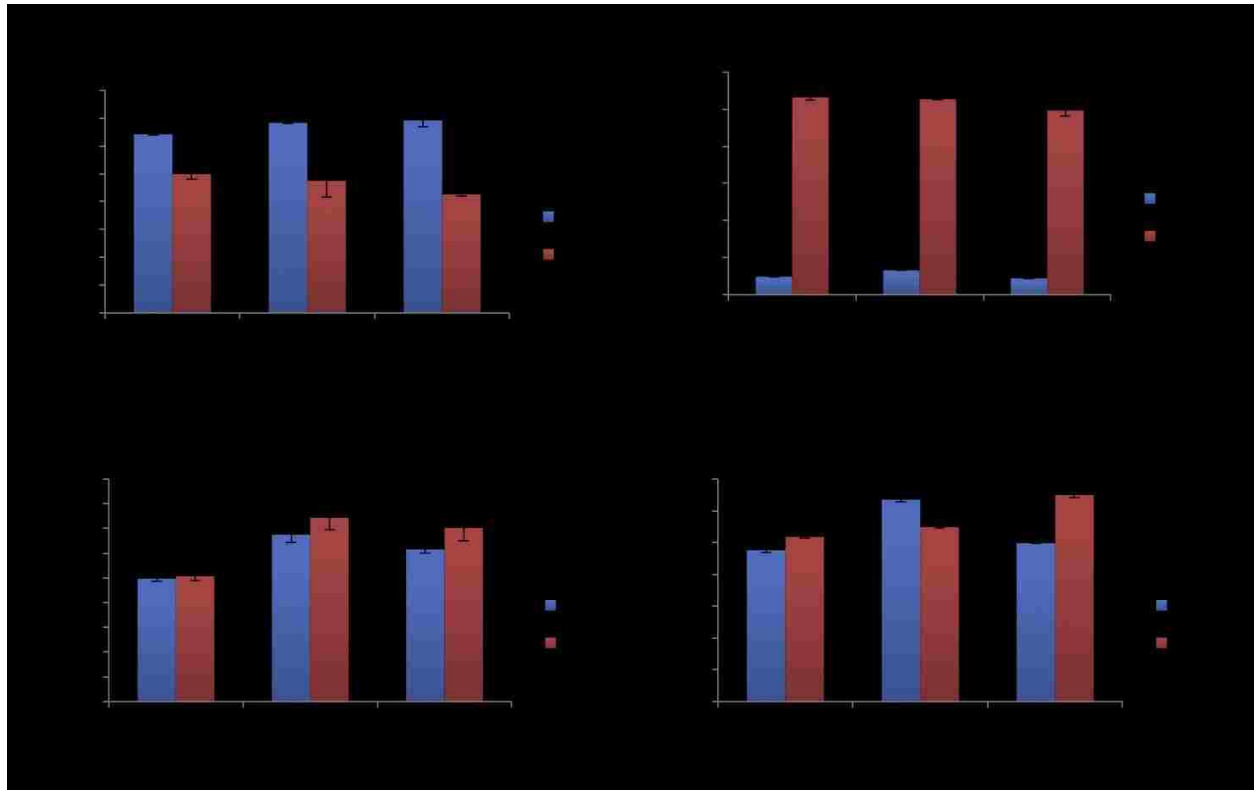
Figure 11: Mineral nutrient content analysis expressed as % dry weight of wild type Col0 and ATag lines lines 440-B4-7 and 441-H1-70 (Control 0 NaCl and Stress 150 mM NaCl). The error bars are showing the 99% and 95% confidence interval of t-test. The data are average of three replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05. (A) Potassium, (B) Sodium, (C) Calcium, (D) Magnesium.

Chapter 3

Identification and Characterization of Salinity Tolerance Genes

from Arabidopsis Activation Tagged Mutant Lines

Abstract

Many crops, selected to produce under optimal field conditions are often faced with high saline environments, either naturally occurring or produced as a consequence of continuous agricultural production. There have been long-term efforts using many genetic strategies to identify salt tolerance genes in various plants species. Gene knockouts are one of the genetic tools by loss of function mutations that can reveal such functions, but this method is mostly not able to reveal the functions of redundant genes or those with a minor phenotype. Gene overexpression analysis, including the use of activation-tagging using Agrobacterium T-DNA and plant transposons, has been used in model plants to identify gain-of-function mutants for genes that have a redundant function, but have a quantitative determined phenotype that can be screened for. In this study, activation tagging using the maize En-I (Spm) transposon system was applied using a collection of about 300 Arabidopsis thaliana mutant lines to identify and characterize activation tagged (I-ATag) salt tolerant candidate genes from several tolerant mutant lines. The genomic DNA flanking sequences of I-ATag insertions of the activating I-element (AIE) were isolated using TAIL PCR then sequenced, and the candidate flanking genes characterized. Two tolerant lines, AIE7 and AIE70, were selected that showed over-expression of adjacent genes which could be candidates for salt stress response and tolerance, caused by the CaMV 35S enhancer present in the AIE enhancing expression of the candidate adjacent genes. The AIE7 mutant line with the activation tagged AT2G41430 genes, annotated as 'Early Response to Dehydration' (ERD) protein family, and AT2G41410, annotated as 'EF-hand calcium binding protein', are candidate genes for salt tolerance.

1.0 Introduction

Salt stress is one of the environmental stress factors that cause significant losses in agricultural land and crop production. A high level of soluble salts in the soil, comprising mainly of NaCl, affects water availability and causes osmotic stress leading to the slower growth of plants. The major physiological perturbation is also caused by salt entering due to the transpiration pull, damaging the cells in transpiring leaves and limiting the growth of plants. There are two phases of growth response affected by salt stress. The first phase effects are minor, in which the plant inhibits the building up of Na^+ and Cl^- ions, and salt is effectively excluded or compartmentalized in vacuoles, with a response quite similar to drought response. The second phase is the major response, wherein the salt is built up in the cell wall and cytoplasm, and causes dehydration of the cell (Munns, 1993).

Tolerance of plants to salt stress can be accomplished by regulating the expression level of the effectors or regulator genes, to re-establish cellular ion homeostasis during salt stress conditions, and promote successful adaptation (Zhu, 2001). The genes that can increase salt tolerance fall into three main categories: transporters that maintain the uptake and efflux of salts, genes that have protective and osmotic functions, and regulatory genes that maintain growth under saline soil by coordinate regulation of plant protective responses. To catalog these, the Arabidopsis Stress Responsive Gene Database (ASRGD) has recorded 139 salt stress responsive genes (Borkotoky et al., 2013) that reveal a number of different stress response mechanisms.

Arabidopsis genes that have been characterized by mutant analysis to be associated with increased salt-tolerance phenotypes include the *sañ*, RS17, RS19, RS20, *pst1* and the *sos* mutants, namely *SOS3*, *SOS2*, and *SOS1* (Quesada et al., 2000; Zhu, 2000). The *sañ* sets of

mutants were the result of extensive screening for mutations in the L*er*, Ws-2, and Col ecotypes of Arabidopsis (Quesada et al., 2000). Mutagens used for these gene backgrounds include EMS, fast neutrons and T-DNA (Quesada et al., 2000). RS17, RS19 and RS20 are mutants (for resistance to salts) selected from Arabidopsis that exhibit the ability to germinate under saline conditions (Saleki et al., 1993). These mutant lines showed tolerance not only to NaCl but also to KCl, K_2SO_4, LiCl and mannitol (Saleki et al., 1993). Another salinity-tolerant mutant found in Arabidopsis is the *pst1* mutant (for *photoautotrophic salt tolerance1*), which has the ability to "detoxify active oxygen species and thus enhances plant tolerance to oxidative stress as well as salt stress" (Tsugane et al., 1999; Zhu, 2000). Given its detoxification ability, the *pst1* mutant was also capable of tolerating other abiotic stresses such as light, heat, freezing, and drought (Tsugane et al., 1999; Zhu, 2000).

Other Arabidopsis mutants found associated with salt-tolerance are the *sos,* or *salt overly sensitive* mutants (Zhu, 2000). There are three *sos* mutants, *SOS1*, *SOS2* and *SOS3*. The *SOS3* gene was shown to encode a Ca-binding protein that has three EF- hands (Liu and Zhu, 1998; Zhu, 2000; Ishitani et al., 2000). The *SOS2* gene encodes a "Ser/Thr protein kinase of 446 amino acids with an estimated molecular mass of 51 kD (Zhu, 2000), and the most recently cloned *SOS1* gene was shown to encode a putative antiporter of sodium/hydrogen ions" (Shi et al., 2000). The *SOS* genes play a significant role in establishing the salinity tolerance pathway in plants. Other genes involved in conferring a salt-tolerance phenotype, in other crop plants such as maize, include the *PMP3* gene, which plays an important role in establishing a successful ion homeostasis mechanism under salt stress (Fu et al., 2012). The *PMP3* gene enhances ion homeostasis by maintaining membrane potential in cells which results in better regulation of ion absorption under saline conditions (Fu et al., 2012).

Another gene involved in salt tolerance is the barley *HVA1* gene which enhances relative water content in leaves, increases leaf and root biomass, and increases plant survival under stressful conditions (Hong and Ho. 1992). The *HVA1* gene was also shown by transformation to increase salt tolerance in maize (Hong and Ho. 1992). Aside from the *HVA1* gene, the gene *mtlD* also confers salt and drought tolerance phenotype in maize, and the combination *HVA1* and *mtlD* imparts higher relative water content in leaves and overall higher plant survival compared to transgenic plants mutated with *HVA1* or *mtlD* (Nguyen et al., 2013). Under saline conditions, plants expressing a combination of the *HVA1* and *mtlD* genes showed fresher and drier shoots and shoot matter as compared to that observed in plants expressing just one of the two genes (Nguyen et al., 2013).

Genes that regulate the activity of protein kinases play a significant role in the development of salinity tolerance traits in plants. This is because protein kinases are largely involved in the signal transduction associated with salt stress and ABA (Shen et al., 2001). In plants, the protein kinase gene *Esi47* from the salt-tolerant species of wild wheatgrass (*Lophopyrum elongatum*) was found to be in the "novel Arabidopsis protein kinase" group, which largely includes serine/threonine protein kinases in plants (Shen et al., 2001, p. 142). To date, there are three (3) *Esi47* homologs described in *Arabidopsis* (Shen et al., 2001). All of these homologs show different mechanisms in providing tolerance to salt stress and ABA response in the leaves and roots of Arabidopsis plants (Shen et al., 2001).

In addition to the functional genes for salt tolerance, transcription factors have also been associated with abiotic stress, including salt tolerance. Transcription factors play an important function in stress signal transduction and the modulation of gene expression during the development of plants (Jin et al., 2013). Specifically, TFs contain DNA domains whose function

is to bind to cis-acting elements located in the promoter region of specific downstream genes (Saibo et al., 2009). TFs function by either inducing or repressing RNA polymerase activity in order to regulate gene expression (Rabara et al., 2014). Given this function, TFs are viewed as master regulators of genes and cellular processes and this ability makes them an ideal candidate for modifying stress tolerance traits in crop plants (Kasuga et al., 2012; Beckett, 2001; Riechmann et al., 2000; Kumar & Bandhu., 2005; Mizoi et al., 2012: Rushton et al., 2010; Shu et al., 2015; Puranik et al., 2012; Wang et al., 2014; Jin et al., 2013). Families of genes that are identified to encode TFs include *AREB*, *DREB*, *WRKY*, *NAC*, and *bZIP* (Kasuga et al., 2012; Beckett, 2001; Riechmann et al., 2000; Kumar & Bandhu., 2005; Mizoi et al., 2012: Rushton et al., 2010; Shu et al., 2015; Puranik et al., 2012; Wang et al., 2014; Jin et al., 2013), shown in Table 1.

Among the important traits regulated by TFs is plant tolerance to abiotic stresses, such as drought and salinity stress (Joshi et al., 2016). The group of genes that is regulated by TFs is termed a regulon, and there are four regulons related to abiotic stress and salinity tolerance (Saibo et al., 2009). These four regulons are CBF/DREB, NAC and ZF-HD, AREB/ABF, and MYC (Saibo et al., 2009).

The CBF/DREB regulon is a group of genes related to the plant's ability to tolerate cold stress (Dubouzet et al., 2003). The CBF/DREB regulon exclusively exists in plants, including those that do not exhibit cold acclimation properties (Dubouzet et al., 2003). The CBF/DREB regulon is activated rapidly and temporarily by cold stress, and the TFs that regulate this regulon also signal the expression of other genes whose functions are related to cold stress response and tolerance (Dubouzet et al., 2003). Moreover, the overexpression of CBF/DREB1 in Arabidopsis

plants increased the survival rate of the plant in response to salt and drought (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999).

The second regulon comprising of NAC and ZF-HD, shows expression when the plant is exposed to dehydration and high salinity stress (Saibo et al., 2009). TFs regulating the NAC and ZF-HD regulons are responsible for activating the ERD1 gene that is associated with dehydration stress tolerance in plants (Nakashima et al., 1997; Tran et al., 2007). But aside from ERD1, the TFs in NAC and ZF-HD regulons are found to activate other genes related to stress tolerance as shown by the finding that overexpression of the NAC regulon results in enhanced drought tolerance in *Arabidopsis*, but without the activation of the ERD1 gene (Tran et al., 2007). The CBF/DREB, NAC and ZF-HD regulons are all ABA-independent, and their expression is unrelated to the presence or lack of abscisic acid (Saibo et al., 2009).

The third regulon, AREB/ABF, contains either AREBs or ABFs that are generally characterized as bZIP TFs capable of binding to the ABRE motif and induce expression of ABA-dependent genes (Saibo et al., 2009). The TFs of the AREB/ABF regulon are also associated with the activation of other protein kinases that depend on the ABA signal transduction pathway (Mustilli et al., 2002; Yoshida et al., 2002). These TFs are capable of regulating stomatal closure in times of drought, high salinity and ABA stress (Mustilli et al., 2002; Yoshida et al., 2002).

The fourth regulon, MYC/MYB, is activated when plants are exposed to drought stress (Saibo et al., 2009). MYC and/or MYB TF binding cis-elements are located in the promoter region of the RD22 gene that is associated with the plant's ability to tolerate drought stress, depending on the presence of ABA signals (Abe et al., 2003). Expression of the RD22 gene through the ABA signal transduction pathway also activates the MYC/MYB TFs, which then

results in enhanced sensitivity to ABA and increased drought tolerance (Saibo et al., 2009). In contrast to the first and second regulons, the third and fourth depend on the presence of ABA (Abe et al., 2003).

The four regulons discussed above are associated with the plant's ability to tolerate abiotic stress. Among the four regulons, the third one shows to have a direct association with high salinity traits in plants. Despite this, it is still necessary to characterize the other regulons and the mechanism of action of their TFs. This is because TFs are often multifunctional and not confined in activating a limited group of genes, as TFs can work in regulating the expression of other gene networks and factors such as protein kinases.

Transposon mutagenesis has been used extensively to screen for salt tolerance genes. Transposon activation tagging approach is a comparatively recent approach that targets inducing gain-of-function of genes, in contrast to the suppression or gene knockout approaches that mostly do not give phenotype because of the redundancy of genes involved in stress response and essential biosynthetic pathways (Marsch-Martinez et al., 2002). Transposon based activation tagging is more efficient compared to T-DNA (Marsch-Martinez et al., 2002). This is likely based on the fact that transposons are inclined toward insertion at multiple locations in the chromosome that are near naturally occurring transcriptional active regions, such as in the introns of the genes or in coding regions, and can contribute to the activation of nearby genes or switch on a number of genes along the chromosomal segment in the range of enhancer activity (to a distance about 10kb) (Marsch-Martinez, 2002). Enhancers work by activating gene expression levels, often maintaining the regulatory temporal and spatial patterns and thus quantitatively increasing the effect of gene activity. This is in contrast to overexpression

constructs that increase gene expression constitutively, thus transposon activation tags maintain the natural regulatory pattern but increase the expression of the tagged gene.

Activation tagging is particularly useful in tracking genes involved in metabolism, enabling the evaluation of the vast repertoire of natural plant compounds that are expressed either in low quantities or at specific sites (Borevitz et al., 2000). Since transposon-based activation tagging (especially the En-I/Spm system) has been effective in generating a high frequency of activated/overexpressed genes, it was regarded as an effective way to increase the expression and function of tagged genes that are involved in the signal transduction or transcriptional regulatory pathways of salinity tolerance. This effort is a follow up of others who have used the *En-I* activation tagging system for identifying drought stress tolerance genes, although salinity tolerance phenotypes have also been activated in these general stress tolerant mutants. Therefore, the Arabidopsis activation tagged mutant lines previously developed (Marsch-Martinez et al., 2002) and used by others in the lab (PhD thesis Shital Dixit), were used in this research in order to identify salt stress tolerant genes in Arabidopsis.

2.0 Objective

The objective of this chapter is to identify and characterize genes from Arabidopsis that confer salt tolerance in plants.

To achieve the objective of this study, a forward genetics strategy of gain-of-function activation tagging via transposons was employed. The maize Enhancer-Inhibitor (En-I) system, also known as the Suppressor-Mutator (Spm) transposon system was used to generate activation tags (AT) in the Arabidopsis genome. The I-ATag transposon used in this study contains the CaMV 35S enhancer, which can be mobilized in the genome by transposition, stabilized, and act as an enhancing element on surrounding genes in the genome (Marsch-Martinez et al., 2002). The I-ATag transposon was proposed to function as a generator of gain-of-function mutants that could be selected in a screen to identify salinity tolerance mutants. Salinity tolerance would be identified in Arabidopsis mutant plants by the phenotypes of enhanced growth or biomass compared to the wild type plants grown under salt treatment. The tagged gene for salt tolerance could then be identified as a gene flanking the I-ATag insertion, which would have enhanced expression.

3.0 Materials and Methods

3.1 Plant Genotypes for Activation Tagging

The methodology used in this study is similar to that described previously for the En-I ATag system in ecotype Columbia (Harb and Pereira, 2011; 2013), using the transformation construct described previously for generation of the En-I ATag system in ecotype Wassilewskija (Ws) (Marsch-Martinez et al., 2002). Arabidopsis Columbia ecotype transformants containing the En-I ATag construct were used for selection of transposed I-ATag activation tagged plants from T3 progeny seeds of putative salt-tolerant and salt-sensitive lines (440-B4-7, 440-E1-15, 440-F2-20, 440-G3-25, 440-B3-34, 440-B4-35, 440-H4-48, 440-C2-54, 440-D1-57, 440-F2-64, 440-G3-68, 440-H1-70, 440-A1-73, 441-E2-83, 441-E4-85, 441-G3-89 and 440-G4-90) as described in the previous chapter on salinity screening. Arabidopsis ecotype Columbia (Col) was used in all experiments as sensitive negative control. The ATag lines were renamed AIE7, AIE15, AIE20, AIE25, AIE34, AIE35, AIE48, AIE54, AIE57, AIE64, AIE68, AIE70, AIE73, AIE83, AIE85, AIE89, and AIE90.

3.2 Selection of BASTA resistant ATag lines

The ATag line seeds were germinated by imbibition with water and stratified at 4 °C in the dark for about 3 days. Later, seeds from respective lines were sown in pots occupied with moist soil (professional growing mix) from Sun Gro Horticulture Company, and then all the pots were kept in the growth chamber at 22°C with 12 hour day/night cycles, and 150 to 200 μmol m-^{2} s-^{1}. Plants were fertilized once a week before salt stress application using the water-soluble fertilizer MiracleGro® All Purpose Fertilizer (24N-8P-16K).

After the seeds germinated, 10 seedlings of each line were sprayed twice a week for 2 weeks with a 0.7 mL/L finale (Basta herbicide contains 150 g/L glufosinate ammonium). The survived seedlings were selected for the genotyping experiments.

3.3 Salt Stress Treatment

For salt treatment, 14-21 day old seedlings at the vegetative stage were separated into two groups; salt treated and untreated control grown in separate trays. The salt-treated plants were maintained in a 150 mM NaCl solution for one week, and later physiological, phenotypic and genetic parameters were measured, along with untreated control plants for comparison.

3.4 Genomic DNA isolation

200 mg of leaf samples were collected randomly from young plants (15-21 days) and DNA was isolated from the samples using a CTAB protocol for DNA extraction (Harb & Pereira, 2011; 2013). Green leaf tissue was ground and homogenized using 500 µl of 2X CTAB buffer (pre-mixed with 2% β-mercaptoethanol and pre-heated at 65^0C). The homogenized tissue was incubated at 65^0C for 30 min with intermittent swirling. The tubes were next cooled briefly, then given an equal quantity (500 µl) of chloroform. Isoamyl alcohol (24:1) was added and the contents were mixed gently. The samples were then centrifuged at 5000 rpm for 15 minutes at room temperature. The upper aqueous layer was collected and transferred into new tubes, and then ice-cold isopropanol was added with the equal amount of upper aqueous. The mixture was incubated at -20 ^{0}C for 30 min, and then samples were centrifuged at 12000 rpm for 1 minute. After precipitation of nucleic acids, pellets were washed with cold 70% ethanol and air-dried.

The pellet was then suspended in 30 µl TE buffer and DNA concentration was measured using the NanoDrop Spectrophotometer. The DNA samples were then stored at -20 °C.

3.5 Thermal Asymmetric Interlaced PCR (TAIL-PCR)

Three reactions of TAIL-PCR were performed to identify genes adjacent to transposons using 20 ng DNA and the primers listed in Table 2 based on the protocol described by Harb and Pereira, 2011; 2013. DNA was isolated as described above from the candidate tolerant and sensitive mutant lines as well as WT Col-0. The primary TAIL PCR reaction consisted of a mix of 2 µl of DNA template, 1× PCR buffer, 1.5 mM MgCl2, 0.1 mM dNTPs, 1 U Taq polymerase, 0.15 µM Int2 primer (which is the furthest I-transposon right-junction (RJ) primer of the transposon insertion with every sample), and 2 µM degenerate primers (AD1, AD2, AD3, AD4, AD5, and AD6) individually in 6 separate reactions in a total of 20ul reaction mixture. The primary round of the Tail PCR is as follows: 1 Cycle denaturation at 94 °C for 2 minutes, 5 Cycles at 94 °C for 1 minute, 62 °C for 1 minute, 72 °C for 2 minutes, 1 Cycle at 94 °C for 1 minute, 25°C for 3 minutes, 72 °C for 2 minutes. Then there are 15 Cycles of the following: 2 cycles at 94 °C for 30 seconds, 65 °C for 1 minute, 72 °C for 2 minutes, 1 Cycle at 94 °C for 30 seconds, 45 °C for 1 min, and 72 °C for 2 minutes. The primary tail PCR products were then diluted 1: 40 with sterile distilled water and the diluted product was used as the template for the secondary TAIL PCR reaction. The secondary tail reaction was comprised of mix of 1µL of the diluted primary round tail PCR product, 1× PCR buffer, 0.1 mM dNTPs, 1 U Taq polymerase, 0.2 µM Irj-201 primer (which is the one inside the RJ - primer of the transposon insertion with every single reaction), and 2 µM degenerate primers (AD1, AD2, AD3, AD4, AD5, and AD6) in separate reactions of a total of 20ul reaction mixture each. The secondary round in the Tail PCR begins with 1 Cycle at

93°C for 1 minute then 13 cycles of the following: 2 Cycles at 94 °C for 30 seconds, 62 °C for 1 minute, 72°C for 2 min, 1 Cycle at 94 °C for 30 seconds, 45 °C for 1 minute, and 72 °C for 2 minutes. The secondary tail PCR reaction products were then diluted using sterile distilled water by 1:10 and used as the template for the tertiary reaction. The tertiary tail PCR reaction contained 1 μL of the diluted secondary PCR product, 1× PCR buffer, 1.5 mM MgCl2, 0.1 mM dNTPs, 1 U Taq polymerase, 0.2 μM DSpm1 primer (which is the nearest to the I-transposon right junction primer of the transposon insertion with every single), and 2 μM degenerate primers (AD primers 1-6) separately in a total volume of 40 μl reaction mixtures. The tertiary tail PCR reaction was performed as follows: 1 Cycle at 93 °C for 1 minute, 20 Cycles at 94 °C for 30 seconds, 45 °C for 1 minute, and 72 °C for 2 minutes. The products of the tertiary tail PCR were then run on a 1% agarose gel and the distinct bands were excised from the gel. Specific fragment bands of interest were then purified and sequenced as explained above, then the sequences were aligned to the Arabidopsis genome using Phytozome 9.1 (http://phytozome.jgi.doe.gov/pz/portal.html) and The Arabidopsis Information Resource TAIR (https://www.arabidopsis.org/index.jsp) for the identification of the position of the Activation tag element insertions and information of the tagged genes.

3.6 Polymerase Chain Reaction (PCR)

Genomic DNA (20 ng) of the mutant lines, was amplified with gene specific and transposon primers, in a PCR reaction mix using a standard PCR program: initial denaturation at 95^0C for 5 minutes, then 29 cycles of 95^0C for 1 minute, T_m (melting temperature) at 58 ^{0}C for 30 seconds, 72^0C for 2 minutes, and final extension at 72^0C for 10 minutes. The PCR product was gel-purified using the EZNA Gel Extraction Kit from Omega Bio-Tek Inc and sequenced using gene

specific primers (Table 3) to verify that the products amplified were the predicted target sequence based on TAIL-PCR results (section 3.5). The sequenced PCR product with many N's and mismatches were additionally cloned into TOPO TA Cloning vector kit (Invitrogen) in order to obtain quality sequence of the tagged gene where the transposon was inserted.

3.9 Analysis of Transposition of Activation Tag Elements using Southern Blot Analysis

Southern blot hybridization analysis was used to identify the copy number of insertion sites in the Arabidopsis mutant genome, with the wild type Columbia-0 used as negative control. For Southern blot analysis, at least 500 ng or (1g genomic DNA) of each plant was digested overnight with *Eco*RI. Digested DNA samples were then loaded and electrophoresed on a 0.8 % w/v agarose gel with ethidium bromide in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA). The separated DNA fragments were transferred to Hybond N+ membranes (Amersham, Buckinghamshire, UK GE Life Sciences Inc.). Restriction digestion with *Eco*RI enzyme enables the differentiation of I-ATag elements in the original full donor site (FDS) of the vector and of the empty donor site (EDS) of transposed ATag lines. In order to distinguish the EDS and FDS lines along with the number of ATag copies, the BAR gene fragment, a part of the ATag element, was used for hybridization. The 513 bp BAR probe was amplified by PCR from a plasmid DNA as a template using primers:

Bar F1: 5'-ACCATGAGCCCAGAACGACGC-3'

Bar R1: 5'-CAGGCTGAAGTCCAGCTGCCAG-3'

The PCR products were then gel purified from the specific band used for making the probe. The membrane was pre-hybridized for two hours in hybridization buffer, and then in the

hybridization buffer with a radioactive ^{32}P-labeled DNA probe that was carried out overnight at 65°C in the hybridization oven. The membrane was washed 3 times at room temperature for 15 minutes using 3 solutions of 2X SDS+ 0.5X SDS, 1X SDS+ 0.25X SD, (0.5X SDS+ 0.125X SDS consecutively at 65°C, respectively. Membrane signals were detected and exposed to X-ray films by autoradiography.

3.10 Genetic Analysis of mutants:

The I-ATag plant lines AIE7, AIE20, AIE25, AIE34, AIE64, AIE68, AIE70, AIE73, AIE83, AIE85 were verified by sequencing the transposon flanking DNA, with transposon specific primer Irj201 and gene specific primers where known. The population of 20 plants of the stable transposed elements AIE7 and AIE70 were examined for visible morphological phenotypic traits and then genotyped. Three replicates of putative mutants used as pollen donors were crossed to the wild type ecotype Columbia (Col-0) to segregate any other background ATag inserts. The F1 progeny were sown and sprayed with Basta herbicide, and the Basta resistant plants were then allowed to self-fertilize to confirm the heritability and dominance of the phenotypic traits. 12 plants of the F2 progeny seedlings were sprayed 3 times with a 0.7 mL/L Finale (Basta herbicide contains 150 g/L glufosinate ammonium) for any phenotypic trait segregation. The genomic DNA samples of the 24 Basta resistant plants of the F2 were tested for homozygosity and genotyped by PCR analysis using two reactions, one reaction with gene specific primers, one reaction with the transposon specific primer Irj201, and one for each direction of the genes specific primer (forward or reverse).

3.11 Gene Expression Analysis

3.11.1 RNA Isolation

Total RNA was extracted from the leaf of all samples using Trizol (Invitrogen). The RNA isolation procedure began by powdering leaf tissue in liquid nitrogen and adding 1 ml of Trizol to the ground tissue. The samples were then incubated at room temperature for 5 minutes and then 200 µl of chloroform was added. The tubes of mixture were vortexed well and the mixed solution was then incubated at room temperature for about 2 min. The tubes were then centrifuged at 12000 rpm for 15 minutes at 4^0C. The supernatant of the samples was then collected and 500 µl of cool isopropanol was added to the collection and mixed, followed by incubation at room temperature for 10 minutes. The tubes were then spun in the centrifuge at 12000 rpm for 10 minutes at $4\ ^0$C, the tubes removed carefully from the centrifuge, the supernatant discarded, 500 µl of 75% ethanol added, and the tubes again centrifuged at 7500 rpm for 5 min at 4^0C. Finally, the pellets were kept to air-dry for about 10 minutes and then 35 µl of nuclease free water wasadded, and the RNA solution stored at -80^0C.

3.11.2 cDNA synthesis

To set up the cDNA synthesis reactions, 4 µg of RNA sample was used for each reaction. RNA samples were treated with 2 µl of Promega RQ1 DNAse 1, 3.5 µl 10 X RT Buffer, 4 µl MgCl2, 0.5 µl RNase inhibitor, and dH2O based on the RNA sample concentration. The final reactions of 34 µl were incubated in the PCR machine at 37°C for 30 min. Afterward, the DNAse 1 was inactivated by adding 1 µl of RQ1 DNA stop solution followed by incubation in the PCR machine at 65^0C for 10 min. The reaction mixture was then immediately placed in ice for 15 minutes, and 5 µl of the following mix was added to the RNA: 0.5 µl 10xRT buffer, 1 µl dNTPs,

1 µl Random Primers, 1 µl Reverse Transcriptase Enzyme, and 1.5 µl dH2O. The final reaction was made to 40 µl. Finally, the RT reaction was carried out at 42°C for 1 hour and 95°C for 5 minutes. The cDNA concentration was then measured using a NanoDrop Spectrophotometer and the cDNA samples were stored at -20 °C. 2 µl of the total RT reaction was used to perform the qPCR with the gene specific primers (Table 4).

3.11.3 Quantitative PCR (qPCR)

To set up the qPCR, 10 µl of the reaction mixture was used, which was comprised of 2 µl cDNA, 5 µl qPCR buffer (GoTaq® qPCR Master Mix, Promega), 0.5 µl of each of the forward and reverse primers, and 2 µl H_2O. The qRT-PCR experiments were conducted using GoTaq® qPCR Master Mix (Promega), gene-specific primers (Table 4), and Ubiquitin used as standard with three biological replicates in a CFX-96 Bio-Rad thermocycler (Bio-Rad). Increasing the temperature (0.5°C 10 s^{-1}) from 55°C to 95°C was used for melt curve analysis. Un-transcribed RNA was also run as negative control2. The fold change in expression of each sample in individual experiments was determined by normalizing the Ct value for each gene against the Ct value of Ubiquitin reference genes, and was calculated relative to the corresponding control using the equation $2^{-\Delta\Delta Ct}$.

3.12 PCR of Transposed element lines

To determine the structure of the En-I transposon cassette after the I-Atag transposed (Marsch-Martinez et al., 2002), primers from the En transposon were designed from sequences flanking the En/Spm transposable element at promoter junction, and the Right Border (RB) of the T-DNA

construct. The primers RGT-35S and RGT-SSU were designed from the terminators of the 35S and SSU in the construct to check if I-ATag transposon was excised from construct or still there, and used to amplify the empty donor site fragment to detect excision of the AT element (Table).

En-1010F- CTGCAGCCAAACACATTTTCGC

En-1474- ACCATGAGTGACACTGTCGAATCC

RGT-35S-TCAACACATGAGCGAAACCC

RGT-SSU- GTTGGTTGAGAGTCTTGTGGCCT

Genomic PCR was performed using the gene specific primers to test if the Activation tag I-ATag elements moved from the original position in the construct. The PCR reaction mix comprised of 1 µl template DNA, 1 µl of each the forward and reverse primers, 10 µl of PCR buffer and 7 µl of sterilized water. The PCR reaction conditions used were initial denaturation at 95^0C for 5 min, followed by 29 cycles of 95^0C for 1 min, 58^0C for 30 seconds, 72^0C for 2 minutes and final extension at 72^0C for 10 minutes. The products of the PCR reaction were run on a 1% agarose gel and then photographed under exposure of the gel to UV light.

3.13 Genotypic and Phenotypic analysis of Candidate genes:

The knockout mutant seeds of the candidate genes for salt tolerance, based on qPCR expression analysis of the ATag flanking genes, were obtained from The Arabidopsis Biological Resource Center (ABRC). The putative candidate genes were AT2G41400, AT2G41410, AT2G41420, AT2G41430, AT2G41440, AT3GG5280, and AT3GG5240, which were grown in the growth chamber and DNA isolated. Genomic DNA samples of the individual candidate lines were used to test for homozygous inserts using gene specific primers listed in Table 5. The homozygous

insert genotypes and the wild type Columbia with 10 replicates were grown and tested for one week for salinity tolerance using 100 mM NaCl, beginning gradually with 50 mM. Then the samples for control and stress conditions were collected and kept in the oven at 70 °C for three days until completely dry. Relative reduction in biomass of the samples was calculated using the equation [(Biomass under control condition) – (Biomass under stress condition) / (Biomass under control condition)]. The data was analyzed using Analysis of Variance (ANOVA) and the t-Test: Two-Sample Assuming Equal Variances.

4.0 Results

4.1 Isolation of ATag transposon flanking DNA by TAIL-PCR:

TAIL-PCR was performed to identify DNA flanking the insertion sites of the transposed elements in the tolerant/sensitive AIE lines that were identified in the previous chapter in screens for salt tolerance. The AIE lines included 13 salt tolerant (AIE7, AIE15, AIE25, AIE34, AIE35, AIE48, AIE54, AIE57, AIE68, AIE70, AIE73, AIE85 and AIE90) and 4 salt sensitive lines (AIE20, AIE64, AIE83, AIE89). Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is an effective technique used to amplify unknown genomic sequences adjacent to known genomic sequences present in the insertion site. In this study three specific nested primers in the AIE transposon were used in combination with six arbitrary degenerate primers in the genome for amplification of the DNA adjacent to the activation tagged element (Figure 1). The transposon specific primer and AD primers are designed to have changes in annealing temperatures with alternating cycles of high and low annealing temperature, leading to increased specificity of amplification of yield products (Singer et al., 2003). The primary reaction has primer Int1, which is the furthest from the transposon insertion site adjacent to the tagged gene. The secondary reaction utilized primer Irj-201, which is located closer to the I-ATag terminus. Lastly, in the tertiary reaction the dSpm1 primer is used, which is typically annealed toward the end of the AIE element junction, adjacent to the tagged gene. Using this method, the specificity of amplifying the target sequence is increased with each reaction, while the non-target sequence is decreased. The products of the tertiary TAIL-PCR reaction amplified using the third specific primer DSpm1 showed the gene-specific flanked sequence in all tagged ATag lines, however not all of the arbitrary degenerate primers with transposons amplified a product. The stringency of AD primers with transposon primer annealing near the gene depends on the mix of different

degeneracy level of AD primers during TAIL PCR reaction, therefore only some AD primers were able to amplify their target sequence. However, the TAIL PCR protocol successfully recovered flanking gene DNA fragments adjacent to the AIE for the tagged ATag lines (Figures 2, 3, 4).

4.2 Sequencing of TAIL-PCR products and position of transposon insertions:

The extracted DNA from TAIL-PCR products after the last primer I-terminal inverted repeat (ITIR-3) was then sent to Eurofins Genomics LLC for sequencing. The DNA sequencing chromatogram data were analyzed and the sequences were identified against the Arabidopsis genome using The Arabidopsis Information Resource (TAIR) resources. The output from the sequencing of tagged genes that were identified in the AIE mutant lines is shown in Table 6. Subsequently, the position of transposon AIE insertions in the Arabidopsis genome of Columbia activation tag mutants were identified using The Arabidopsis Information Resource (TAIR) and Phytozome v12.1 resources. Based on BLASTN results, the coordinates and direction of candidate surrounding genes located up to 10 kb upstream and 10 kb downstream of the ATag, and their distance from the 35S enhancer in the AIE insertion was calculated, and models for the mutant structure were drawn (Figure 5).

The range of flanking candidate genes was chosen based on previous studies of Activation tagged genes, in which the expected and verified enhancer activity of the 35S CaMV enhancer on a gene can range from 10 kb upstream to 10kb downstream of the insertion site (Weigel et al., 2000; Marsch-Martinez, et al., 2002). In most mutant lines the ATag element was found to have

inserted in the coding region (AIE7, AIE20, AIE70, AIE73, AIE85, AIE89 and AIE90) and for some mutants the insertion was in 3` UTR (AIE34 and AIE83) (Figure 5).

4.3 Characterization of ATag mutant lines:

The Arabidopsis Information Resource (TAIR) database was used for collecting genetic and expression data of the candidate genes amplified with TAIL PCR. The activation tagged gene with gene loci number, function, and expression from various tissue types is summarized in Table 1. Based on the position of transposon insertions in the Arabidopsis genome the results showed that the activation elements were located in the coding region of genes for most of the mutant lines. In such cases, there are multiple possible scenarios that can cause the salt stress phenotypes. First, only homozygous lines or knock-outs of the tagged gene can contribute to the phenotype. Secondly, there can be multiple undetected insertions of transposons in the genome contributing to the phenotype. Last, the insertion in the heterozygous state with the activation tag enhances the activity of the nearby genes and contributes to the salt stress phenotype. In order to distinguish between the multiple possibilities, a systematic genetic and molecular approach was taken to understand the role of the activation tag for salt tolerance.

4.4 Southern Blot Analysis of Transposition of ATag elements:

In order to test whether there are multiple I-ATag inserts in the mutant genome, a Southern blot for genomic DNA was performed. The Southern blot analysis with a BAR gene probe on the activation tag lines showed a number of single or multiple copy inserts with the Basta gene probe

(Figure 6). The results of the Southern blot analysis revealed that most of the ATag lines along with Col-wild type shared a common band. However, one lines contained one or more additional bands, suggesting multiple copies of transposed I inserts in the tagged lines (Figure 7). The tolerant line AIE7 contained a single insertion with a size of 12.000 kb. Single inserts were found in other activation tag lines but their estimated sizes were different: AIE34 (11.900 kb), AIE2 (11.000 kb), AIE70 (4.100 kb), and AIE83 (1.900 kb). However, the AIE73 ATag line comprised of two copies of BAR inserts, one of 11.000 kb and the other of 4.000 kb. The Southern blot analysis provided a number of restriction fragments hybridizing to the I-ATag element, which indicate the number of AIE insertion copies present at one or more loci in the mutant line genome. The lines with more inserts present in their genome could have multiple complete AIE element insertions. On the other hand, the lines with one insert indicate the presence of a single AIE insertion, which is most likely contributing to the salt-tolerance phenotype. The mutant lines with multiple insertions had to be crossed with the wild-type Col in order to segregate out the insert contributing to the salt tolerance phenotype.

4.5 Segregation analysis of the Activation Tag lines:

Since the Southern blot showed one insertion in the AIE7 and AIE70 lines and the tagged genes showed some candidate genes 10kb upstream or downstream that could be involved in salt tolerance, further genetic analysis of these lines was conducted to verify the T3 generation of Basta resistance segregation ratios. In the 20 plants from the T3 progeny of AIE7 and AIE70 lines sprayed with Basta, all of the seedlings survived, suggesting that the original T2 plant was homozygous for the insert, or there was another I-ATag insert in the genome. However, genomic

PCR performed with gene-specific and transposon primers of both of the lines (AIE7 and AIE70) showed that they were heterozygous for the I-ATag insert. This suggests that the ATag line was probably homozygous, but with an En-transposase in the background giving rise to the wild-type fragment due to excision products in some cells, suggesting transposase activity in somatic cells (Figure 8, 9, 10, 11). The lower band represents the empty donor site of the target gene and the larger band includes the ATag transposon. The alternative explanation for none of the progeny being homozygous is that the homozygous embryos could be lethal.

4.6 Genetic Analysis of Activation tagging lines AIE7 and AIE70:

In order to understand the genetic segregation data and conclude if the resultant phenotype is because of the ATag insert near the potential target gene, the pollen from the AIE7 T3 line and the AIE70 T3 line was crossed with the wild type (Col0). The F1 progeny of AIE7 survived Basta application, but the AIE70 progeny were completely dead. This indicates that the AIE7 was successfully crossed with the wild type but the line AIE70 was not. Therefore, AIE7 progeny were selfed and the F2 progeny were sprayed with Basta herbicide. The results of selfed progeny from eight F2 plants showed Mendelian segregation after Basta treatment. The segregation analysis of 8 F2 progeny shows that two F2 progeny were completely Basta sensitive (20 plants per progeny), four F2 progeny showed Mendelian segregation (20 plants per progeny) and two showed all Basta resistant (20 plants per progeny) (Figure 12).

The PCR analysis of the genotyped 24 plants of the F2 progeny (12 from all Basta resistant progeny and 12 from segregating progeny) showed that all plants were heterozygous (using gene specific forward and reverse primers in one reaction) (Figure 13), and the transposon

Irj201 primer with the reverse direction of gene specific primers in another reaction (Figure 14). These results again suggest that the transposase is in the background (upper band in Figure 13) and is causing distortion in Mendelian segregation as observed in PCR analysis.

In order to further test for the presence of the En transposase, the En-F and En-R primers with positions from En sequence as shown in Figure 17 were used, although technically the remnant cassette should have been deselected with the SU1 negative selectable marker (Marsch Martinez et al., 2002). However, out of 24 F2 progeny, 21 show amplification with the En primer, suggesting that the cassette is still segregating in the line and the En-transposase is active and can still destabilize the I-ATag element in some cells (Figure 15). The presence of the SU1 marker gene was also checked using the primers RGT-35S and RGT-SSU (SSU = RuBisCo small subunit terminator) from the terminators of the 35S and SU1 in construct redrawn from Marsch-Martinez et al., (2002) (Figure 16, 17). The PCR results confirm that there is absence of SU1 in the remnant cassette as expected for successful selection against presence by spraying with the pro-herbicide R7402 (Marsch-Martinez et al., 2002).

In conclusion, the complete cassette of the En-IATag-SU1 gene was not completely deselected with the negative selection of the R7402 spray, as during Agrobacterium transformation the region from the right border at far right side of T-DNA cassette shown in Figure 17, (SSU marker for R7402 resistance) was probably truncated. The results from the F2 progeny screen show one plant (#11) with no amplification with the En-F and En-R primers, but show amplification with the gene specific primer and transposon. This suggests that this plant is heterozygous and is probably stable, as it does not have the En transposase. Thus, the screening of the progeny of this line (plant #10) should be able to show Mendelian segregation.

The salinity screen results of the 18 F2 progeny of the crosses of Col0 to AIE7 displayed a significant decrease in the relative reduction of plant biomass with a mean of ~0.2 gm, compared to the wild type Col-0 mean of ~0.44 gm in response to salinity stress treatment (Figure 18).

4.7 Expression Analysis of Tagged Genes and Neighboring Candidate Genes in Response to Salt Stress:

To study the expression of the most likely activated candidate genes by the ATag element of the AIE7 and AIE70 lines for their role in salt tolerance, primers were designed based on insertion sites and the genome sequence of the genes. The primers were designed specific to the tagged genes and to the adjacent genes spanning 10kb upstream and 10kb downstream of the ATag insertion site. For AIE7, the putative AIE insertion was in gene AT2G41400, and the adjacent genes are AT2G41410, AT2G41415, AT2G41420, AT2G41430, AT2G41440 upstream and AT2G41390 and AT2G41380 downstream. For AIE70 the putative insertion was in gene AT3G50280 and the adjacent genes are AT3G50270, AT3G50260, AT3G50250, AT3G50240 upstream and AT3G50290, AT3G50300 and AT3G50310 downstream.

Total RNA was isolated from the mutants AIE7, AIE70, and wild-type Col0 for control and stress treatment, cDNA was synthesized, and qPCR performed using UBQ10 (AT4G05320) as a reference control gene for expression. The gene expression analysis using qPCR is displayed for AIE7 in Figure 19 and for AIE70 in Figure 20. The analysis of the results shows that line AIE7 shows no expression for the gene AT2G41400- Pollen Ole e 1 allergen, and the unknown gene AT2G41415 in response to salt stress and control conditions. Based on the Arabidopsis eFP

browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), the AT2G41400 gene is only expressed in the seed embryo but not in the shoot, which is used here for the gene expression analysis. However, the expression of some adjacent genes was highly induced in response to salt stress: AT2G41410-Calcium-binding EF-hand family protein (~3.5-Fold), AT2G41420- Proline-rich family protein (~1.8-Fold), AT2G41430- Early Responsive to Dehydration 15 (~12.1-Fold), AT2G41440- unknown protein (~7.8-Fold), AT2G41380- Methyltransferase activity (~7.11-Fold), and AT2G41390- Pollen Ole e 1 allergen (~3.9-Fold).

In the case of the line AIE70, the expression of the insertion tagged gene (AT3G50280-HXXXD-type acyl-transferase family protein) was same in both control and stress conditions, showing no expression. Genes AT3G50270 and AT3G50230, which belong to the same family of HXXXD-type acyl-transferase, AT3G50310- that encodes a member of MEKK subfamily, and AT3G50250 that involves in elemental activities, showed no expression in either conditions of control and stress. On the other hand, the expression for some adjacent genes was significantly up-regulated in stressed plants (AT3G50240- that is involved in cell wall organization was increased ~1.6-Fold, AT3G50260- that encodes a member of the DREB subfamily increased ~0.2-Fold, and AT3G50270 a HXXXD-type acyl-transferase family protein increased by ~1.2-Fold, suggestive of candidate genes for the phenotype. The higher expression for these adjacent genes could be a›ttributed to the presence of the CaMV 35S enhancer in the AIE element inserted in the tagged gene. Although there is no upregulation of the I-ATag insertion-tagged genes in AIE7 the AIE70 lines as expected, these lines function as stress conditional overexpressors for neighboring genes under salt treatment as shown by the qPCR data.

4.8 Transposon Activation Insertion Tagging Candidate Genes Observation:

The PCR analysis of the activation tagged lines, and specifically lines AIE7 and AIE70, showed that the AIE were inserted in the coding region of genes AT2G41400 and AT3G50280. It is also evident that AIE activates the expression of the tagged genes from as far as 10 kb distance, although inserted within the genes AT2G41400 and AT3G50280, and the respective Arabidopsis ATag lines display salt tolerance phenotypes. This suggests that activation of one or more of the adjacent genes are responsible for the salt tolerance phenotype. Primarily considering the case of the chromosome 2 insertion, i.e. in gene AT2G41400 that has the AIE insertion in line AIE7, the neighboring candidate genes are within a range of 20 kbp away, a distance also shown to be accessible for activation by the the 35S-enhancer (Marsch-Martinez et al., 2002). The genes AT2G41410, AT2G41430 and AT2G41440 were found to be ~1.961 kb, ~5.547 kb and ~ 6.66 kb upstream from the IATag insertion site to the gene promoter respectively, which suggests that the genes can potentially induce salt tolerance to the tagged plant (Figure 25).

The insertion I-ATag tagged gene identified on chromosome 3, adjacent to the AT3G50280 gene (which is predicted to code for an HXXXD-type acyl transferase family protein in line AIE70) is likely not responsible for the gain-of-function in salt tolerance. The tolerance phenotype of the AIE70 line is likely due to I-ATag mediated enhancement, expressed by adjacent candidate genes caused by the activation tag transposon insertion. The candidate tagged genes in the AIE70 line are the AT3G50270 gene at a distance of ~5.985 kb upstream from the IATag insertion site to gene promoter, and AT3G50240 which is around 12.347 kb downstream from the IATag insertion site to gene promoter. Therefore, the over-expression of these candidate genes in the activation tagged line are probably involved in the salt tolerance phenotype of the ATag Arabidopsis line.

4.9 Salt Screening for Candidate Genes for salt tolerance:

An alternative method of studying the function of the candidate genes for salt tolerance can be by an analysis of the knockout mutant phenotype of the candidate gens in response to salt. Therefore, knockout insertion mutants were identified from the Arabidopsis resource TAIR, and the mutant lines prepared for testing potential knockout phenotypes. The knockout lines segregating for the T-DNA insert were grown and used for genotyping with gene and T-DNA specific primers. The T-DNA lines for the genes AT2G41400, AT2G41410, AT2G41420, AT2G41430, AT2G41440, AT3GG5280, and AT3GG5240 showed that most of the plants were heterozygous, so homozygous lines would have to be selected for in the next generation (Figure 21).

However, only the knockout line of the gene AT2G41430 was homozygous and the T-DNA insertion elements were present in this genome of this line (Figure 22). Therefore, the knockout mutant KO-AT2G41430 line was tested for salt stress response. Interestingly, the salt screening of the KO-AT2G41430 line exhibited salt stress-sensitive phenotype compared to wild-type Col0, showing a significant increase in the relative reduction of plant biomass in the T-DNA line compared to the wild type Col-0 (Figure 23, 24). These results support the fact that this candidate gene was activated by the I-ATag transposon insertion, and the line showed a gain of function phenotype function of salt tolerance.

5.0 Discussion

The development of abiotic stress tolerant crops through genetic methods of selection or engineering is increasingly one of the most important solutions used to combat the huge losses due to abiotic stresses such as high salinity conditions. For this, active research has to be done to identify more genes at a genome-wide scale that can impart stress tolerance to offer multiple convenient solutions for crop improvement. It is evident that plants are triggered at the genetic level against these abiotic stresses and respond to signals from salt, cold or drought stresses. An analysis of the signals and tolerance responses will provide an insight in understanding such genes and their functions, which in turn will contribute to the development of tolerant lines in the future that will have the inherent genetic ability to fight off the inhibitory effects of such stress conditions. In this study, the genes that have been identified from Arabidopsis thaliana activation tagged lines are expected to be involved in tolerance against the high salt concentration and expressed in distinct plant parts for carrying out various activities. In this study around 23 candidate genes have been identified in the Arabidopsis ATag mutant lines that might be involved in response and tolerance to salt stress, in some organ or condition, which when activated by the 35S-enhancer provide salt tolerance at a whole plant level.

The salt tolerance phenotype, especially for the tolerant ATag lines AIE7 and AIE70 which have been selected for the genetic analysis described here, are expected to be regulated by the candidate genes described in the tolerant lines that show higher expression than wild-type ecotype Columbia in the presence of salt treatment. For genetic segregation analysis, the heterozygosity of the tolerant lines AIE7 and AIE70 was confirmed by the genetic analysis since T3 progenies were found to be heterozygous, although there were no wild type or homozygous plants found in the lines. The genetic analysis of crossing AIE7 to the Col0 wild type was

confirmed by the evidence of heterozygous plants in the progeny population, as all the plants were tolerant to Basta. This revealed the presence of only heterozygotes and the possibility that the homozygous progeny might be lethal, or of low frequency. The salt tolerance phenotype observed in these heterozygotes also means that this gain-of-function phenotype is contributed to by overexpression of the adjacent gene(s).

Through this activation tagging approach, it was observed that a large number of adjacent and nearby genes are also activated, and the tolerance exhibited may be attributable to a combined action of a number of these genes. In our findings, multiple nearby genes at chromosomes 2 and 5 of the lines AIE7 and AIE70 were found to be highly expressed, which might impart tolerance of the mutant strains to salinity. This enhanced expression of the multiple genes adjacent to the I-ATag insert is likely to be due to the influence of the CaMV enhancer elements present in the I-ATag insertion, which can activate genes more than 10 kb upstream and downstream of the insertion. Our results are consistent with several studies of activation tagging. In a previous study for T-DNA activation tagging (Weigel et al., 2000) it has been stated that genes at a distance of 3kb from the 35S enhancer are likely to be activated and contribute to a unique phenotype. However, in the En-I transposon based activation tagging, the 35S enhancer was shown to have the ability to activate genes on right and left sides of the AIE insertion to a distance of around 10kb adjacent to the insert (Marsch-Martinez et al., 2002).

Another suggestion by previous reports is that the multiple CaMV enhancers presented in Ac/Ds elements can activate one or two genes at the same time in Ds lines (Moin et al., 2016; Mathews et al., 2003). A recent study of salt tolerance screened 70 Ac/Ds activation tag lines from *Oryza sativa* ssp *indica* rice plants in the T3 generation. In the Ds-16 line one activation tagged gene was identified as a salt stress tolerant gene (LOC_Os01g08790) which showed high

expression level in response to 150 mM NaCl. It was also shown that this gene was activated by the tetramer of the CaMV 35S enhancer (Manimaran, et al., 2017). Similarly, the salt tolerance phenotype of the tolerant lines AIE7 and AIE70 lines are regulated by candidate genes that have high induction in response to saline condition, since enhancers can enhance both constitutive and regulated expression of genes.

Transposon activation tagging candidate genes observation:

The gene loci AT2G41400, AT2G41410, AT2G41430 and AT2G41440 in the activation tagged line AIE7 are closely located on chromosome number 2, which encodes proteins that are targeted in the extracellular region, and the majority of their functions are still not well defined (Lin et al., 1999). These genes are candidates that could probably exhibit the tolerance characteristic similar to that expressed by the mutant line AIE7 against salt stress. AT2G41400 is a pollen allergen that has been found to not be expressed in AIE7 tolerant lines. On the other hand, adjacent genes were found to be highly expressed in response to salt stress, suggesting that they might have cis elements in their promoters that respond to salt stress and that are enhanced in transcriptional activity in the I-ATag line. These candidate genes also show association with the salt tolerance function based on their documented function (Figure 25).

First, AT2G41410 is a calcium binding protein in the plasma membrane that has been related to cell cycle regulation during stresses (Ascencia-Ibanez et al., 2008; Wang et al., 2008). Calcium modulation is a well-established salt tolerance feature that employs CDPKs, calmodulins and CBL-CIPKs (calcineurin B-like protein- CBL interacting protein kinase) for protection against salinity, and has been studied in this biological function, thus suggesting the

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significant role of AT2G41410 in salt tolerance (Kader and Lindberg, 2010). The calcium binding EF-hand here specifically contains the calcium-binding site. Calcium ions play an essential role in the maintenance of ionic homeostasis by regulating the potassium to sodium ratio, which at high levels is detected by the salt overly sensitive pathway (SOS) (Munns, 2005). Here the SOS1, SOS2 and SOS3 are the main components of the pathway, where SOS3 detects the high calcium level in shoots then deals with the sodium ion toxicity (Huang et al., 2012). Out of the three SOS mutants, studies using the SOS3 mutant have proven the presence of calcium binding domains on SOS3 processing three EF hands (Yang, 2009). This function associates the gene AT2G41410 with the SOS pathway that plays a significant role in salt tolerance, which could enable swift ionic stability and maintain the K^+/Na^+ homeostasis suppressing the deleterious effects of high sodium ions and its toxicity.

Secondly, AT2G41430, which was also found to be highly induced under salt stress in the line AIE7, is well characterized and expresses cytoplasmic cysteine-less hydrophilic proteins during various biotic and abiotic stresses (Sukweenadhi et al., 2015). The ERD products have been found to play a significant role during drought, light and cold, as hydrophilic proteins without cysteine residues are expressed under stress responses (Aalto et al., 2012). The ERD 15 proteins have the most notable functional and structural identities because of their ability to respond to not only one pathway but also various pathways (Aalto et al., 2012).

Third, the AT2G41440 gene encodes a MADS-box protein involved during pollen germination as well, and is expressed specifically in the nucleus and involved in nitrogen and carbon regulation through small RNAs and mRNAs in the Arabidopsis roots (Wang et al., 2008; Vidal et al., 2013). RNA interference has been a recent regulatory focus, and miRNA expression

profiling has been used for the analysis of *miRNAs* that are involved in tolerance and sensitive responses of plants towards stresses (Peng et al., 2014).

The gene loci AT3G50240, AT3G50270, AT3G50280 and AT3G63210 have also been found to be involved in cell regulation via the transcription factor IIIC subunit 5 (AT3G49410 in nucleus), the kinesin related protein (AT3G50240 in chloroplast), the HXXXD-type acyl transferase family protein (AT3G50270 and AT3G50280 in chloroplasts) (Zhu and Dixit, 2012; Kong et al., 2015). The tolerant lines here highly expressed the responsive genes AT3G50240, AT3G50270 and AT3G50260 involved in chloroplasts, which is one of the more recent studied organelles for response toward high salt conditions and attributed via cellular mechanisms like reactive oxygen species (ROS) based scavenging, signaling via abscisic acid, salicylic acid or jasmonic acid biosynthesis and protein turnover (Suo et al., 2017).

The kinesin and transferase (transferring amino acyl groups) activity proteins associated with the gene loci AT3G50240, AT3G50270 and AT3G50280 have been found to cause localized programmed cell death that can be attributed to the involvement of MAP kinase pathways, dehydration response element binding factor 2 (DREB2) and elevated abscisic acid (ABA) biosynthesis in response to salt stress (Ascencio-Ibanez, et al., 2008; Liu et al., 2007). The identification of these tolerant loci by this activation tagging approach suggests that the strong enhancers led to high coordinate expression of all of these closely knit genes through gain-of-function, such that their combined regulatory function features might confer a high salt tolerance characteristic to the tolerant Arabidopsis ATag lines AIE7 and AIE70 compared to the wild type. While the enhanced expression of these genes needs the presence of the transposed enhancer in the ATag line, it is possible that high 'coordinate regulation' of this gene cluster might be a means to naturally evolve tolerance under selection in populations in nature.

Candidate Gene AT2G41430 knockout mutant:

The analysis of the candidate gene AT2G41430 knockout mutant showed increased sensitivity to salt stress, revealing the function as necessary for tolerance to salt stress. It is obvious that the transposon activation tagging element increased the expression of AT2G41430 to above normal levels, which enabled the exhibition of the salt tolerance phenotype. However, the major advantage of the activation tag method is the display of a gain in function phenotype, which is not exhibited in the knockout mutant and directly suggests an application that would otherwise have to be tested in overexpression transformants. The AT2G41430 dehydration-induced protein (ERD) genes are highly induced when they experience drought stress (Kariola et al., 2006). AtERD15 is also a member of this locus and its miRNA silencing enhances ABA signaling which is a central regulator for salinity tolerance and further increases the plant salt and drought tolerance capacity by stomatal closure and regulation of water relations (Aalto et al., 2012). ABA has been characterized as a potential hormone for ABA dependent and independent signaling during salt stress for conferring tolerance to the plant, which is essential in determining the extent of plant adaption to environmental stresses (Yamaguchi-Shinozaki, and Shinozaki, 2006). Thus, since the AT2G41430 gene was found highly induced by salt stress in AIE7, it could be involved in the ABA signaling pathway and thereby confer salt tolerance.

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Table 1: Transcription factors and genes associated with salt stress.

Name	Abbreviation	Function	Group of plants	References
NAM/ATAF/CUC transcription factors	NAC	Biotic and abiotic stress control. Plant tolerance response such as drought and salinity	Arabidopsis, Rice, Grape, Soybean	(Kasuga et al., 2012)
Basic Leucine Zipper	Bzip	Seed formation and abiotic stress response tolerance to salt, osmotic and drought stresses	Arabidopsis, Rice	(Beckett, 2001
Apetala2/Ethylene Response Factor	APR/ERF	Response to abiotic stress, such as salinity stress	Arabidopsis, Rice, Grapevine, Soybean	(Riechmann et al., 2000)
Comes from WRKY domain	WRKY	Transcriptional regulator of biotic and abiotic plant stress response	Arabidopsis, Rice, Pinus, Soybean, Papaya, Poplar, Sorghum, Barley	(Kumar & Bandhu., 2005)
Trihelix	Trihelix (GT-factors)	Salt stress tolerance	Arabidopsis, Rice	(Mizoi et al., 2012)
Abscisic Acid (ABA)	ABA	Drought, salinity stress, and ABA signaling.	Arabidopsis, Rice	(Rushton et al., 2010)

Table 1 (Cont.)

Name	Abbreviation	Function	Group of plants	References
APETALA2 (AP2)	DREB1A/CRT	Response to Salinity Stress	Arabidopsis, Rice	(Shu et al., 2015)
Nuclear Transcription Factor	NF-Y	Drought and salinity stress signaling and ABA	Arabidopsis, Rice	(Mizoi et al., 2012)
SOS Pathway	SOS	Salt tolerance Pathway	Arabidopsis, Rice	(Puranik et al., 2012)
A Populous emphatic SOSI	PeSOSI	Response to salt sensitivity	Arabidopsis	(Wang et al., 2014)
GTL1(GT2-IIKE-1)	GT2-IIKE-1	Downgrades drought tolerance.	Arabidopsis, Rice, Soybean, Papaya	(Jin et al., 2013)

Table 2: List of primers used in TAIL PCR analysis.

Primer ID	Primer Sequence (5`-3`)	IUPC code
Int2	CAGGGTAGCTTACTGATGTGCG	
Irj-201	CATAAGAGTGTCGGTTGCTTGTTG	
DSpm1	CTTATTTCAGTAAGAGTGTGGGGTTTTGG	
ITIR-3	CTTACCTTTTTTCTTGTAGTG	
AD1	TG(A/T)G(A/T/G/C)AG(A/T)A(A/T/G/C)CA(G/C)AGA	**TGWGNAGWANC ASAGA**
AD2	(G/C)TTG(A/T/G/C)TA(G/C)T(A/T/G/C)CT(A/T/G/C)TGC	**STTGNTASTNCTN TGC**
AD3	CA(A/T)CGIC(A/T/G/C)GAIA(G/C)GAA	**CAWCGICNGAIAS GAA**
AD4	TC(G/C)TICG(A/T/G/C)ACIT(A/T)GGA	**TCSTICGNACITW GGA**
AD5	A/T)CAG(A/T/G/C)TG(A/T)T(A/T/G/C)GT(A/T/G/C)CTG	**WCAGNTGWTNGT NCTG**
AD6	AG(A/T)G(A/T/G/C)AG(A/T)A(A/T/G/C)CA(A/T)AGG	**AGWGNAGWANC AWAGG**

Table 3: List of primers for gene identification.

Primer ID	Forward Primer	Reverse Primer
AT5G15200	TCTGCGTGCCCTAGTTATTC	AACCCAAAGGTTTAGCAATC
AT5G43185	GCTGCATTGATCTTCCCAAA	ACATTTCACCAACAATGA
AT3G63210	GTCTTAGCAGTGAGCGAGATTG	TGCAGGCGAGAAAGGTTATG
AT4G04330	TGGAGTCATCTTCTTCACTC	AGGGCTAAGTTCATGTGACG
AT2G41400	ATTGAATGTCCAGGCTCAA	AACCAGGGCTGAGAAATGTT
AT3G50280	ATGGCCGACGTAAC	ATACTGTTACTCGGTCCAGC
AT1G64940	GTTACTTATCTTGGGCTCGC	TCACCATATTCGCCACATAG
AT4G17970	GACGAAGTGGATGGTTCT	ACTAGCAACGACGCAAAC
Bar	ACCATGAGCCCAGAACGACGC	CAGGCTGAAGTCCAGCTGCCAG
En-1010	CTGCAGCCAAACACATTTTCGC	
En-1474		GACGAAGTGGATGGTTCT
RGT-35S	TCAACACATGAGCGAAACCC	
RGT-SSU		GTTGGTTGAGAGTCTTGTGGCCT

Table 4: Primers for qPCR analysis.

Primer ID	Forward Primer Sequence	Reverse Primer Sequence
AT3G50280	GACGGCTCTGGTGCTAAAT	TTGACACCGTTCATGGGATAG
AT3G50240	CTCTTCGGAAATGGCTGGATAA	GTGCAGCTCGTACTTGAATTTG
AT3G50250	CGGTGGTTGTGGTTGTCC	CCCACCACTAACACCACTAA
AT3G50260	CTTTGGCTCGGCTCTTACTC	GGAAGTTGAGCGTAGCAGTT
AT3G50270	CCTGATGGTTCTGTTCCTGATT	CGTCCTTCATCTCGGTAACTTG
AT3G50290	CGCTTTCCGCACATTTATGG	TTGTCAAGCGGAGGGTTTAG
AT3G50300	TGGGAGATTCATCAGCCTCTA	TCTTGCCCTCTCTGTCTCTATC
AT3G50310	GTTGGTGATGAGTTACCGAAGA	CATCTCAGCCGTCCATCTTT
AT2G41400	TGTTCAATCCCGCCAACA	CGCCCGGTGGATAGATAAAG
AT2G41380	GGTGAGGTTTGTAACGGAGAA	CTTCCATCTCAGCCGTCAAA
AT2G41390	TTCGTGGTGTCGTGTATTGTAG	CTTGAGCGAGGGTTGAGTTT
AT2G41410	GACGTGGATCGTAACGGAAA	TTCCATCAACCGTCGCTATC
AT2G41415	GTCTGCATACTGTTGCTCTCTC	CGCAAATTTCATGGACGCATAC
AT2G41420	TCAGCAACAACAGAGCAGTC	TCAGAAGCAAGCATCCAAGAG
AT2G41430	CGACTTGGTACCCTGATTACTG	GACCTCCACCATTCTCATTCTC
AT2G41440	GGGAGATGTTCGTCGGATAATG	CATGCTCTTCCGCTGATAAGA
AT3G63210	GTCTTAGCAGTGAGCGAGATTG	TGCAGGCGAGAAAGGTTATG
AT5G43185	GCTGCATTGATCTTCCCAAA	ACATTTCACCAACAATGA
UBQ10	CGGATCAGCAGAGGCTTATTT	CGACTCCTTCTGGATGTTGTAAT

Table 5: Sequences of specific Primers used in PCR analysis for knockout mutants genes.

Primer ID	Forward Primer Sequence	Reverse Primer Sequence
AT2G41400	ACTTCTCATGGCTTCACTCTTC	ATCACCGATGGCATAGTTAGC
AT2G41410	ACGTCTCTTCCGTACCAAATC	TTCCATCAACCGTCGCTATC
AT2G41420	TGGCTTTCTAGAAGGATGGTTAG	AGAGAGAGAGAGACTCCAATCAG
AT2G41430	CAACGTAGGTTCTGGTGAATGA	CGTACAGCTGCCGGAATAAA
AT2G41440	CAGATGAAGAGGAAGCGAAGAG	AGCCCGTAGAGCTCGTAATA
AT3G50280	GATGGCTCTGTTCCTGACTTT	TTGTCAAGCGGAGGGTTTAG
AT3G50270	CCTGATGGTTCTGTTCCTGATT	CGGTGGTTATGGCTGATGAA
AT3G50240	TGGGCTGATTCCTCAAGTTATG	CCCATGCTATTCTGCCTAAGT

Table 6: Summary of the Candidate genes and their probable roles exhibiting tolerant / sensitive nature to Arabidopsis Activation tagging lines.

ATag Line	Tolerance/ Sensitivity	Gene Loci	Documented Functions	Reference
AIE73	Tolerance	AT1G64940	Cytochrome P450 mono-oxygenases catalyzing redo reactions and secondary metabolite production	(Goyal et al., 2016; Dai et al., 2007).
AIE7		AT2G41400	Pollen Ole e 1 allergen a extension family protein function are still not well defined	(TAIR, 2017; Lin et al., 1999).
AIE7		AT2G41410	Calcium binding proteins involved in cell cycle i.e. growth & pollen germination	(Ascencia-Ibanez et al., 2008; Wang et al., 2008).
AIE7		AT2G41415	Encodes a Maternally expressed gene (MEG) family protein	TAIR (2017).
AIE7	Tolerance	AT2G41430	CTC-interacting domain 1 (cys-less hydrophilic protein) and salt tolerance up-regulated on interaction with a biotic agent	(Sukweenadhi et al., 2015; Aalto et al., 2012).
AIE7		AT2G41440	Regulated Nitrogen & Carbon cycles through small RNA and mRNA	(Wang et al., 2008; Vidal et al., 2013).
AIE7		AT2G41380	Methyltransferase activity	TAIR (2017).
AIE7		AT2G41390	Pollen Ole e 1 allergen & extension family protein. function are still not well defined.	TAIR (2017).
AIE7		AT2G41420	Proline-rich family protein involved in megasporogenesis	TAIR (2017).

Table 6: (Cont.)

ATag Line	Tolerance/ Sensitivity	Gene Loci	Documented Functions	Reference
AIE89	Sensitivity	AT3G49410	TFIIIC for pre-transcription complex for class III genes	(Zhu and Dixit, 2012; Kong et al., 2015)
AIE70	Tolerance	AT3G50240	Kinesin related protein	(Zhu and Dixit, 2012; Kong et al., 2015)
AIE70		AT3G50270	Acyl transferase family protein	
AIE70		AT3G50280	HXXXD-type acyl-transferase family protein	
AIE70		AT3G50250	Elemental activities (catalysis or binding)	
AIE70		AT3G50260	Encodes a member of the DREB subfamily	
AIE70		AT3G50290	HXXXD-type acyl-transferase family protein	
AIE70		AT3G50300	HXXXD-type acyl-transferase family protein	TAIR (2017)
AIE70		AT3G50310	Encodes a member of MEKK subfamily & Osmotic stress response	TAIR (2017)
AIE83	Sensitivity	AT3G63210	MARD1 affecting ABA signaling & inducing dormancy/ senescence	(Zhu and Dixit, 2012; Kong et al., 2015)
AIE20	Sensitivity	AT4G04330	ATRbcX1 involved in synthesis of large subunit of Rubisco	(Kolesinski et al., 2013)
AIE89	Sensitivity	AT4G28830	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein performing methylation and act as methyltransferases	(TAIR, 2017; Panjabi et al., 2008)

Table 6: (Cont.)

ATag Line	Tolerance/ Sensitivity	Gene Loci	Documented Functions	Reference
AIE89	Sensitivity	AT4G17970	Aluminium activated malate transporter performing stomata movements and sulfate transport	(Malcheska et al., 2017; Medeiros et al., 2016)
AIE34	Tolerance	AT5G15200	Ribosomal small subunit structural component meant for mRNA binding	(Turkina et al., 2011; Ascencio-Ibanez et al., 2008)
AIE85	Tolerance	AT5G43185	Expressed protein whose function is not known	TAIR (2017)
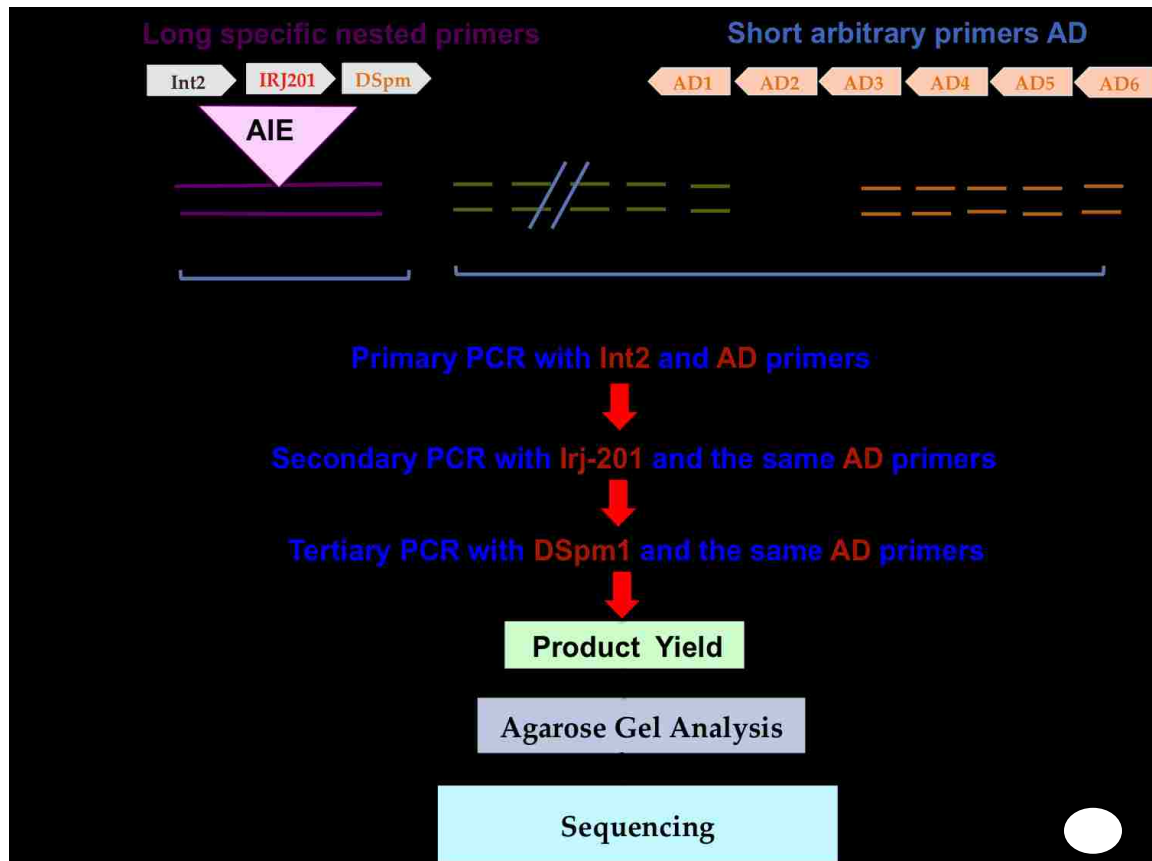
Figure 1: Schematic outline represents the procedure of amplification of flanking target genomic DNA of the Activation tag mutant lines using Thermal Asymmetric Interlaced PCR (TAIL-PCR). The I-Tag transposon (T-DNA) with nested primers (Int2, IRJ20, DSpm) primers shown along with the different short arbitrary (AD) primers used for amplification.
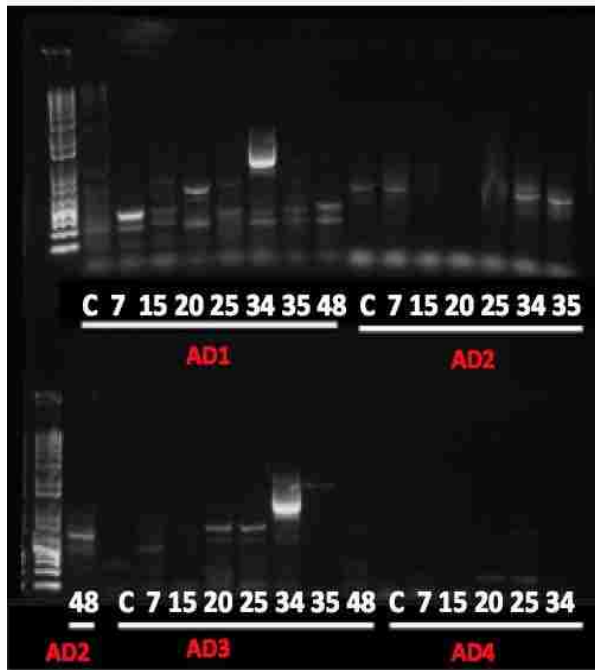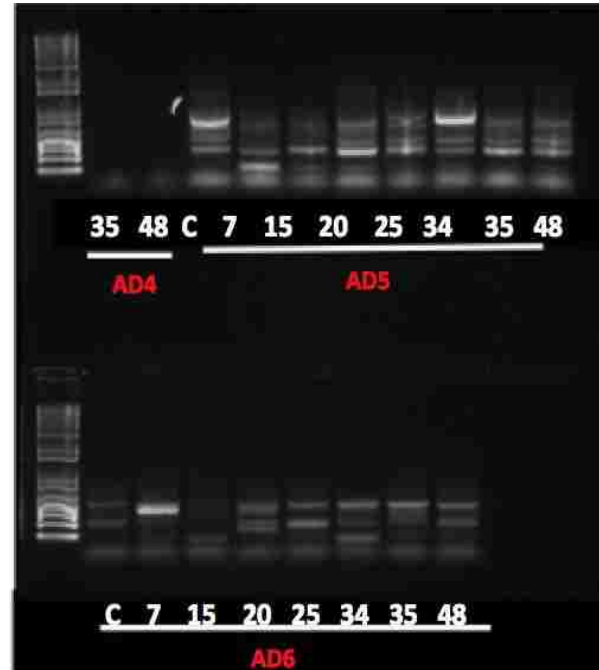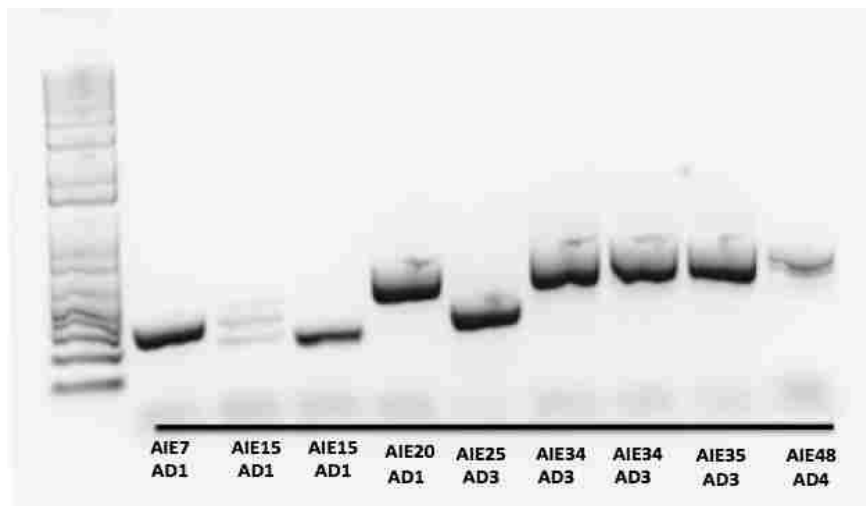
Figure 2: (A&B) Agarose gel analysis of tertiary TAIL-PCR products of Arabidopsis ATag lines (Col0, AIE7, AIE15, AIE20, AIE25, AIE34, AIE35, AIE48) that were positive for ATag construct using six arbitrary degenerate primers and specific primer DSpm1. **(C)** Selected positive plants for the ATag construct for identification of tagged genes adjacent to transposons by excising out the bands of tertiary TAIL-PCR using ITIR-3 primer for sequencing. M= 1kb plus DNA ladder.

A
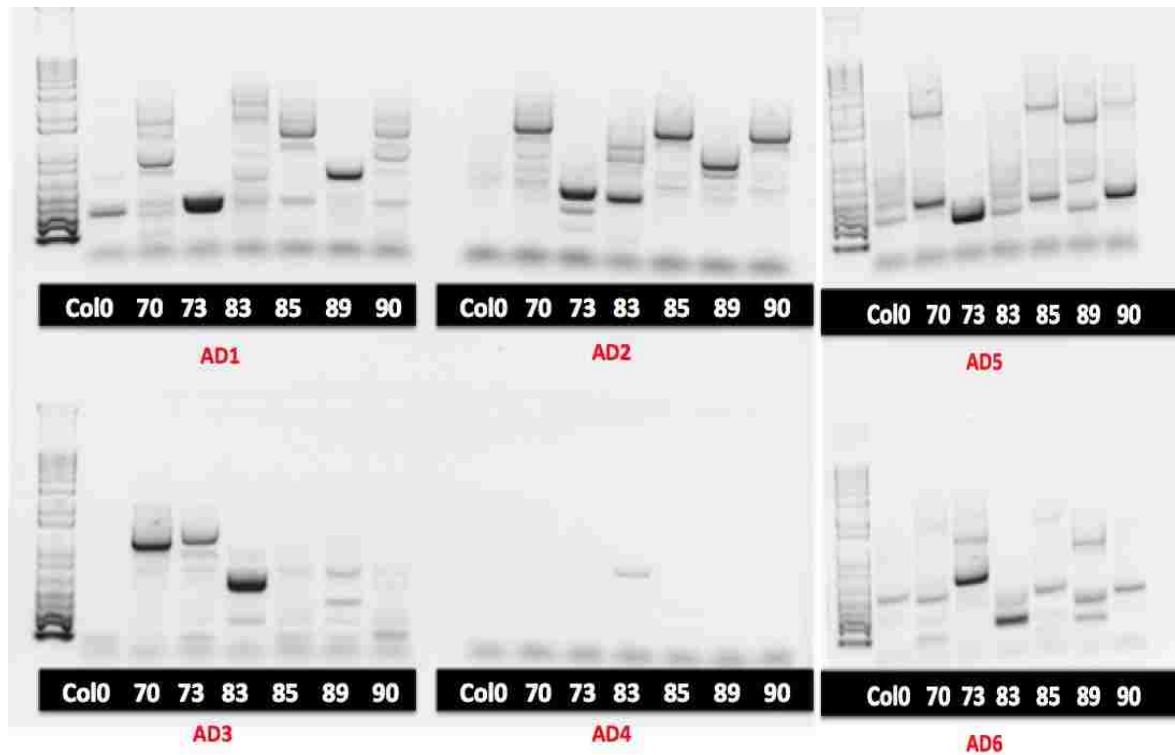
B
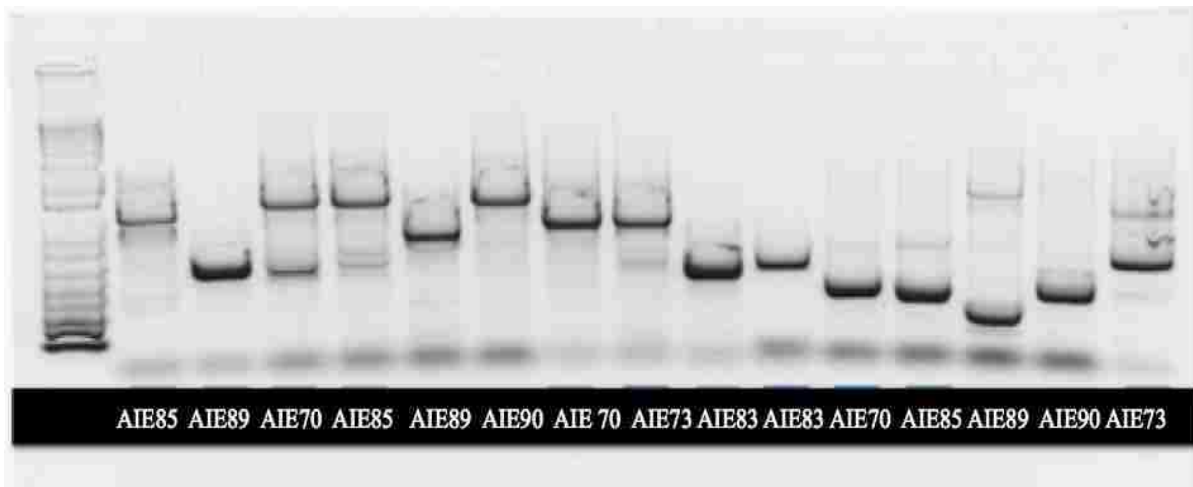
Figure 3: **(A)** Agarose gel analysis of tertiary TAIL-PCR products of Arabidopsis ATag lines (Col0, AIE70, AIE73, AIE83, AIE85, AIE89, AIE90) that were positive for ATag construct using six arbitrary degenerate primers and specific primer DSpm1. **(B)** Selected positive plants for the ATag construct for identification of tagged genes adjacent to transposons by excising out the bands of tertiary TAIL-PCR using ITIR-3 primer for sequencing. M= 1kb plus DNA ladder.
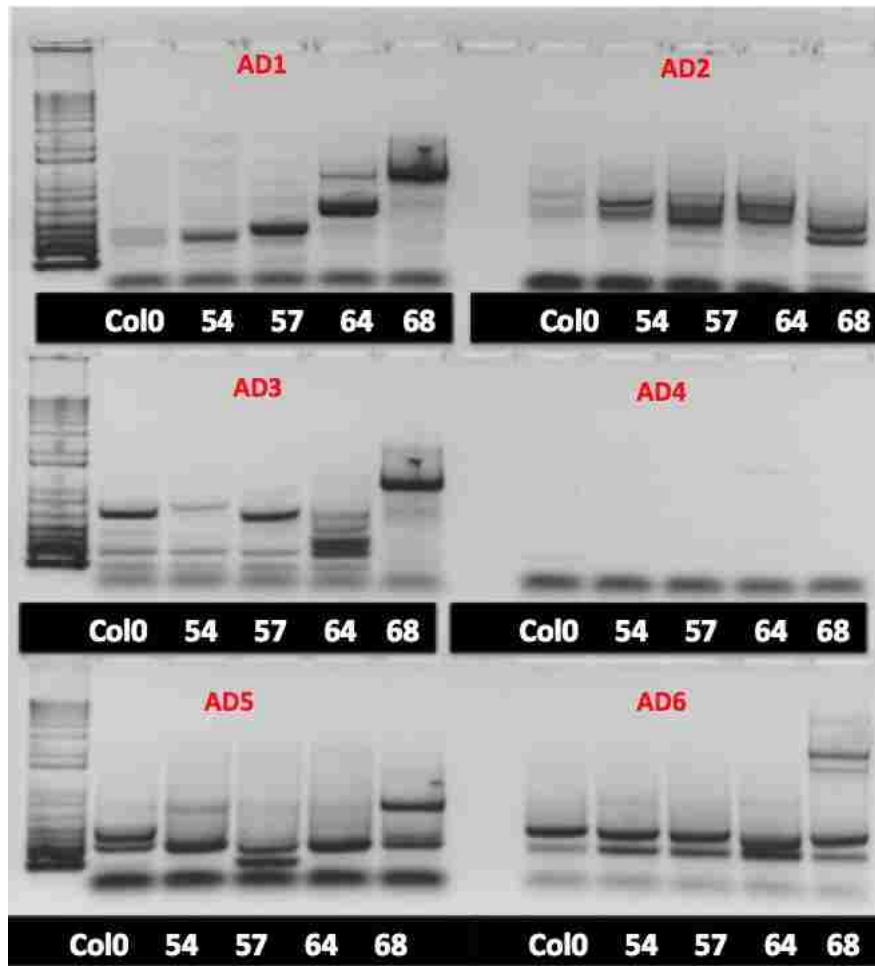
Figure 4: Agarose gel analysis of tertiary TAIL-PCR products of Arabidopsis ATag lines (Col0, AIE54, AIE57, AIE64, AIE68) that were positive for ATag construct using six arbitrary degenerate primers and specific primer DSpm1. M= 1kb plus DNA ladder.
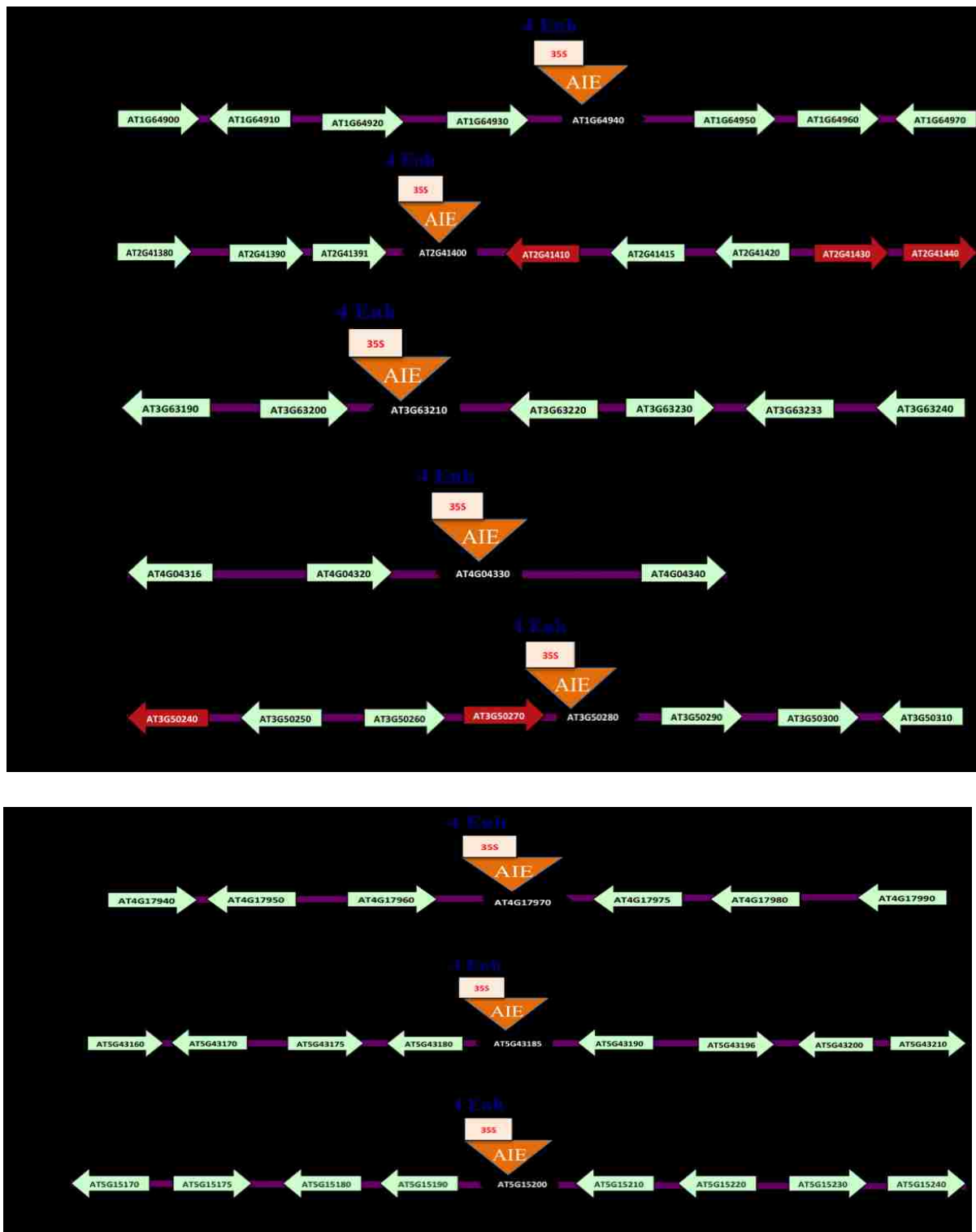
Figure 5: Schematic illustration representing the position of transposon (AIE) insertion in Arabidopsis genome in Columbia activation tag mutants. The arrow shows the coordinates and direction of candidate genes for salt tolerance based on TAIR genome sequence annotation in the ATag lines (A- H). The black arrow refers to the main gene of ATag insertion and the other arrows are the neighboring candidate gens for salt tolerance 10kb upstream and 10kb downstream with their distance from 35S enhancer in the AIE.
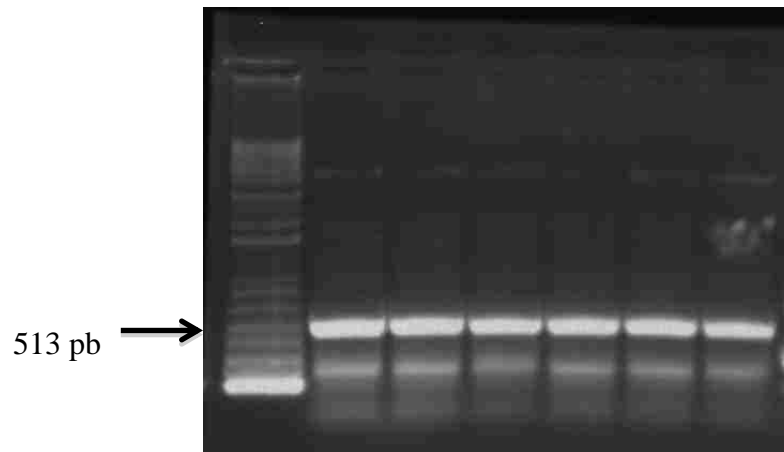
Figure 6: PCR analysis of plasmid DNA obtained from cloning the PCR products. Lanes indicating putative clones were inoculated in kanamycin selective medium for plasmid isolation. Isolated plasmids were used for PCR using Bar gene primer forward and reverse (513 bp). M=1kb DNA plus ladder.
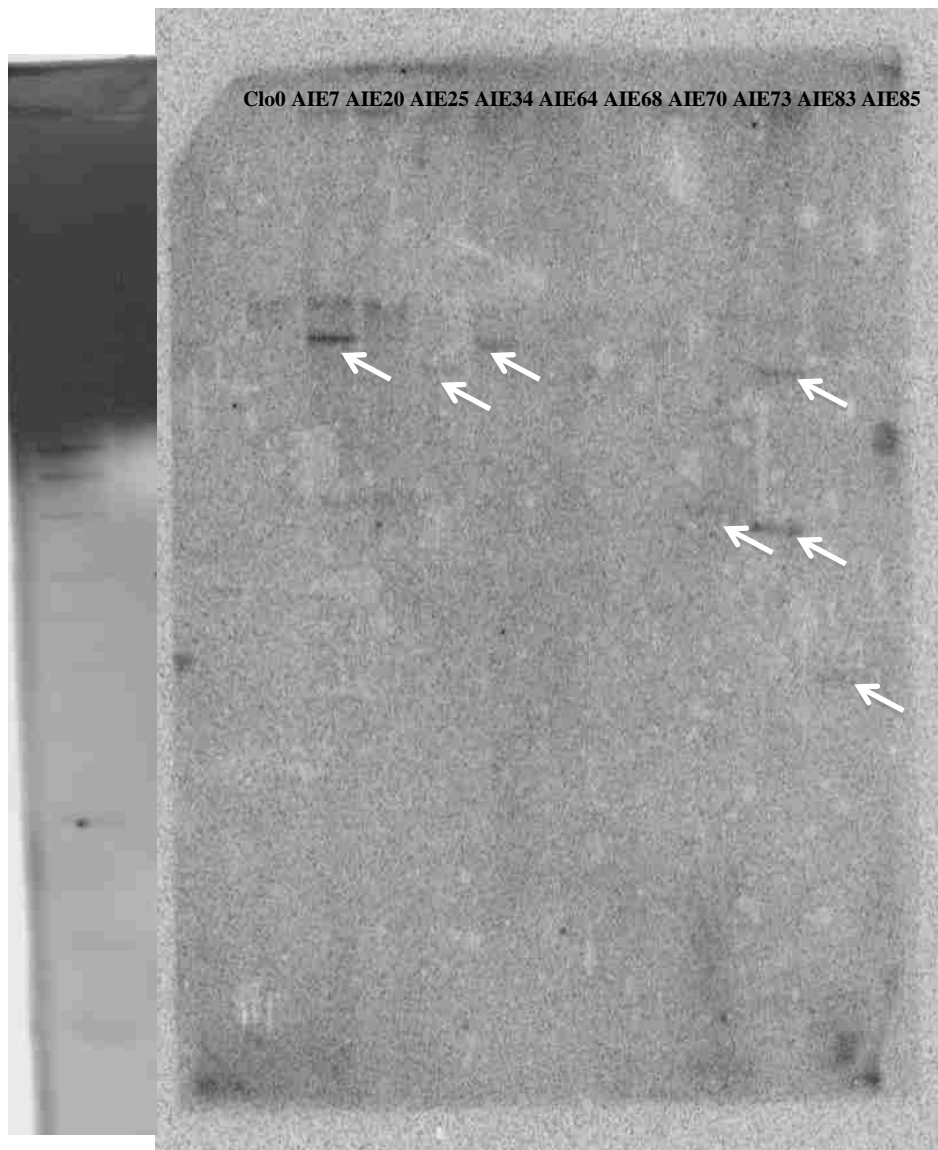
Figure 7: Southern blot analysis with a BAR gene probe of transformants plants and the wild type Clo0, AIE7, AIE20, AIE25, AIE34, AIE64, AIE68, AIE70, AIE73, AIE83, and AIE85. The bands indicate independent insertions with size. Ladder is presenting the size in kilo base. AIE7- 12000 kb, AIE34-11900 kb, AIE25- 11000 kb, AIE73- 2 bands (11000 kb - 4000 kb), AIE70- 4100 kb, AIE83- 1900 kb. M= 1kb DNA ladder.
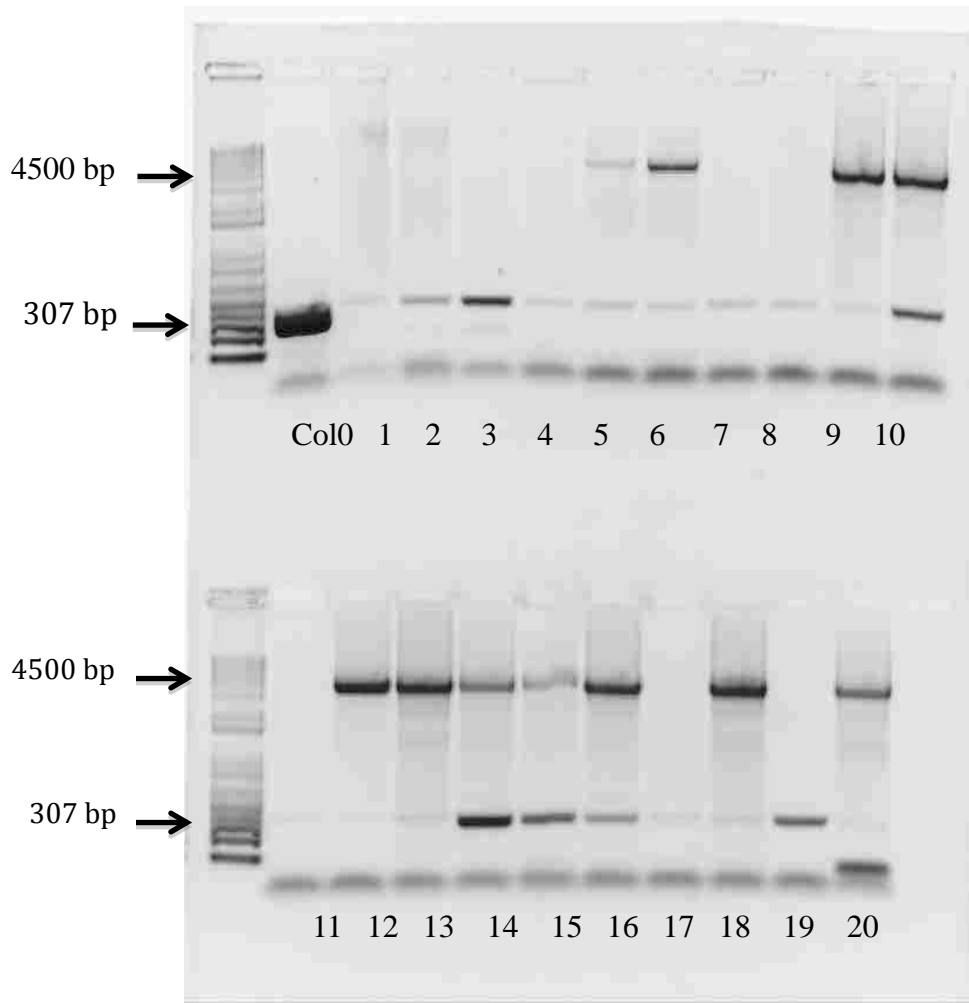
Figure 8: Genomic PCR analysis using AIE7 gene specific primers (307 bp) for 20 AIE7 transgenic plants of T3 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. HT- indicates heterozygous. M= 1kb plus DNA ladder.
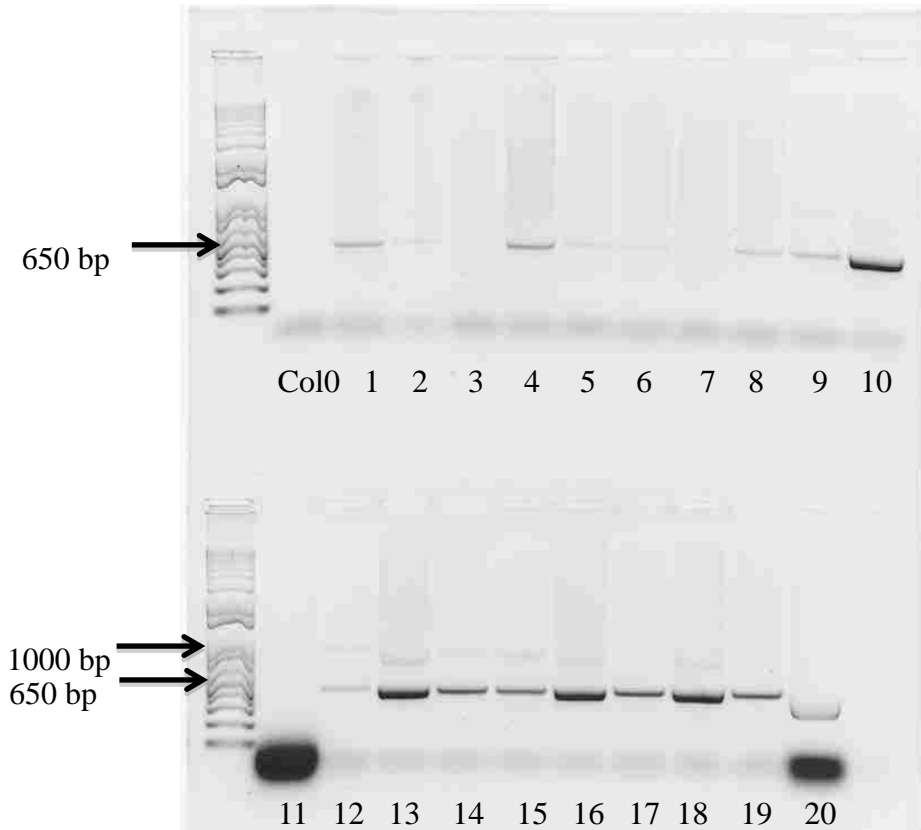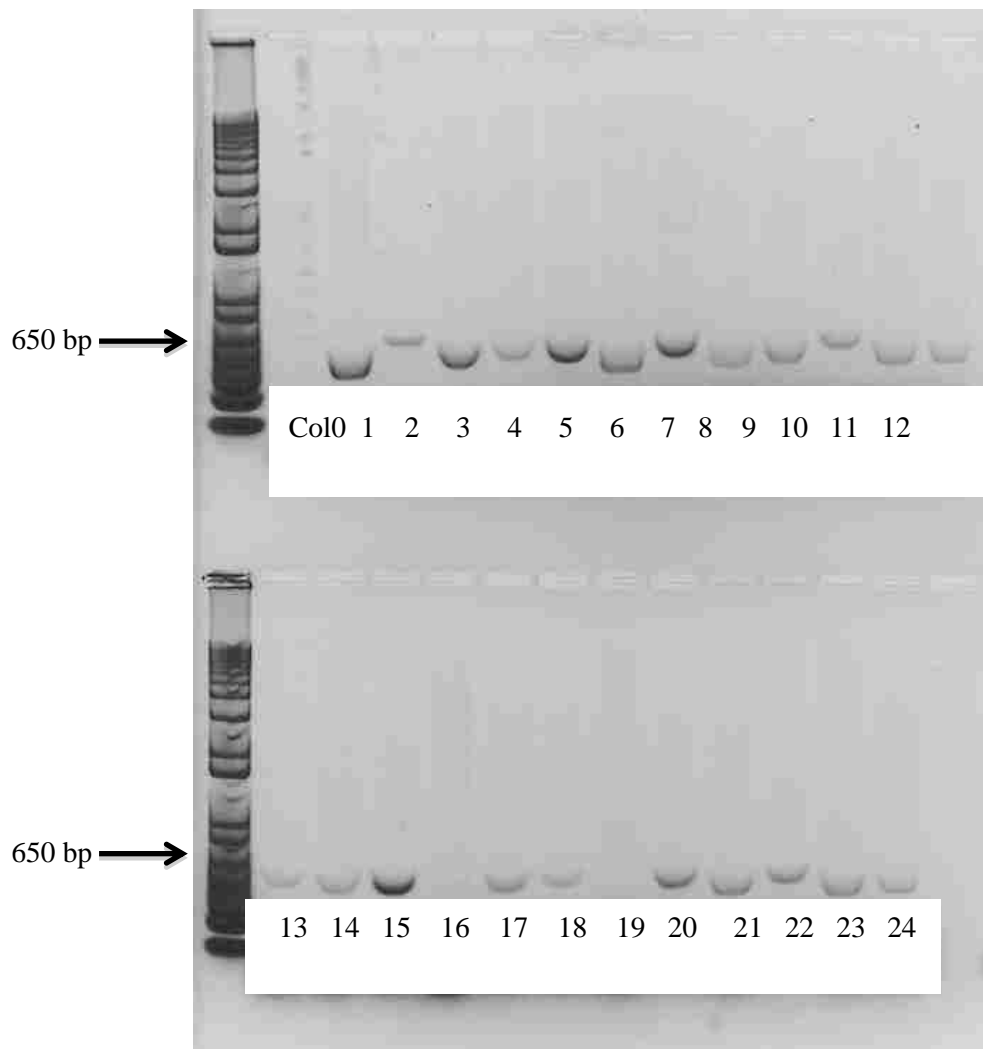
Figure 9: Genomic PCR analysis using AIE7 gene specific primers (Reverse) and Transposon specific primer (Irj201) for 20 AIE7 transgenic plants of T3 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.
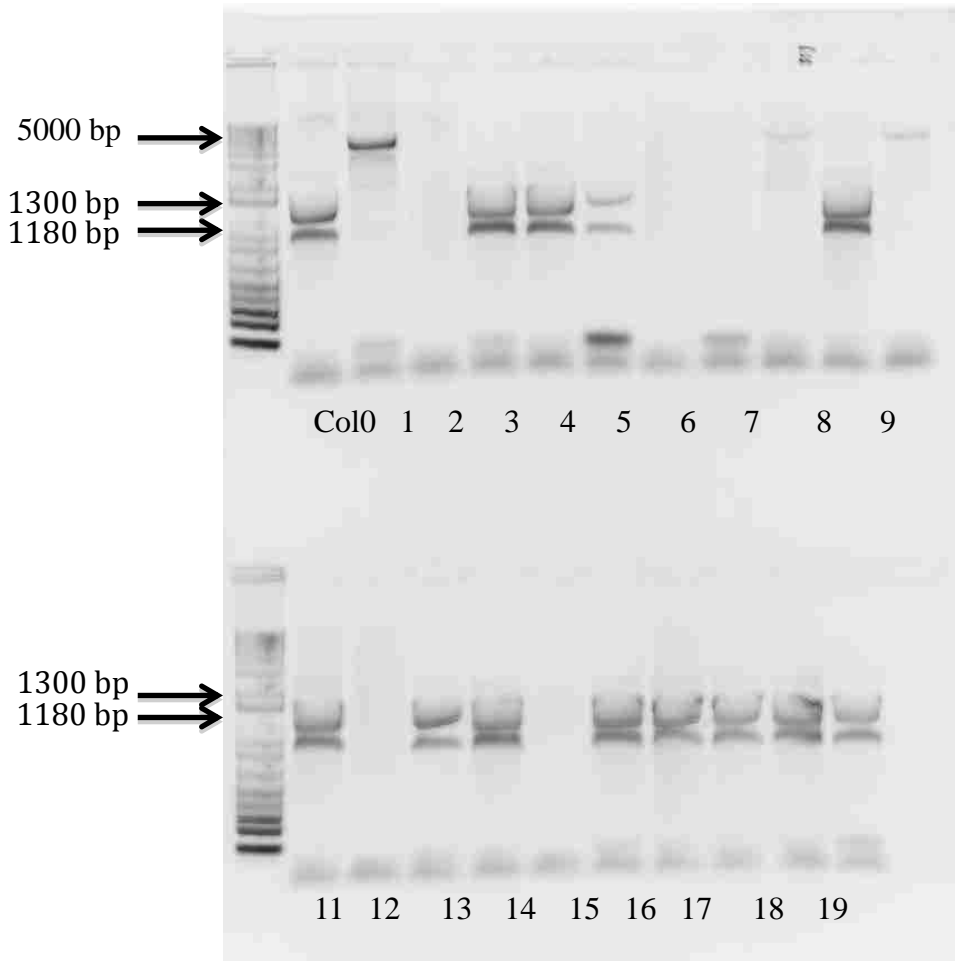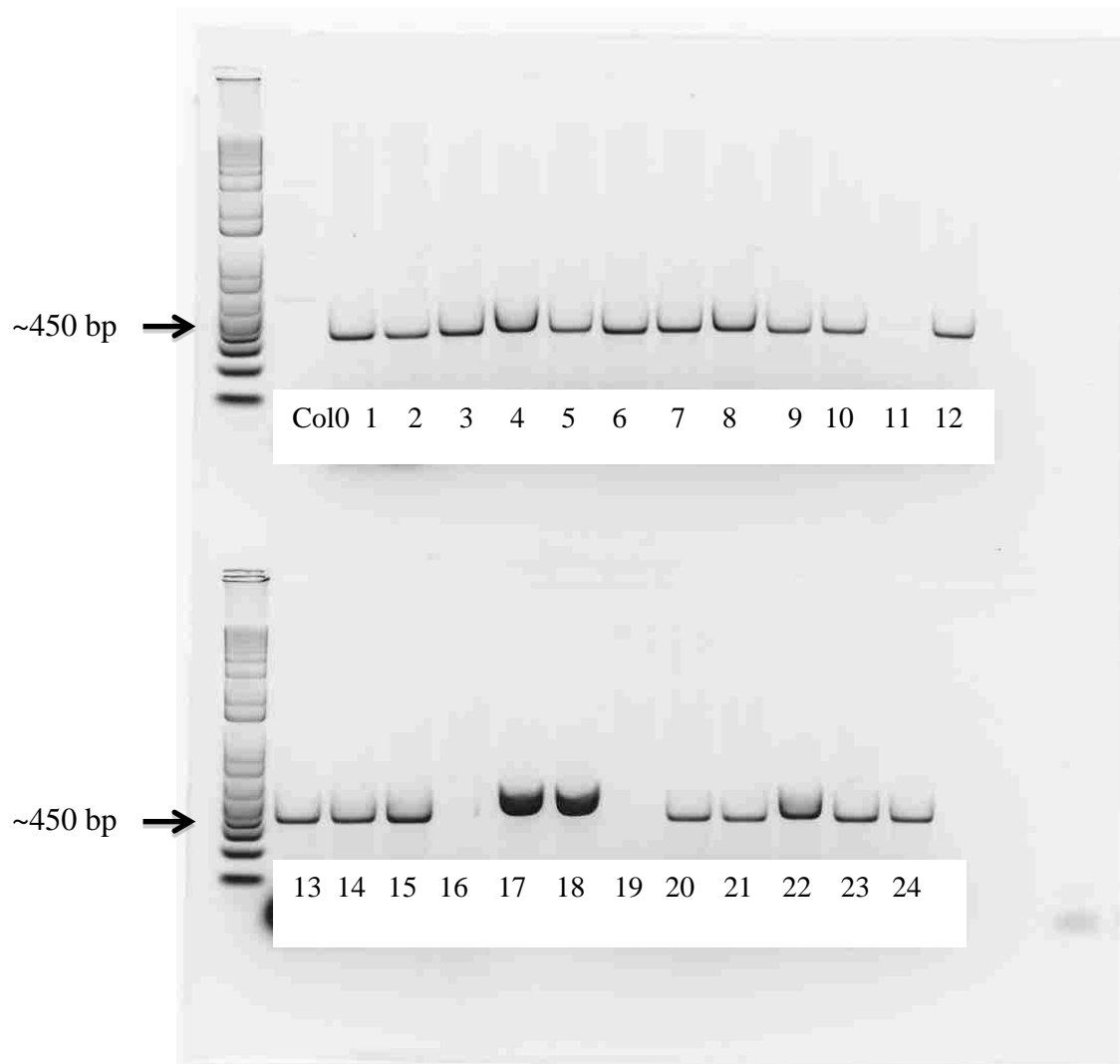
Figure 10: Genomic PCR analysis using AIE70 gene specific primers (1300 kp) for 20 AIE70 transgenic plants of T3 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. HT- indicates heterozygous. M= 1kb plus DNA ladder.
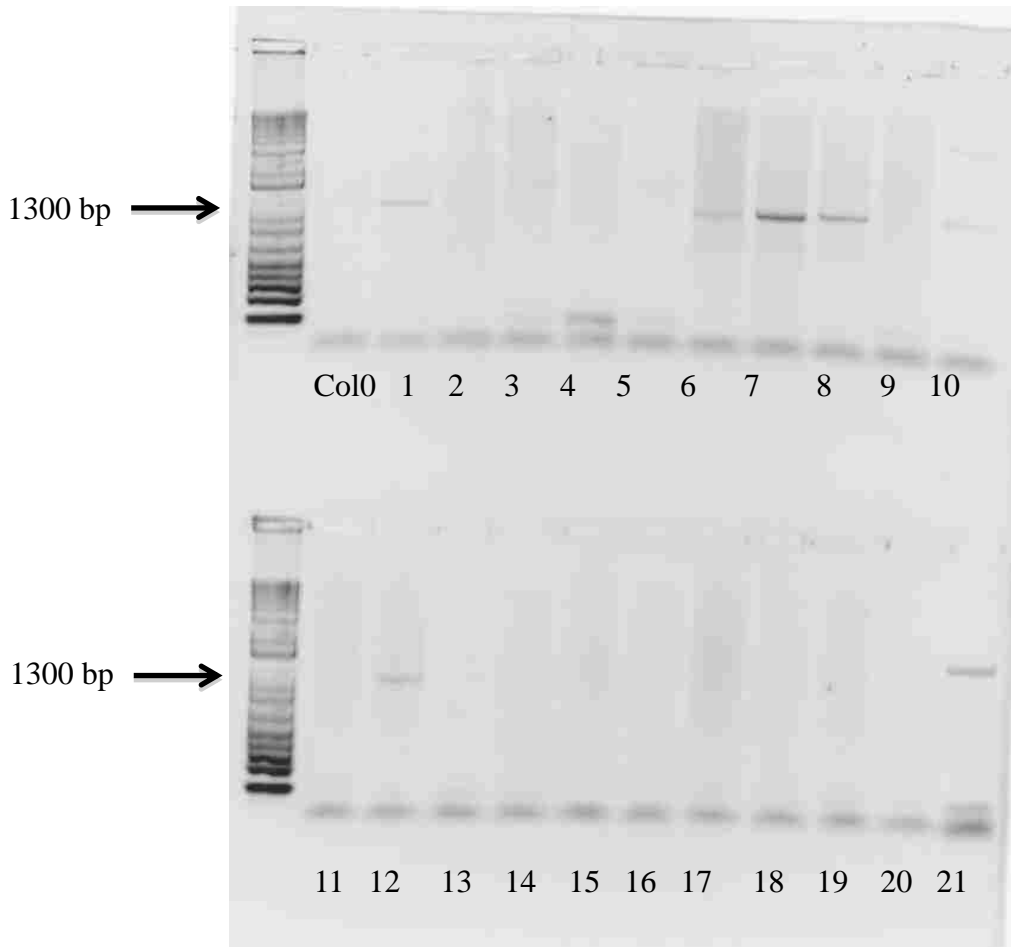
Figure 11: Genomic PCR analysis using AIE70 gene specific primers (Reverse) and Transposon specific primer (Irj201) for 20 AIE7 transgenic plants of T3 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.
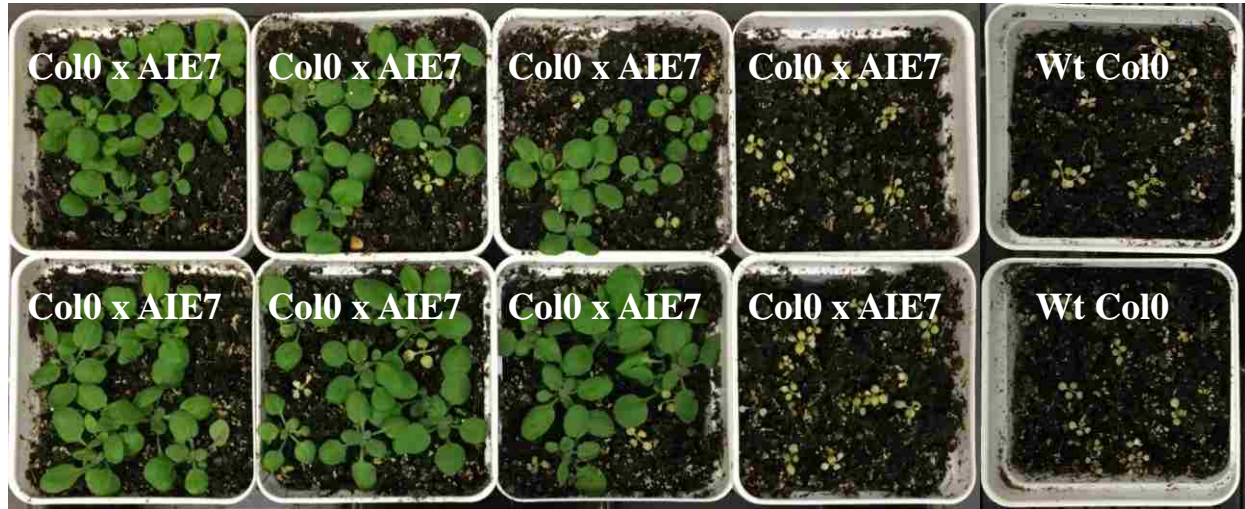
**Figure 12:** Morphological phenotypes segregation of of F2 progeny of crossing Columbia activation tag mutant lines **(A)** AIE7, **(B)** AIE70 with the wild type WT (Col0) after Basta Herbicide application.
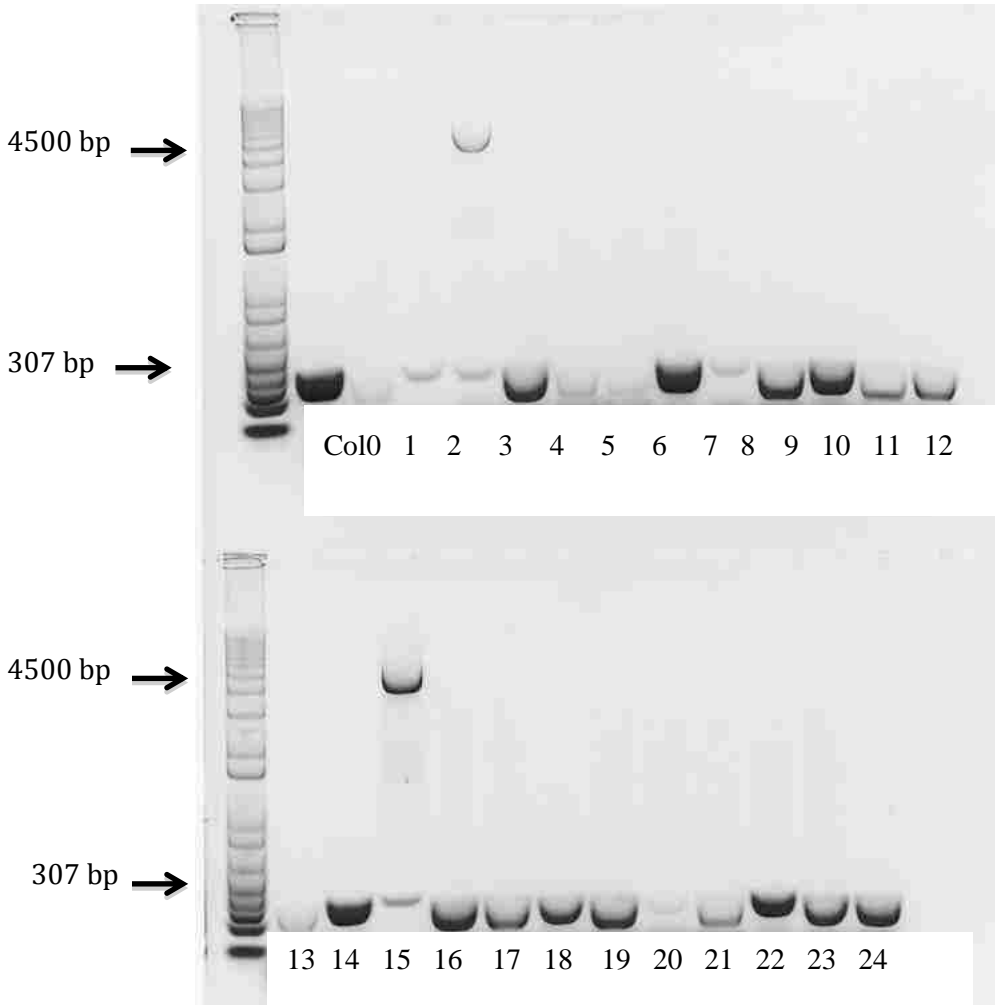
Figure 13: Genomic PCR analysis using AIE7 gene specific primers (307bp) for 24 Col0xAIE7 transgenic plants of F2 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.

Figure 14: Genomic PCR analysis using AIE7 gene specific primers (Reverse) and Transposon specific primer (Irj201) for 24 Col0xAIE7 of F2 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.

Figure 15: Genomic PCR analysis using En-F and En-R primers on 24 Col0xAIE7 F2 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.
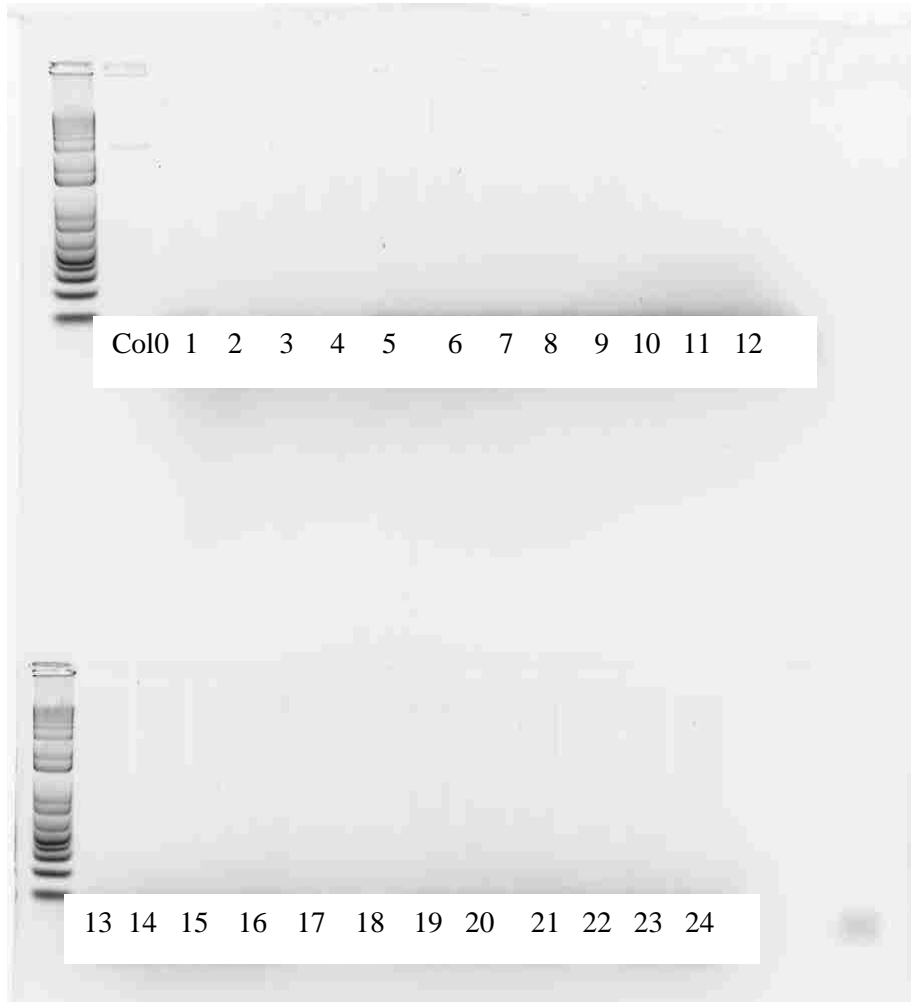
Figure 16: Genomic PCR analysis using using the primers RGT-35S and RGT-SSU on 24 Col0xAIE7 F2 progeny sprayed with Basta herbicide. Wild type Col0 was used as a positive control. M= 1kb plus DNA ladder.
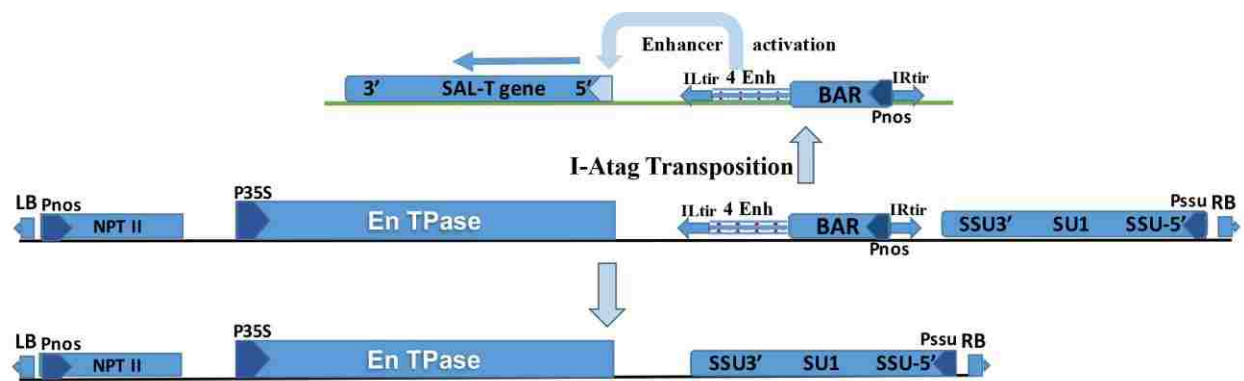
Figure 17: Schematic representation of the Activation-Tag lines using the En-I transposon system for generation of Salinity Tolerant (SAL-T) mutants by gain-of-function, adapted from Marsch-Martinez et al., (2002). The elements of the construct are as follows: T-DNA LB (Left border) and RB (right border); P35S CaMV35S promoter; *En*TPase, *En* immobile transposase; *I*-element left (ILtir) and right (IRtir) terminal-inverted repeat; 4-Enh (tetramer of the CaMV 35S enhancer). Selectable marker: positive selectable marker BAR (glufosinate/Basta resistance) and negative selectable marker SU1 (Pro-herbicide R740).
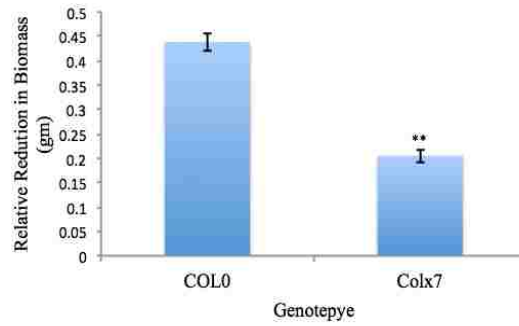
Figure 18: Relative reduction in biomass (RB) of F2 progeny of crossing Columbia activation tag mutant lines to the wild type Col0 (Col0xAIE7 line) compared to WT Col0. Bars represent ± SE, N= 10. The data are average of ten replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05.
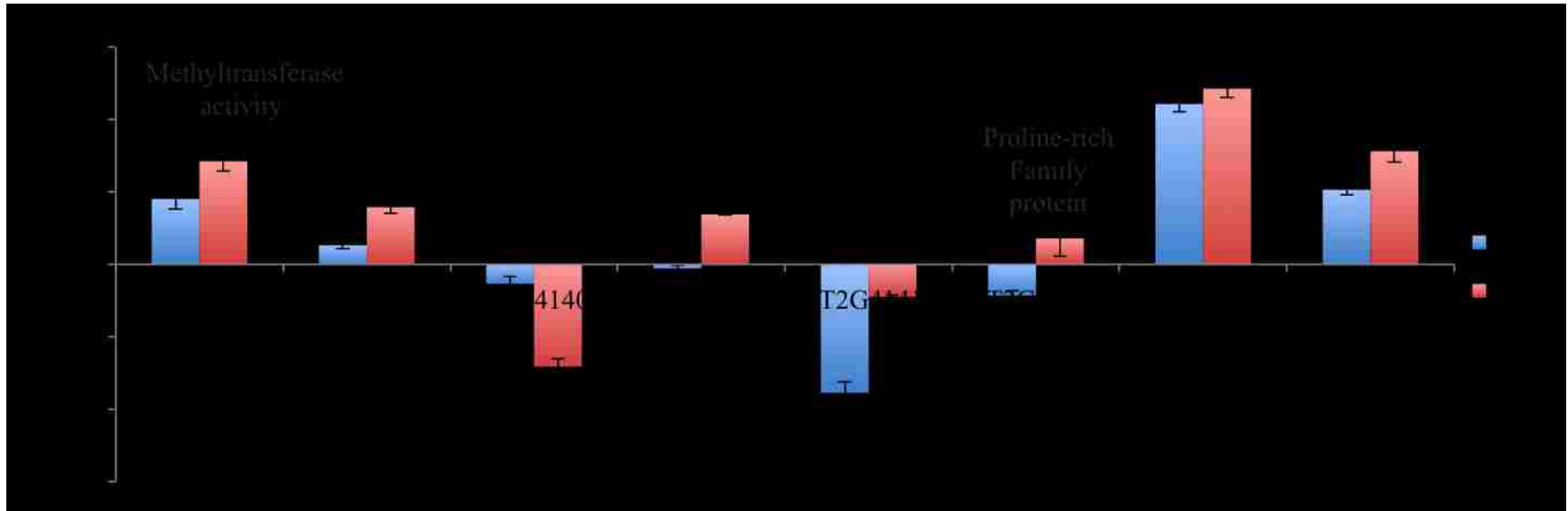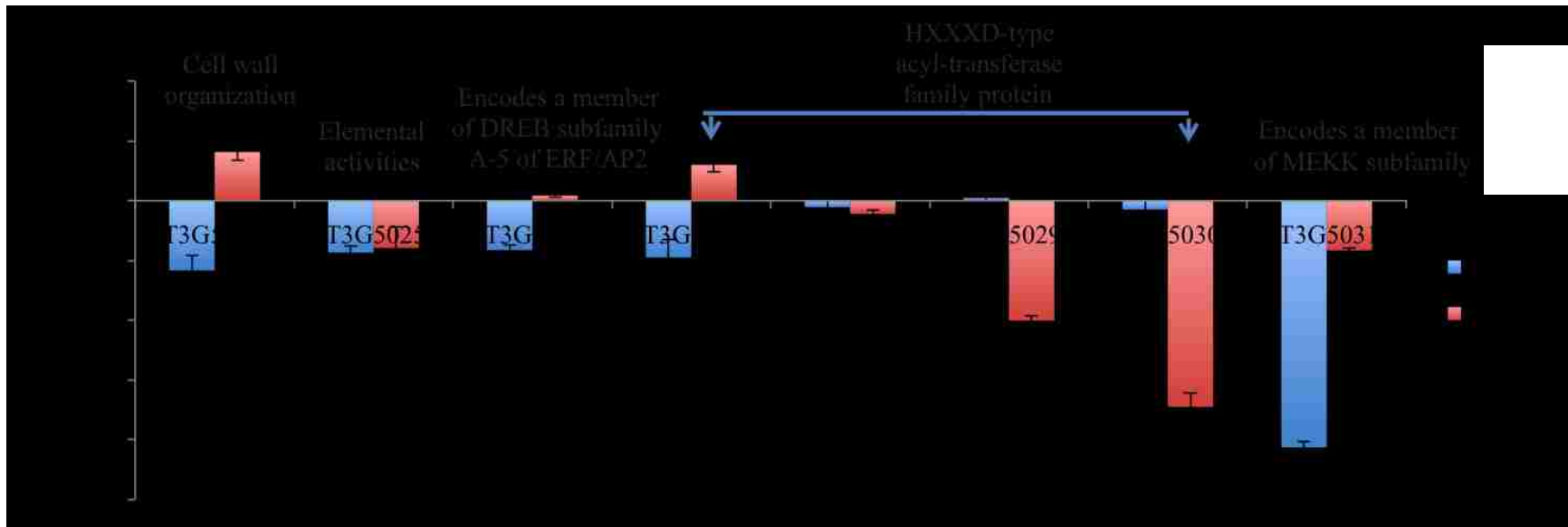
Figure 19: Expression analysis of tagged genes line AIE7 and the surrounding candidate genes (A-H) under control 0 NaCl and salt stress 150 mM NaCl conditions. Bars represent ± SE, N= 2. The data are average of two biological replicates.
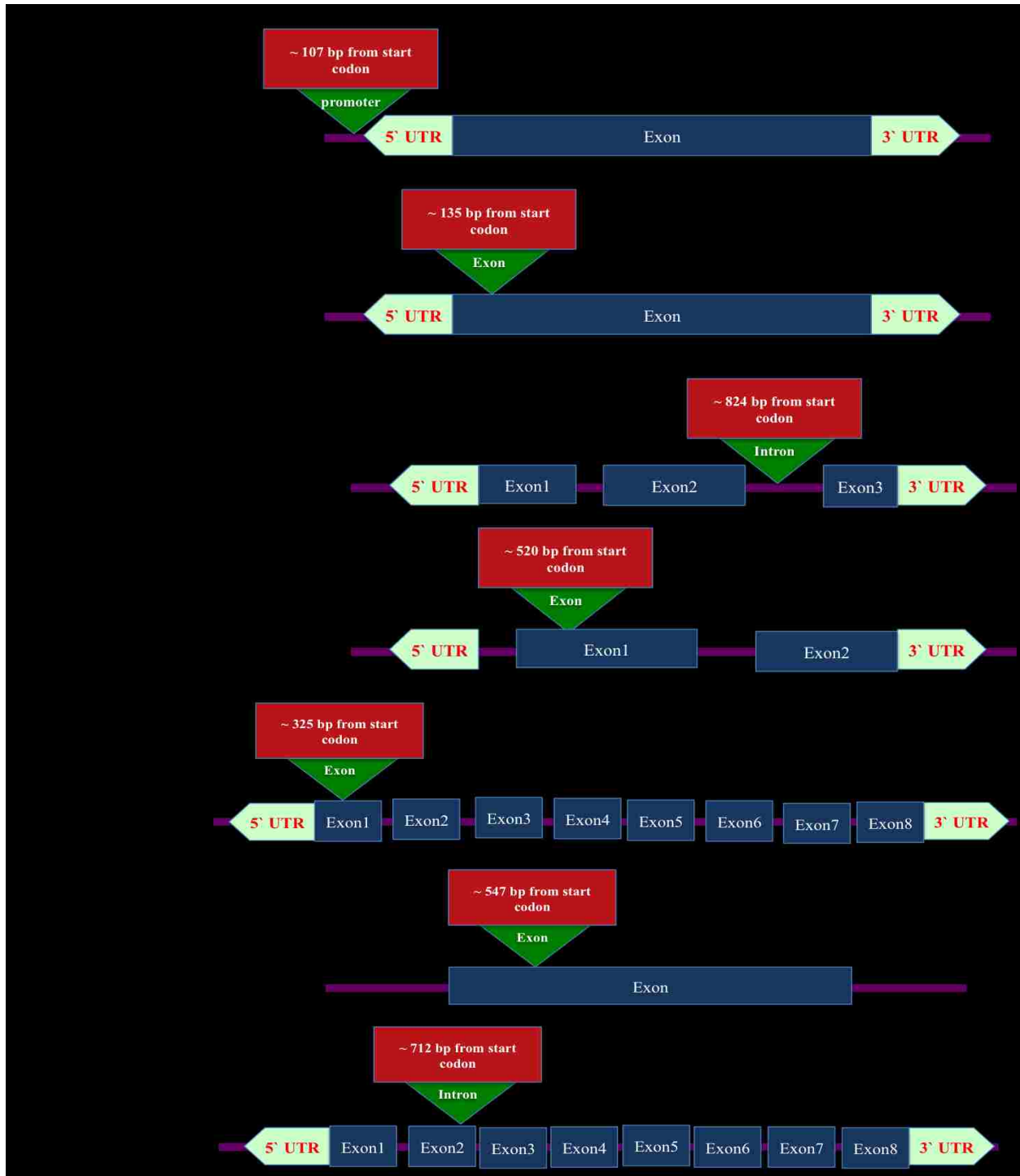
Figure 20: Expression analysis of tagged genes line AIE70 and the surrounding candidate genes (A-H) under control 0 NaCl and salt stress 150 mM NaCl conditions. Bars represent ± SE, N= 2. The data are average of two biological replicates.

Figure 21: Diagram of TDNA insertion elements in knockout mutants plants for candidate genes for salt tolerance for lines AIE7 and AIE 70, **(A)** AT2G41400, **(B)** AT2G41410, (C) AT2G41420, (D) AT2G41430, (E) AT2G41440, **(F)** AT3GG5240, **(G)** AT3GG5280.
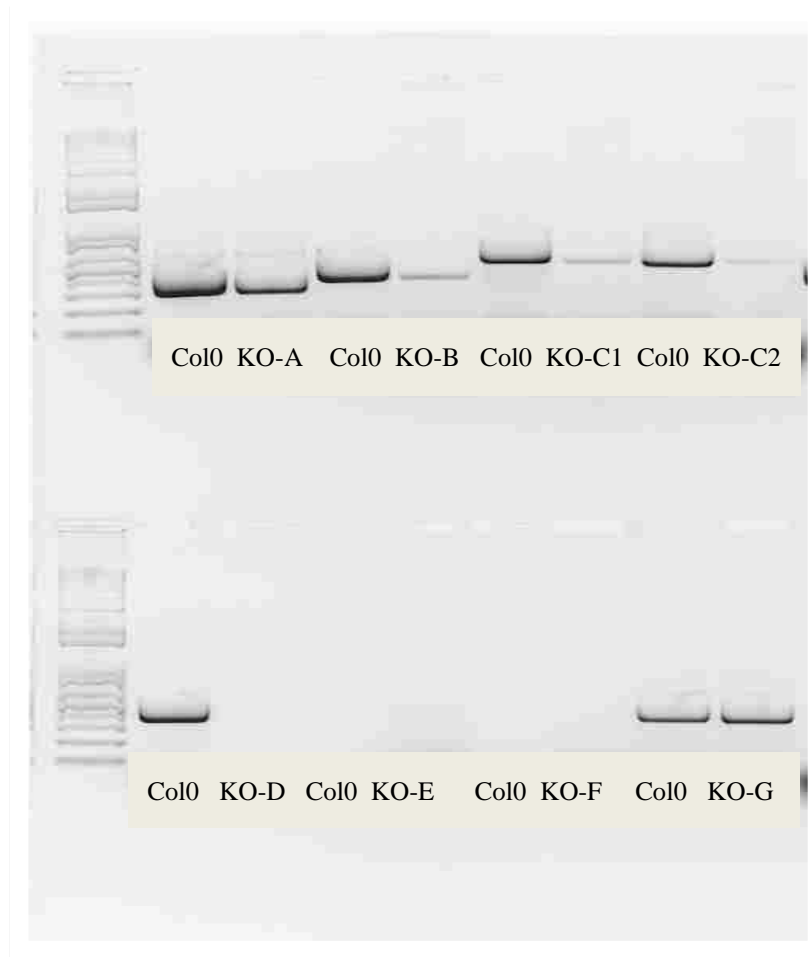
Figure 22: PCR segregation analysis for present or absence of TDNA insertion elements in knockout mutants plants for candidate genes for salt tolerance for lines AIE7 and AIE 70, **(A)** AT2G41400-**451 bp**, **(B)** AT2G41410-**578bp**, (C) AT2G41420-**723bp**, (D) AT2G41430-**520 bp**, (E) AT2G41440-**591bp**, **(F)** AT3GG5240-**1165 bp**, **(G)** AT3GG5280-**556 bp**, wild type Col0 was used as a positive control.

A

Control

Stress

B

Control

Stress

Figure 23: Response wild type and AT2G41430-KO mutant line to control condition (0 NaCl) and salt stress condition (150 NaCl). **(A)** Wt Col0, **(B)** AT2G41430-KO.
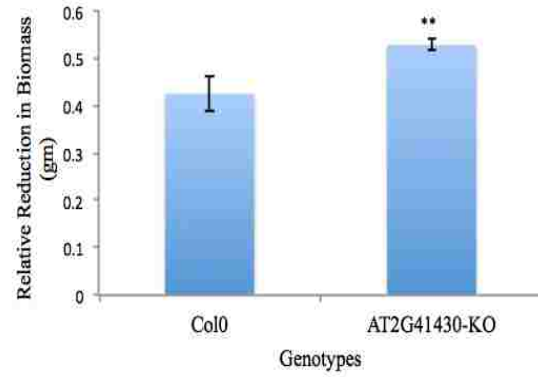
Figure 24: Relative reduction in biomass (RB) of AT2G41430-KO mutant lines compared to WT Col0. Bars represent ± SE, N= 10. The data are average of ten replicates, with ** indicating significance at p-value ≤ 0.01, * indicating significance at p-value ≤ 0.05.
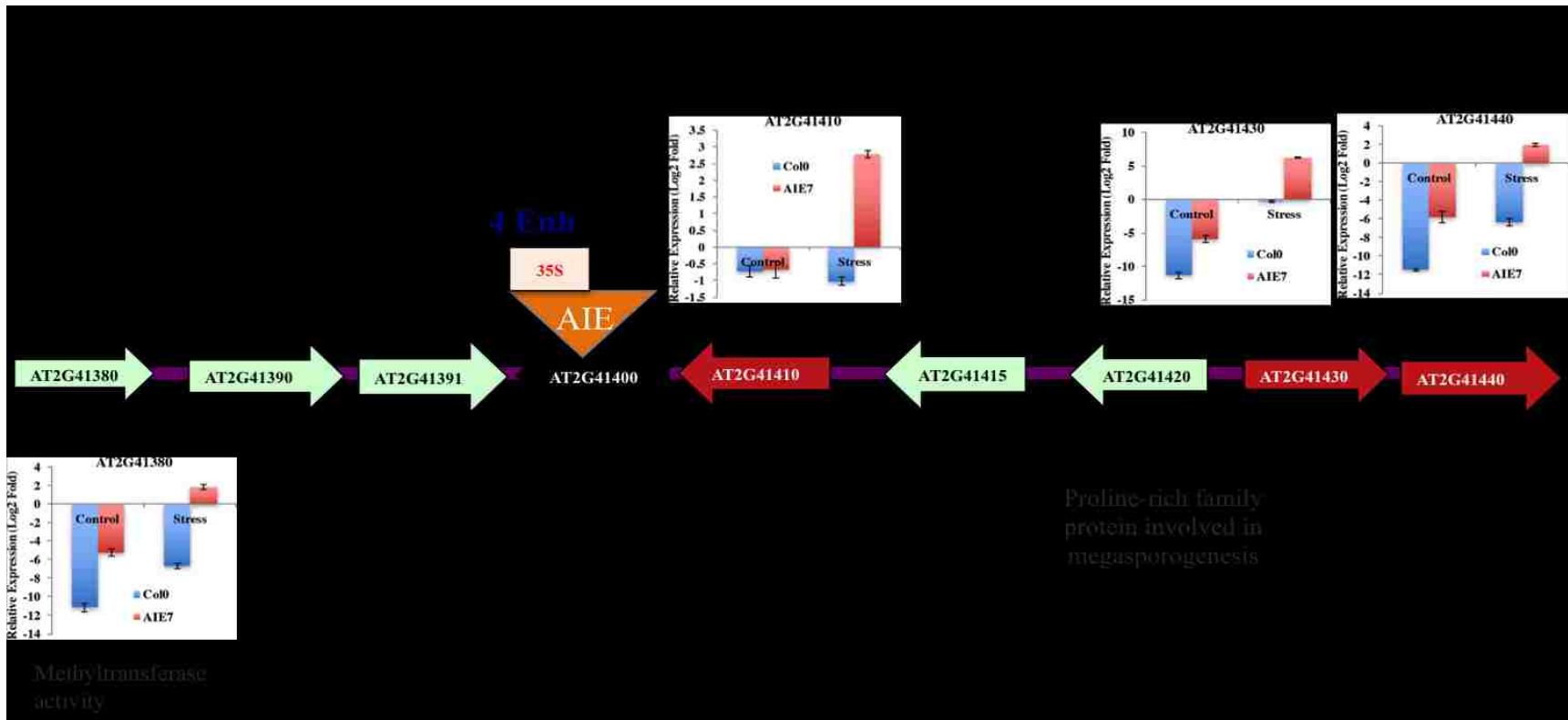
Figure 25: Schematic illustration representing the position of transposon (AIE) insertions in the Arabidopsis genome in the Columbia activation tag mutant line AIE7. The arrows indicate the coordinates and direction of candidate genes for salt tolerance based on TAIR genome sequence annotation in the ATag line (AIE7). The black arrow refers to the gene at the ATag insertion site and the other arrows showing neighboring candidate genes for salt tolerance, 10kb upstream and 10kb downstream their distance from the 35S enhancer to the AIE, their function, and their expression in response to salt stress.

Conclusion

The genes identified during expression analysis through qPCR of the AIE7 and AIE70 tolerant Arabidopsis mutant lines surprisingly suggest that there is a plethora of potential functions in the plant genome that need to be evaluated further using activation of genomic regions (with methods such as this transposon activation tagging system) which may lead to gain of function due to sets of nearby gene loci. This approach uses strong enhancers for regional activation of promoters enhancing local gene expression, as well as the systematic analysis of mutant phenotypes that are obtained, possibly due to coordinate expression of multiple genes on the chromosome which are not usually traceable for normal salt stress responses. This function is attributed to the possibilities provided by an efficient activation tagging system, whereby nearby genes may get activated, effecting the expression of coordinate expression of closely linked genes, much like a regulon, that then exhibit the tolerance phenotype in the plant.

Activation tagging approach has high prospects, as targeting just one gene locus can yield a specific interaction. That being said, as revealed here the transposon based activation tagging system can reveal hidden dominant gain-of-function genetic interactions that are not often found to be expressed naturally but are unveiled in the presence of the strong promoters that activate nearby loci as well. Such revelations are very useful as they can be genetically incorporated in the economically significant crops in the form of a cassette comprising promoters, enhancers, transcriptional regulatory machinery and desired effector genes, to naturally defend against external stress factors. In some ways this system is much like bacterial operons, where several genes involved in a function are linked together and closely regulated. In higher organisms, they are separate so that multiple regulatory factors can be involved in their regulation independently. By coordinating regulation as a unit they are effective in combining multiple functions needed

for the expression of a complex trait. It is extremely beneficial in the biological field to use overexpression of gene functions that are feasible to control and exhibit enhanced traits, compared to those involving gene knockouts or suppression. Along with this, such an approach will also open up pathways for evaluating inter-chromosomal interactions that may be happening during natural responses against salt stress. The information gathered about gene loci from activation tagging approaches can then be evaluated further for studying the expression of linked genes under varied situations using transcriptome or proteomic profiling.

Finally, just as Barbara McClintock termed these transposable elements as controlling elements, and while their functions in causing insertion mutations were found more spectacular, there remained a concept of the original description 'controlling elements' in the background while the mutagenic status was found more engaging. Just the same way, Peter Peterson's analysis of this transposon system suggested the mobile element name 'Enhancer' in the 50's before enhancers were described, suggesting a regulatory role which did not stop at the single gene level but affected chromosomal domains and batteries of regulated genes. The feature that remains is to use this method of coordinate regulation and potentially obtain enhanced traits due to domains of genes with similar functional roles.