FUNCTIONAL STUDY OF COMMON BEAN ISOLIQUIRITIGENIN 2'-O-METHYLTRANSFERASE GENE UNDER SALT STRESS

by

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ABSTRACT

Soil salinity has been one of the major problems in agriculture for many years. Nearly 20% of all cultivated land and half of irrigated land of world soil are saline and these levels are expected to rise in the future which will result in further crop yield reduction. *Phaseolus vulgaris* L. (common bean) is a legume crop with great value in the world with its nutritional and agricultural features, yet it is fairly susceptible to salt stress. Thus to understand the underlying mechanism of salt stress tolerance in plants become a necessity to eventually develop salt tolerant varieties. Our previous studies on transcriptome analysis of common bean under salt stress has revealed a major differential expression in transcripts of secondary metabolism which undoubtedly plays role in both biotic and abiotic stress responses. Further bioinformatics analysis on the transcriptome data has pointed out many salt responsive genes. A secondary metabolism gene, Isoliquiritigenin 2'-O-methyltransferase (ChOMT) has emerged as a prominent gene in salt-tolerance responses from the *in silico* analysis of salt induced transcripts in a salt-tolerant common bean variety. Overexpression of common bean ChOMT gene in *Arabidopsis thaliana* model enhanced salt tolerance of transgenic plants possibly by creating an impact on (i) accumulation of organic solute content and architectural change in root tissues to compensate adverse effect of osmotic stress and increase in the chance to absorb and conduct water to ensure biomass, and (ii) protection of seed integrity via increase in the viability and vigor of seeds.

Our results suggest that ChOMT can be a good candidate gene to improve crops for salt stress tolerance.

ÖZET

Ekilebilir alanlarda tuz oranlarının artışı yıllardır tarımın gelişmesinin önündeki en büyük sorunlardan biri olmuştur. Dünyadaki tarım alanlarının yüzde 20'si tuz stresi altındadır ve bu miktarın gelecekte artması üretim miktarlarında daha fazla düşüşe neden olacaktır. Baklagiller familyasının bir üyesi olan fasülye bitkisi dünyada besinsel ve tarımsal değerleriyle öne çıkmaktadır ve tuza karşı duyarlılık göstermektedir. Bu yüzden fasulye bitkisinin tuz koşullarında verdiği tepki mekanizmalarının anlaşılması, dayanıklı çeşitlerin geliştirilmesi için bir gereklilik olmuştur. Fasülye bitkisinin tuz stresi altındaki transkriptom analizi strese dayanıklılıkta ikincil bitki metabolizmasının önemini göstermiştir. Transkriptom verisinin biyoinformatik analizi tuzlulukta etkin olan bir çok geni ortaya çıkarmıştır. Tuza dayanıklı bir fasulye çeşidi olan İspir'in transkriptomundaki tuz tarafından indüklenen genlerin *in siliko* analizi, bitki ikincil metabolizmasının bir parçası olan, isoliquiritigenin 2'-O-methyltransferase (ChOMT) adlı genin tuza dayanıklılık tepkilerinde önemli bir yeri olabileceğini göstermiştir. Bu genin Arabidopsis bitkisinde anlatımının arttırılması transgenik bitkilerde muhtemel olarak (i) organik çözünen miktarının artması ve kök yapısının değişmesine neden olarak ozmotik stressin etkilerini kompanse etmiş ve bitkinin suya ulaşımı kolaylaştırarak biyokütle korunumunu sağlamış ve de (ii) tohum bütünlüğünü koruyarak çimlenme oranını arttırmıştır. Sonuçlar ChOMT geninin tuza dayanıklı tahıllar geliştirilebilmek için önemli bir aday gen olabileceğini ortaya koymuştur.

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LIST OF ACRONYMS

bp	Base pairs
gr	Gram
kb	Kilobase
M	Molar
Cl^-	Chloride ion
g	Gravitational force
L	Liter
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
Na^+	Sodium ion
°C	Centigrade degree
s	Second
µg	Microgram
µl	Microliter
NaCl	Sodium chloride
ChOMT	Isoliquiritigenin 2'-O-methyltransferase

OD	Optical density
ROS	Reactive oxygen species
ORF	Open reading frame
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
SAM	S-adenosyl-L-methionine
UV	Ultraviolet
Nr	Non-redundant
FDR	False discovery rate
EC	Electrical conductivity
dS/m	DeciSiemens per meter
KEGG	Kyoto encyclopedia of genes and genomes
GI	GenInfo
SAM	S-adenosyl-L-methionine
MT	Methyltransferase
COG	Clusters of orthologous groups
Uni-Prot	Universal protein resource
RPKM	Reads per kilobase of transcript per million reads mapped
Diff.	Difference

1. INTRODUCTION

1.1. Abiotic Stress and Importance of Water

Any non-living factor in a given environment which has a negative impact on the organism can be defined as abiotic stress factor. Factors like cold, heat, salinity, drought, ultraviolet light and heavy metals cause dysfunction of the biochemical nature of an organism and generate instability (Figure 1.1).

The primary reason of crop failure worldwide is majorly caused by abiotic stresses resulting in a dramatic decrease of more than 50% in yields for most of the major crops [1]. Only an estimated 3.5% of the land on Earth is not affected by an abiotic stress factors [2].

Among abiotic stresses, majority of the factors such as heat, cold, drought and salinity result in similar responses on plants mainly causing decrease in photosynthesis and growth, hormonal imbalances, oxidative damage and stress-related molecule aggregation. These responses are generally the consequence of dehydration of plant tissues [3, 4]

Liquid water, undoubtedly is the most basic and indispensable requirement for life to flourish and thrive, at least in our world. It is for sure the most crucial necessity for a plant since 90% of the fresh weight of most plants are comprised of water. Its unique biophysical features like high dielectric constant, high surface tension and high heat of vaporization provides an ability to solvate diverse number of ions, minerals and molecules while protecting liquidity over a broad range of temperature. Besides, water serves as a reactant in various biochemical reactions, one of the most significant being the main electron donor molecule in photosynthesis. Moreover, water is the primary constituent of cell turgor maintenance in physiological aspect [2].

Today, world with over 35% arid or semiarid land surface area experience inadequate precipitation, thus availability of water is a major limitation to plant productivity [5].

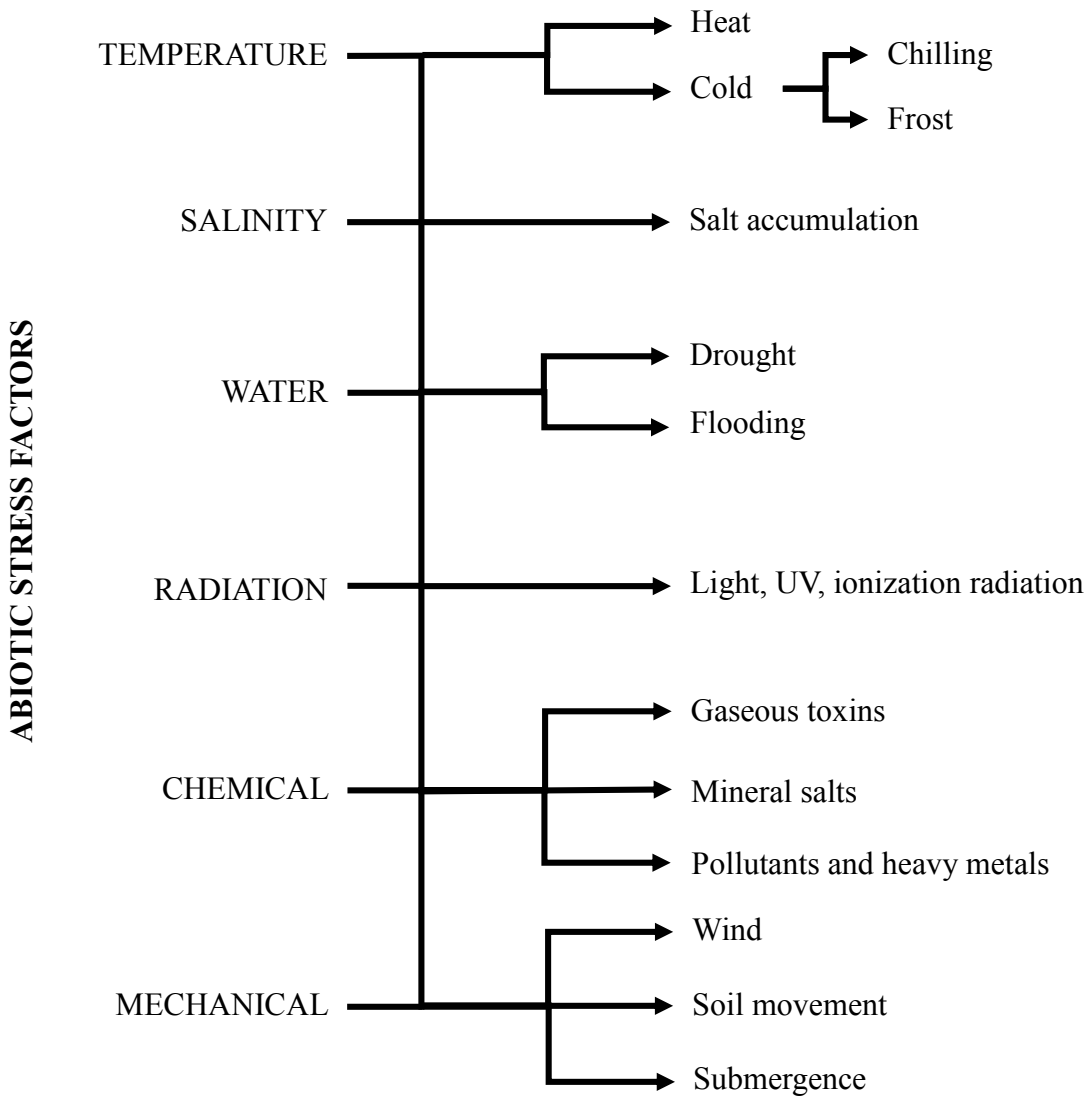

Figure 1.1. Abiotic stress factors and main stress signals

1.2. Soil Salinity and the Effects of Salt Stress on the Plant Biology

Effects of drought stress and salt stress on plants overlap with each other on the basis of physiology. Decreased soil porosity resulting from the altered texture of the soil due to deposited high salt concentration gives rise to reduced aeration and water conductance. The resulting low water potential zone makes it difficult for the plant to obtain the water and the nutrients, causing both hyperosmotic and hyperionic stress that can lead to death of the plant [6].

A soil featuring an electrical conductivity (EC) of 4dS/m or more than 40mM NaCl is considered as saline [7]. Abundance of salt on Earth creates a major environmental stress and is a substantial constraint to global agricultural productivity on which the sustainability of increasing human population depends. Nearly 20% of all cultivated land and half of irrigated land of world soil are saline [8] (Figure 1.2). Estimations suggest that throughout the mid-21st century the loss of arable land will reach up to 50% due to salinization [9].

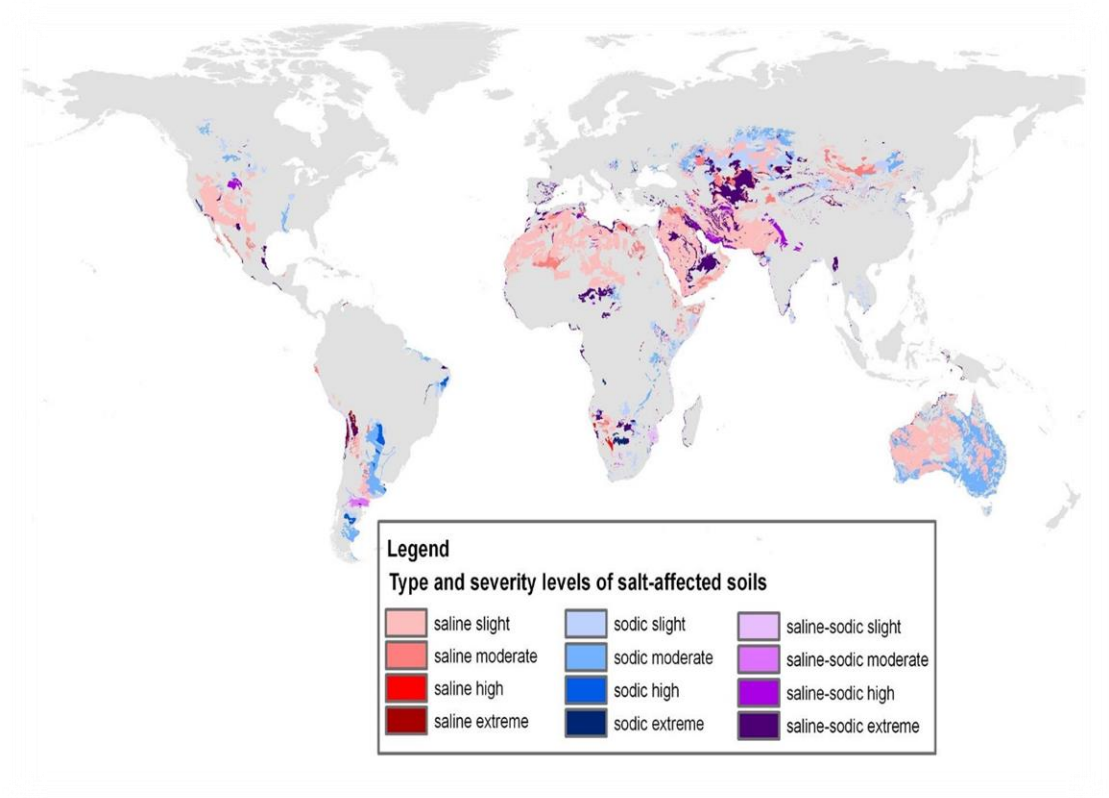

Figure 1.2. Salt and sodium accumulation levels of the arable land on the global extent. Nearly 1.1 global hectares (Gha) are saline as indicated by FAO Harmonized World Soil Database [10].

High salt stress causes several instabilities on the plant cells: Increase in Na^+ influx disrupts the optimum membrane potential which in turn increases the uptake of Cl^- creating an ionic disequilibrium. High concentration of Na^+ has toxic and noxious effects on the protein functioning [11]. Additionally Na^+ causes several different imbalances like

membrane disorganization, disruption of cell cycle, reduction in growth, osmotic imbalance, and reduction in photosynthesis leading to generation of reactive oxygen species [12, 13].

In salt-sensitive plants, drastic effects of these imbalances reflected as permanent reduction of shoot, and to lesser extent, root growth even within hours [14]. Prolonged stress conditions establish water potential imbalance between the two sides of the plasma membrane, resulting turgor reduction below the yield threshold (minimum cell turgor pressure for growth) which cease the growth completely [15]. Plants lose their biomass and photosynthetic capacity as the leaf turgor pressure drops in response to osmotic stress [16].

1.3. *Phaseolus vulgaris* L. on the Subject of Agriculture and Soil Salinity

Common bean (*Phaseolus vulgaris* L.) contains rich source of minerals, vitamins, and dietary proteins, thus plays an essential role in human nutrition. Besides being nearly half of the consumed and the most extensively produced grain legume in the world, it is beneficial and vital in agriculture by forming root nodules with nitrogen fixing bacteria thus increasing the nitrogen content of marginal lands for reclamation [17]. In average more than 17 million metric tons of common bean was produced annually in between years 2002-2012 in the world. While China with annual production of nearly 13 million metric tons was the leading country, Turkey was in the third position in the world with more than 575 thousand tons of production. Turkey was also the second most profited country from common bean export after China in those years (http://faostat3.fao.org/).

Common bean is salt-susceptible and the productivity of this grain legume can drop by 20% even at a saline soil with 1dS/m of electrical conductivity [18]. Majority of common bean production occurs in middle Blacksea region in Turkey; however, the soil of this region can be considered as saline even in unirrigated seasons (nearly 2dS/m). Kızılırmak river as being the major water source for the irrigation of this region, the soil salinity level can reach up to 2-4dS/m [19, 20]. Globally, agriculture of common bean is also under distress of saline soil. For example more than 20% of common bean cultivation areas in the Middle East are influenced by soil salinity where common bean is a vegetable crop of a great value [21].

The nutritional and agricultural values of common bean makes it a necessity to understand the underlying parameters of salt-responses of this plant. The genetic diversity

among common bean cultivars has shown the presence of salt tolerant varieties [22]. Comprehension of the differential responses given to salinity stress between the tolerant and susceptible varieties emerges to be a very important approach in the path of producing improved varieties.

1.4. How Do Plants Cope with Salt Stress?

Plant responses to abiotic stress are culminations of complex and dynamic processes depending on the actions and interactions of various genetic elements, cellular compartments and cofactors [23].

The effects of high salinity starts with the perception of the stress signal by membrane, cytoplasmic and nuclear bound receptors. Process continues with the plant signal responses like expression of transcription factors and stress-related genes, production of signaling proteins and generation of secondary molecules [14].

Plants reduce the rate of cell expansion in leaves and roots, and avoids water loss through stomatal closure as it encounters with the osmotic stress produced by high salinity [24]. As a result, the growth and development slow down. These regulations are also controlled by several plant hormones like, giberellins, saliciylic acid and abscisic acid which are proved to be involved in the regulation of stomatal conductance, growth rate and overall stress responses [25].

Additionally both hyperosmotic and hyperionic effects of salt stress leads to escalation in the concentration of ROS such as superoxide (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) in the cells which in turn causes oxidative damages in cells, majorly affecting the photosystems [26]. Increased oxidative stress triggers production of ROS scavengers like superoxide dismutases and glutathione peroxidases [6].

On the other hand, osmotic stress makes the plants accumulate ions and organic solutes. In spite of its degrading toxicity and extrusion of it from tissues, plants still accumulate Na^+ in their cells for the sake of maintaining the osmotic balance and preventing dehydration [12, 27]. Most of the salt tolerant plants have very efficient Na^+ secretion metabolisms for the regulation of internal salt concentrations [21] which means survival in high saline soils depends on the Na^+ accumulation ability and maintenance of high tissue-

Na^+ concentrations [12]. Plants deal with excessive Na^+ ions that make it to the shoots by either accumulating them in water filled vacuoles or excreting them from the leaves through modified structures like bladders and trichomes, yet the functionality of most enzymes are impaired as the intracellular Na^+ concentrations reach up to 100mM [28]. Thus plants employ specific molecules called compatible solutes to reset the impaired osmotic balance, so they can keep the ionic toxicity low by avoiding Na^+ ions.

Accumulation of compatible solutes (osmoprotectants) is a basic strategy most plants resort under abiotic stress for metabolic acclimation [29-31]. Compounds such as proline, glycine betaine and polyols are accumulated by many plants in remarkable amounts in the face of salt stress [32, 33]. Besides their well-known roles in osmotic protection [34, 35], they also have functions as stabilizing the photosystem II complex, low-molecular-weight chaperone, ROS scavenging, maintaining membrane integrity and protecting the structure of proteins [36-38].

Plants also employ vast constitution of pathways and small molecule products which referred as secondary metabolism, against all kinds of abiotic and biotic stresses. However, both synthesis of compatible solutes and formation of defensive secondary metabolites need excessive energy, thus reduces carbon to biomass production dramatically and result in a greatly reduced growth [39, 40].

In conclusion plants feature several distinctive mechanisms against osmotic and ionic stresses produced by high salt concentration, but all together these solutions to tolerate stress demands most of the energy that plant holds and halts development. Therefore understanding the most effective pathways in each stress condition, and generating crops that utilize such pathways in an enhanced fashion are prerequisites for developing efficient stress tolerant plants.

1.5. Plant Secondary Metabolism

Higher plants produce a wide variety of secondary metabolites from primary metabolites (e.g., carbohydrates, lipids and amino acids). The denomination ‘secondary’ was given to emphasize that these metabolites are not directly required for the primary processes like growth and development. These natural products are known to possess significant

functions in plant defense mechanisms in the cases of both biotic (herbivores, microbial pathogens) and abiotic stresses (UV exposure, drought, salinity, cold etc.). Secondary metabolites are separated into three major groups as viz-terpenes, phenolic compounds and nitrogen/sulfur-containing compounds which in overall make up more than 100,000 known metabolites mostly believed to be involved in biochemical defense mechanisms that have been evolving throughout millions of years [41].

Upsurge of secondary metabolites in diverse environmental stresses have been reported in numerous studies. For example deficiencies of sulfur, magnesium and potassium are known to rise the concentration of phenolics. Roots increase the release of phenolic acids in the case of low iron levels [27]. Calcium is known to take role in many plant abiotic stress responses including salinity [6]. Reports have shown that flavonoid anthocyanins accumulate in response to salt stress [42]. Moreover, formation of polyamines and phenyl amides increase dramatically in bean and tobacco under abiotic stress which implies the antioxidant roles of secondary metabolites [43]. Certain stresses, such as pathogen attack, UV-irradiation, high light, wounding, nutrient (nitrogen and phosphate) deficiencies, temperature and herbicide treatment often increase the accumulation of phenylpropanoids.

Essential roles of secondary metabolites in defense and adaptation to environment have been known and studied for a long time. Until the recent studies [44] they were considered as insignificant for the maintenance of life processes. However recent studies clearly indicate that they have important roles in plant growth, development and communication.

Osmotic and ionic stresses resulted from excessive salt in the environment creates a wide balance shift on the concentrations of many specific secondary metabolites in plants (Table 1.1) thus understanding the constituents of plant secondary metabolism is essential in the sense of stress physiology.

Table 1.1. Examples of secondary metabolites accumulated in plants in the case of salt-stress [44 and references therein]

Secondary metabolite	**Plant species**
Sorbitol	*Lycopersicon esculentum*
GABA	*Sesamum indicum L.*
Flavonoids	*Hordeum vulgare*
Jasmonic acid	*Lycopersicon esculentum*
Polyphenol	*Cakile maritima*
Tropane alkaloids	*Datura innoxia*
Anthocyanins	*Grevillea spec.*
Trigonelline	*Glycine max*
Glycinebetaine	*Trifolium repens*
Polyamines	*Oryza sativa*
Glycine betaine	*Triticum aestivum*
Sucrose and Starch	*Cenchrus pennisetiformis*

1.5.1. Flavonoids

Flavonoids are phenolic secondary metabolites that are found abundantly in many kind of plants and they consist of seven major subgroups: Chalcones, anthocyanins, pro-anthocyanidins, condensed tannins, flavones, flavonols, flavandiols and aurones [45]. It is known that flavonoids tend to increase in concentration in the face of environmental stresses like pathogen attack, wounding, temperature changes, UV-irradiation and drought [46]. Diverse and broad biological functions have been embraced by more than 6000 diverse flavonoids identified comprising protection against various biotic and abiotic stresses as well as displaying actions in fertility, coloration of flowers and signaling with symbiotic nitrogen-fixing bacteria during induction of nodulation [47].Some flavonoids are known to be very

effective on assuring protection from cellular stress conditions by chelating ROS generating metals via the Fenton reaction and scavenging free radicals. Moreover there is compelling evidence on the regulatory roles of flavonoids on polar auxin transport pathway [48] which is known to take part in several stress responses via regulating stomatal opening and partitioning nutrients under stressful growth conditions [49, 50].

1.5.2. Isoliquritigenin

As a chalcone class flavonoid compound, isoliquiritigenin (2′,4′,4′-trihydroxychalcone) [51] exhibits a dual benzene ring structure linked by an α, β-unsaturated carbonyl group [52]. It is found in several plants such as *Glycyrrhiza uralensis* (licorice) [51], *Sinofranchetia chinensis* [53], *Allium ascalonicum* [54], *Dalbergia odorifera* [55] and *Glycine max* L [56] Isoliquiritigenin demonstrates various and valuable pharmacological activities such as anti-inflammatory [55], anticancer [57], antiangiogenic[58], antiallergic [59] as well as antioxidant effects [60]. Interest on pharmacologic activities of isoliquiritigenin on human health ascended rapidly in the recent years yet, its role on plant stress defense and regulation of metabolism have been highly disregarded.

1.5.3. Methylation and Methyltransferases

Methyl group addition to biochemical molecules like neurotransmitters, lipids, nucleic acids, hormones and proteins alters the physiochemical aspects of the molecules. Broad functions of methylation encompass detoxification, signal transduction, protein repair and sorting, biosynthesis, metabolism and nucleic acid processing [61].

Methylation reactions are performed by numerous different methyltransferase enzymes thus methylation can yield variety of functions. Distinct families of MT enzymes have been discovered and studied. Catalysis of the majority of methylation reactions are performed by S-Adenosyl-L-methionine (SAM)-dependent MTs [62] in which SAM acts as a methyl group donor.

O-methyltransferases are SAM-dependent MTs that constitute a large family of enzymes which catalyze O-methylation of phenolic secondary metabolites such as

flavonoids. The acceptor phenolic compounds turn into their methyl ether derivatives and the reaction gives S-Adenosyl-L-homocysteine as a byproduct which in turn is used in methionine cycle to produce SAMs again.

SAM binding region is conserved among this enzyme family, yet active sites possess a general variety for binding and positioning of diverse family of substrates. As plants are known to synthesize a vast amount of phenolic compounds, it is appreciable that plant OMTs harness a significant substrate specificity [63]. The underlying chemical mechanism of methyl transfer reactions is shared by many OMTs, yet their selectivity differs both on the basis of the stereochemistry of the acceptor compound and their phenolic hydroxyl group substitution patterns [63].

Transferase mediated chemical and enzymatic substitution reactions that mostly happen on the phenolic rings create a diversity on the basis of structure and function for flavonoid compounds. Modification of the flavonoid backbone with methyl groups by transferases alters the reactivity, solubility and interaction with cellular targets of the flavonoid thus modulates its physiological activity [64, 65]. O-methylated flavonoids are reported to be significant effectors not only on plant growth and development but also plant-environment communication by regulating symbiosis, auxin transport and phytoalexins [66-68]. Moreover, experiments on salt-tolerant barley [69], salt-tolerant sweet-potato [70] and ice plant [71] have demonstrated that OMTs were highly expressed in saline conditions and may possess a prominent role in salt-tolerance.

Isoliquiritigenin (2', 4, 4'-trihydroxychalcone) 2’-O-methyltransferase (ChOMT) is a SAM- dependent methyltransferase which catalyzes the methylation of 2’-hydroxyl group of isoliquiritigenin producing 2'-O-methylisoliquiritigenin (4, 4’-Dihidroxy-2’-methoxychalcone). Studies on *Medicago sativa* [72] have shown that ChOMT is primarily expressed in the roots and nodules, and its product 2'-O-methylisoliquiritigenin was reported to be the most potent nodulation-gene inducer for symbiotic nitrogen-fixing bacteria, *Rhizobium meliloti* [73]. Therefore, ChOMT is thought to have a key role in the formation of the nitrogen-fixing root nodules in this plant.

Highly limited knowledge about ChOMT comes from studies on *Medicago sativa* plants. Although isoliquiritigenin is found in both legume [73] and non-legume [53, 74] species, isoliquiritigenin 2’-O-methyltransferase was detected only in certain legume species

such as *Glycine max* and *Medicago sativa* [72]. There is still much to be studied about the effects and the regulation of this very specific methyltransferase in the cases of biotic and abiotic stresses since it modulates the actions of isoliquiritigenin, a very potent and promising antioxidant agent for the stress physiology.

1.6. Biotechnological Approach on Functional Analysis for Stress Tolerance

Genetic alteration and expression regulation of candidate genes to generate stress tolerant crops have been the most commonly utilized techniques in recent years. Proteome analyses have shown that plant stress tolerance improves considerably due to accumulation of relevant proteins in the cellular organelles under abiotic stress. On this basis, overexpression studies can be considered as a powerful and an effective technique for the generation of transgenic plants for enhanced stress tolerance [75, 76]. Recently performed overexpression studies using candidate genes with roles in the regulation of osmotic homeostasis such as *atDREB1A* transcription factor in *Arachis hypogaea* [77], *S-adenosly methionine decarboxylase* in *Arabidopsis thaliana* [78], *vacuolar H^+-pyrophosphatase* in cotton [79], and *pyrroline-5-carboxylate synthetase* (*P5CS*) in wheat [80], and also genes that have role in ROS scavenging mechanisms such as *WRKY70* transcription factor in *Arabidopsis* [81] and *superoxide dismutase 2* (*SOD2)* in rice [82] have been successfully used in development of stress tolerant plants.

Recent developments in genetic approaches have allowed us to generate a bigger picture of stress response pathways induced by several different abiotic stresses which supported the hypothesis that, plants employs common set of response mechanisms for various stress factors [83]. Therefore upon application of recent technologies, exploration and functional characterization of the stress-related genes even for a unique plant or for a unique stress factor may build up a fine foundation for the general understanding of stress-pathways for all plant types.

1.6.1. RNA-sequencing and Transcriptomics

A transcriptome is the full set of messenger RNA (mRNA) transcripts and their quantities expressed by a cell, specific to an environmental condition, developmental stage,

and affiliated tissue type. The transcriptome exhibits a dynamic structure in contrast to stable genome and in close interaction with its environment. Thus understanding characteristics of transcriptome is crucial for the evaluation of the molecular constituents and the functional elements of distinctive cells in different conditions [84].

Gene expression profiling has become practical for understanding the effects of biotic and abiotic stresses on plants, as the high-throughput approaches have been developed. Recent years have proved that RNA-sequencing is an influential tool for expression profiling to assess the stress relevant genes of model plants and important agricultural crops. Although studies on transcriptome assemblies of legume species [85-88] have gained momentum with appearance of publications regarding the effects of alkaline conditions, salt stress and drought on expression profiling of soybean [89], *Medicago truncatula* [90], alfalfa [91] and chickpea [92] respectively, there has not been enough focus yet on the effects of abiotic stress factors in common bean. Study of transcriptome analysis of Ispir variety common bean, under saline conditions by our group [31] has been the first publication, providing not only valuable input to the literature, but it also contributed to the general knowledge on stress related genes especially from legume species perspective. The results of transcriptome analysis has constituted the basis for the studies presented in this thesis.

2. PURPOSE

Analysis of differentially expressed genes in the transcriptome of salt-tolerant common bean root tissues have created certain insight about potential candidate genes that may play role in salt stress tolerance mechanisms. Further analysis of these candidate genes in their function on salt tolerance responses has commenced especially on those genes which were upregulated upon stress in different common bean varieties.

In this project, *Arabidopsis thaliana* has been used as model organism for overexpression of the selected candidate gene 'isoliquiritigenin 2'-O- methyltransferase' (ChOMT) of common bean which displayed a significant upregulation in root tissues in our transcriptome analysis. Despite of the specificity of this enzyme to legume species and its suspected importance in stress physiology, the scarcity of the knowledge on its role in stress responses has been a driving force in our choice to evaluate its function. Phenotype assessments were performed upon measurements of physiological parameters to determine the functional effects of the candidate gene in overexpression transgenic lines under control and saline conditions to evaluate its impact on tolerance.

3. MATERIALS

3.1. General Enzymes, Kits and Reagents

Table 3.1. List of general enzymes, kits and reagents

Name	Model
Plasmid Miniprep Kit	740615 Nucleospin Plasmid Quickpure, Macherey-Nagel, Germany
First Strand cDNA Synthesis Kit	K1622, Thermo Scientific (RevertAid), USA
DNA Ladder	SM0311, GeneRuler 1 kb DNA Ladder, Fermentas, USA
DNA Loading Dye	B7021S, Fermentas, USA
LR Clonase	11791, Gateway® LR Clonase II Enzyme Mix, Life Technologies, USA
BP Clonase	11789, Gateway® BP Clonase II Enzyme Mix, Life Technologies, USA
qPCR Master Mix	K-6252 Accupower 2X GreenStar Master Mix, Bioneer, Korea
DNA Polymerase	EP0711, DreamTaq Green DNA Polymerase, Thermo Scientific, USA
	F-530S, Phusion High-Fidelity DNA Polymerase, Thermo Scientific, USA
	F-130, Phire Plant Direct PCR Kit, Thermo Scientific, USA
Magnesium Chloride	25mM MgCl2 Solution (Promega, USA)
dNTPs	10mM PCR Nucleotide Mix (Promega, USA)
Genomic DNA Extraction Kit	69106 DNeasy Plant Mini Kit, Qiagen, USA
RNA Extraction	15596-026, TRIzol Reagent, Invitrogen, USA
Gel Extraction Kit	K0691, Genejet Gel Extraction Kit, Thermo Scientific, USA

3.2. Chemicals, Plastics and Glassware

Chemicals were purchased from either *Applichem* (Germany), *Merck* (Germany), *Duchefa* (Netherlands) *or Sigma-Aldrich* (USA) unless stated otherwise; tips and tubes from Axygen (USA), falcon tubes from *BD Biosciences* (USA) sterile plates from *Interlab* (Germany). Glasswares were purchased from *VWR* (USA). For sterilization, all glassware, tips and tubes were autoclaved at 121 ^{o}C for 20 minutes before use.

3.3. Equipments

Table 3.2. List of used equipments

Name	**Model**
Agarose Gel Electrophoresis	MINICELL PRIMO EC320 Electrophoretic Gel System, Thermo Scientific, USA
Autoclaves	Model MAC-601, Eyela, Japan
	Model ASB260T, Astell, UK
Balances	AY123, Satorius, Germany
Centrifuges	5453000 MiniSpin Plus Benchtop Centrifuge, Eppendorf, Germany
	Allegra X-22, Beckman, USA
	J2-MC Centrifuge, Beckman, USA
	J2-21 Centrifuge, Beckman, USA
Deep Freezers (-20°C)	A2021-D, Arçelik, TURKEY
Deep Freezers (-80°C)	Forma 860-ULT, Thermo Scientific, USA
Ice Machine	Scotsman Inc. AF20, ITALY
Magnetic Stirrer	0004810000 IKA RCT basic Safety Control, Germany
Scanner	EPSON GT-20000 Scanner
Thermal Cycler	C1000 Thermal Cycler, Bio Rad, USA

Table 3.2. List of used equipments (cont.)

Thermal Cycler	Runik Thermal Cycler, Sacem Life Technologies, TURKEY
Incubator	EN500 Nüve, TURKEY
Microwave	MD55I, Arçelik, TURKEY
Gel documentation system	Gel Doc XR System, Bio Rad, USA
Micro-centrifuge	Himac CT15RE, Hitaci Koki, Japan
Microplate Reader	680, Bio Rad, USA
Oven	Gallenkamp 300, UK
pH Meter	HI 83141, Hanna, USA
Pipettes	Pipetman Classic, Gilson, USA
Power Supply	164-5050 PowerPac Basic, Bio Rad, USA
	EC250-90, Thermo Scientific, USA
Refrigerator (4°C)	MFAA1, Hotpoint Ariston, Italy
Rotors	JS-7.5 Beckman, USA
	JA-14 Beckman, USA
Spectrophometer	NanoDrop1000, Thermo Scientific, USA
Vortex	NM110, Nüve, Turkey
Gartengold Torf	Substrate1, SAB, Germany
Plant Growth Chamber	JSPC-960, JSR, Korea
Real Time PCR System	PikoReal96, Thermo Scientific, USA

3.4. Buffers and Solutions

Table 3.3. General buffers and solutions

Name	Ingradients and Concentrations
LB Medium	10g/L Tryptone
	5g/L NaCl
	5g/L Yeast Extract
LB Agar	10g/L Tryptone
	5g/L NaCl
	5g/L Yeast Extract
	15g/L Agar
YEP	10 g/L Peptone
	10g/L Yeast Extract
	5g/L NaCl
	10g/L Bactoagar
MS Medium (0.5x)	2.2g/L Murashige Skoog Basal Medium
	10g/L Agar
Kanamycin	50mg/ml in ddH2O
Rifampicin	50mg/ml in DMSO
Hygromycin B	15mg/ml in ddH2O
Spectinomycin	50mg/ml in ddH2O
Gentamicin	30mg/ml in ddH2O

Table 3.4. Modified Hoagland solution for common bean growth

Macronutrients	**Concentrations (mM)**
$MgSO_4.7H_2O$	1.4
KH_2PO_4	0.02
$CaNO_3$	2.8
KNO_3	1.8
Micronutrients	**Concentrations (µM)**
H_3BO_3	20
$MnSO_4$	1.1
$CuSO_4.5H_2O$	0.2
$NaMoO_4.2H_2O$	0.1925
$ZnSO_4.7H_2O$	0.5
NaFe(III) EDDHA	1

Table 3.5. Hydroponics nutient solutions for Arabidopsis growth

Germination Medium		**Basal Nutrient Solution**	
Macronutrients	**Concentration (mM)**	**Macronutrients**	**Final conc (mM)**
$CaCl_2$	0.75	NH_4NO_3	2
KCl	1	KNO_3	3
$Ca(NO_3)2.4H_20$	0.25	$CaCl_2$	0.1
$MgSO_4.7H_20$	1	KCl	2
KH_2PO_4	0.2	$Ca(NO_3)2.4H_20$	2
$MgSO_4.7H_20$	0	$MgSO_4.7H_20$	2

Table 3.5. Hydroponics nutient solutions for Arabidopsis growth (cont.)

KH_2PO_4	0	KH_2PO_4	0.6
NaCl	0	NaCl	1.5
Micronutrients	**Final conc (µM)**	**Micronutrients**	**Final conc (µM)**
NaFe(III)EDTA	50	NaFe(III)EDTA	50
H_3BO_3	50	H_3BO_3	50
$MnCl_2.4H_20$	5	$MnCl_2.4H_20$	5
$ZnSO_4.7H_20$	10	$ZnSO_4.7H_20$	10
$CuSO_4.5H_20$	0.5	$CuSO_4.5H_20$	0.5
Na_2MoO_3	0.1	Na_2MoO_3	0.1

3.5. Biological Material

3.5.1. Plant Material

Common bean (Ispir and TR43477 varieties) seeds were provided by Prof. Yıldız Dasgan from Çukurova University. *Arabidopsis thaliana* (Col-0 variety) seeds were provided by Dr. Giorgia Batelli, CNR-IGV, Italy.

3.5.2. Bacterial Strains

Escherichia coli, DH5α strain was used for cloning experiments. *Agrobacterium tumefaciens*, GV3101 strain that is resistant to rifampicin (100µg/ml) (in genome) and gentamicin (30µg/ml) (in helper plasmid) was used for transformation experiments.

3.5.3. Plasmids

Gateway® destination vector pDONR207 and binary vector pMDC32 were obtained from Dr. Giorgia Batelli, CNR-IGV, Italy and binary vector pGWB411 was obtained from Dr. Nakagawa T. from Shimane University, Japan.

3.5.4. Primers

3.5.4.1. Real Time Primers.

Table 3.6. List of qRT-PCR primers for selected pre-candidate genes

Gene Name	Sense/Anti-sense Primer (5'-3')	Amplicon size (bp)	Annealing Temp. (C°)	Nr-ID
BURP-domain containing protein	CTCCACCTTTTCCACCAAC	157	56	188531129
	CTTCCCACTACTCCTATTCC			
Ferric reductase	CAGAGTCAAGCATCAAGT	163	54	302633356
	GCAATAATCCCAGCTACAT			
Isoliquiritigenin 2'-O-methyltransferase	TCCACAACAACCACCTTC	237	54	359806350
	AATCTCCAAATACCCTTCC			
Sulfate transporter	TGATCCCATTGCAAATCC	151	53	357489361
	TCCCCCCATAAAACCAAC			
Vicilin-like antimicrobial peptides 2-3	ACTCCAACAAACTCGAAACA	234	55	357483349
	CACATACCACTCGGACCA			

Table 3.7. List of qRT-PCR primers for internal reference genes

Name	Sense/Anti-sense Primer (5'-3')	Amplicon Size (bp)	Annealing Temp. (°C)	Genbank Number
Insulin degrading enzyme	GCAACCAACCTTTCATCAGC	156	56	FE702602.1
	AGAAATGCCTCAACCCTTTG			
Actin 11	TGCATACGTTGGTGATGAGG	190	58	CV529679.1
	AGCCTTGGGGTTAAGAGGAG			

3.5.4.2. Cloning Primers.

Table 3.8. Primary *attb* cloning primers for Gateway® Cloning

Primer Name	Sequence (5'-3')	Annealing Temp. (°C)
pv_c_iso-liq_Fwd	AAAAAAGCAGGCTTCATGGGGGAATCCTATGTTGT	62
pv_c_iso-liq_Rev	CAAGAAAGCTGGGTCTTACTTGTAGAATTCCATCA	52

Table 3.9. Secondary *attb* cloning primers for Gateway® Cloning

Primer Name	Sequence (5'-3')	Annealing Temp. (°C)
*attb*2nd_step _F	GGGGACAAGTTTGTACAAAAAAGCAGGCT	54
*attb*2nd_step _R	GGGGACCACTTTGTACAAGAAAGCTGGGTC	

3.5.4.3. Sequencing Primers.

Table 3.10. List of sequencing primers

Vector/Gene	**Primer Name**	**Sequence (5'-3')**
pDONR207	pDONR207-F	TCGCGTTAACGCTAGCATGGATCTC
	pDONR207-R	GTAACATCAGAGATTTTGAGACAC
Isoliquiritigenin 2'-O-methyltransferase	Iso_liq_seq_F	GAAGGAAGCAGTGATTGA
	Iso_liq_seq_R	ATGGAAGGGTATTTGGAG

4. METHODS

4.1. Plant Growth Conditions

Ispir (salt-tolerant) and TR43477 (salt-susceptible) varieties of common bean *(Phaseolus vulgaris* L.) were grown and salt treated in hydroponic conditions to collect root samples. Hydroponics system was generated by black storage boxes containing nutrient solution connected to an air pump for continuous oxygenation of the solution. The seeds of the plant materials were sterilized in 5% hypochlorite solution for 5 minutes and rinsed three times with distilled water. Germination was done in plug trays containing vermiculite under a 16-h light/8-h dark photoperiod at 24°C/20°C cycle with 50-70% relative humidity. Trays were watered daily with 1X Modified Hoagland nutrient solution (Table 3.4) until the plants reached to fully expanded foliage stage. Four seedlings from each variety were wrapped with sponges around their shoots and placed into the holes on the two separate boxes containing nutrient solution that was aerated continuously. Salt-treatments were carried on in the same conditions with the relevant transcriptome study on salt-tolerant common bean performed by Hiz *et al*., (2014) to obtain correlated results. Gradual step acclimation method was employed to prevent osmotic shock [93]. After five days post transfer, the plants in one box was left as control group, and the other plants were subjected to gradual NaCl treatment starting with 50mM first day, increased to 100mM on the second day and set to 125mM on the third day. In total, the plants were grown under 125mM NaCl for three days before they were sacrificed for root tissue sample collection.

Arabidopsis thaliana plants (Col-0 ecotype) were grown at 22°C with 16/8 hours light-dark cycle and 50-60% relative humidity in JSPC-690 plant growth chamber. Turf was used as soil and the watering was done with half strength Basal Nutrient Solution [94] twice a week until most of the cliques were dry. When the plants were fully dried, the seeds were harvested with the help of sieves to exclude the seed coats and other contaminants.

4.2. Candidate Gene Selection

The selection of salt responsive candidate genes were based upon bioinformatics analysis and literature search on the basis of RNA-Seq results that was performed by Mahmut Can Hiz as part of his ongoing Ph.D thesis study.

RNA sequencing of the total RNA samples from leaf and root tissues of Ispir variety (extracted from both salt treated and control plants) was performed by Beijing Genomics Institute (Shenzen, China) using Illumina HiSeqTM 2000 system. The *Phaseolus vulgaris* reference genome (http://phytozome.jgi.doe.gov/) was not available at the onset of the study, therefore *de-novo* assembly method was employed. Assembled sequences in their final forms were referred as unigenes. Annotations, sequence orientations and protein coding region predictions determined by aligning the assembled sequences to NCBI non-redundant (NR) protein, Uni-Prot protein, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Cluster of Orthologous Groups (COG) databases [31]. RPKM (reads per kilobase per million) based method [95] was used to assess the relative gene expression levels of the treated samples in comparison to control samples.

4.2.1. Bioinformatics Analyses

Selection of a prominent candidate gene for this study from a long list of transcripts was performed after several steps of eliminations. Further bioinformatics analyses were carried out especially on upregulated transcripts in roots; non-regulated or downregulated genes were disregarded. Normalized RPKM values were represented in $\log_2$ base for the expression of the fold-change levels of the unigenes in treatment conditions. Default stringency cut off points (log2 (RPKM tr/cont) ≥ 1, *P*-value ≤ 0.05 and False Discovery Rate; FDR < 0.001) for defining the significant expressional regulation on the sequencing data, have produced an upregulated unigenes list that was comprised of 1237 transcripts. Increasing the stringency with new cut off points (log2 (RPKM tr/cont) ≥ 2, False Discovery Rate < 0.0001) was effective both on removing the transcripts that were not significantly upregulated and reducing the number of false positives. The new defining conditions created a list which was further narrowed down by removing the transcripts with low number of total raw fragments (≤ 100); that were not characterized or found in the Nr-database; with a gene

length less than 750bp and with an Nr-score for similarity to the its match in Nr-database less than 500.

Increased stringency produced a list of 60 transcripts (Table B.1) which were further analyzed for candidate gene selection. Transcripts with no definitive annotation (such as hypothetical and uncharacterized proteins) or that were known to be part of extensively studied metabolisms were eliminated from the list.

A total of 5 transcripts (Table 4.1) were selected as pre-candidate genes for transgenic studies after the remaining transcripts were searched in literature and public databases for prominent functions and characteristics in abiotic stress related pathways and responses. Full expression patterns of those genes in the roots of both Ispir and TR43477 varieties in control and salt treated conditions were compared to each other by qRT-PCR (Section 4.2.2). A flowchart for bioinformatics analyses was presented in Figure 4.1.

Table 4.1. Selected pre-candidate genes list

Gene ID	**Gene length (bp)**	**Raw read #**	**Fold dif. (log2)**	**FDR**	**GI number**	**Nr-score**	**Annotation**
Unigene23432_All	1232	1462	3.84	4.4E-289	359806350	577	isoliquiritigenin 2'-O-methyltransferase
Unigene4622_All	1121	232.5	3.54	2.61E-42	357489361	525	Sulfate transporter
Unigene5066_All	1324	2121.5	2.33	1.6E-227	188531129	684	BURP domain-containing protein
Unigene623_All	1457	218	5.57	2.94E-56	357483349	600	Vicilin-like antimicrobial peptides 2-3
Unigene8946_All	795	774.5	2.06	4.89E-68	302633356	521	Ferric reductase

The final elimination step was performed according to results of deep literature search and qPCR analysis together with KEGG Pathway enrichment analysis [31] for most enriched and regulated pathways in response to salt treatment. ORF prediction of selected candidate gene was performed via ORF prediction tool of CLC Main Workbench, from both our transcriptome data and previously released database of *Phaseolus vulgaris* transcriptome (phytozome.net), on the basis of the homology to each-other and similarity to their first match on the Nr-database.

Translated protein sequence of the candidate gene was examined for shared conserved region similarities by Pfam domain search with its closest relatives which were identified from the homology alignment tree that was constructed with 36 different plant species including Arabidopsis using CLC Main Workbench Alignment Tree construction tool.

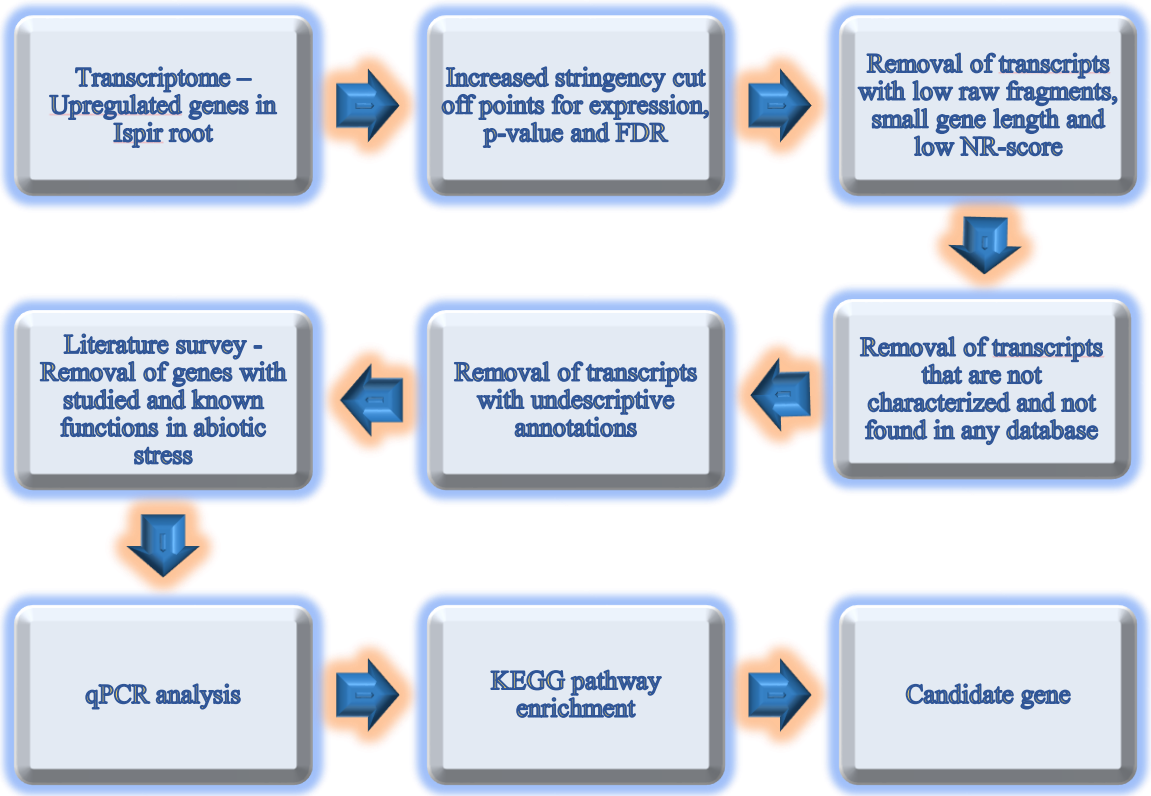

Figure 4.1. Bioinformatics flow-chart for candidate gene selection.

4.2.2. Quantitative Reverse Transcription (qRT) PCR Analyses of Pre-Candidate Genes

4.2.2.1. cDNA Library Preparation with RT-PCR. Sampling of the root tissues for both control and salt treated common bean plants (Ispir and TR43477 varieties) was performed at the fifth day of the treatment. After samples were frozen in liquid nitrogen, they were stored at 80°C up to RNA extraction. Total RNA extractions were carried out with 100mg mortar and pestle homogenized tissue, using TRIzol® Reagent (Invitrogen, USA) according to manufacturer's instructions. Quantification of the sample RNA concentrations was performed in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). Quality and integrity of the RNA samples were determined by 1% denaturing agarose gel electrophoresis.

Single stranded cDNA library construction for each sample was performed using 1µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions with the preference of random hexamer primers. The generated cDNA sample was diluted to 5ng/µl on the basis of total RNA quantity.

4.2.2.2. qPCR Analyses. CLC. Main Workbench Primer Design Module was used for gene-specific primer designation for qPCR. Parameters for the designed primers were given at Table 4.2.

Table 4.2. qPCR primer design parameters

Parameter Name	**Limitations**
Primer Length	max:22
	min:18
Amplicon Length	max: 250
Melting Temperature	max:60
	min:50

Table 4.2. qPCR primer design parameters (cont.)

GC content	max:60
	min:40
Max melting temperature difference	3
Max hydrogen bonds between pairs	15
Complementarity to the template	No mispriming allowed
Max hydrogen bonds between pair ends	6

qPCR analyses were performed with the selected pre-candidate genes (Table 4.1) for the verification of the expressional levels obtained from the RNA-seq. Actin-11 [GenBank: CV529679.1] and insulin degrading enzyme [GenBank: FE702602.1] genes of common bean were used as internal references as they were reported preserve a stable expression level under salt treatment in common bean [96]. Four samples for both control and salt treatment conditions from each varieties (Ispir and TR43477) were used for analyses as biological replicates that consist of three qPCR reactions as technical replicates. PikoReal 96 Real-time PCR system (Thermo Fisher Scientific, Wilmington, DE) was used in performing qPCR analyses with 10µl working solution which were prepared with AccuPower 2X Greenstar master mix (Bioneer, Daejon, Korea) with the addition of 2µl cDNA sample and 0.25 pmol gene-specific forward and reverse primers. Cycle conditions of qPCR reactions were given in Table 4.3. Calculation of relative expression levels was performed using $2^{\Delta\Delta}$Cq method [97] and the results were normalized to geometric average of the Ct values of the reference genes. Significances on relative expression levels of the genes between control and treated samples were calculated by the application of Student's *t*-test.

Table 4.3. qPCR cycle conditions

Temperature (°C)	Step	Time	Cycle
95	Initial Denaturation	5'	1
95	Denaturation	10"	40
Primer Specific	Annealing /Extension	60"	

4.3. Gateway® Cloning and Bacterial Transformation

4.3.1. Plasmids

pDONR 207 plasmid was used as a donor vector for the preparation of entry clone in Gateway® cloning (Invitrogen, USA) system. Two vectors were used as destination vectors; one with N-terminal FLAG tag (pGWB412) (Figure A.3a) and the other with no tag (pMDC32) (Figure A.3b). Both destination vectors contain two copies of 35S cauliflower mosaic virus (CMV) promoter for constitutive expression in plant.

4.3.2. Adapter Addition and Preparation of Expression Clone

The adapter addition procedure to the ORF of the selected candidate gene was partitioned in to two as the adapter sequences were too long to be added in a single-step reaction. First PCR reaction was performed with primary *attb* primers which contain first 15bp of the *attb*1 and *attb*2 adapters for forward and reverse primer respectively. The remaining of the primers were gene specific and 20bp long. PCR cycle was built specifically as a two-step reaction: The first step had a lower annealing temperature for just the gene specific parts to engage with the cDNA and increase the quantity; the second step had a higher annealing temperature for higher pairing specificity for the whole 35bp primer and more cycles to increase the quantity of the product. The product of primary PCR reaction was run in 0.7% agarose gel and the size selected product was gel-extracted for the second PCR.

The second PCR reaction was carried out with secondary *attb* primers that had no gene specific parts but all adapter sequences, *attb*1 and *attb*2, for forward and reverse primer respectively. The PCR product with adapter sequences at both ends was subjected to 0.7% agarose gel electrophoresis and the desired bands were excised and purified from the gel with gel-extraction. Both PCRs were performed in 50µl reaction mixes with four replicates to increase the product amount obtained from gel-extraction. The cycle conditions of primary and secondary PCR reactions were given in Table 4.4.

Table 4.4. Primary and secondary PCR cycle conditions

Primary *attb* adapter site addition PCR			
Temperature(°C)	**Step**	**Time**	**Cycle**
98	Initial Denaturation	3'	1
98	Denaturation	20"	11
52	Annealing	30"	
72	Extension	1'15"	
98	Denaturation	20"	20
72	Annealing/Extension	1'20"	
72	Final Extension	5'	1
Secondary *attb* adapter site addition PCR			
Temperature(°C)	**Step**	**Time**	**Cycle**
98	Initial Denaturation	3'	1
98	Denaturation	20"	13
45	Annealing	30"	
72	Extension	1'15"	
98	Denaturation	20"	20

Table 4.4. Primary and secondary PCR cycle conditions (cont.)

72	Annealing/Extension	1'20"	20
72	Final Extension	7'	1

4.3.3. Vector insertion with BP and LR cloning

The insertion of the candidate gene with adapter sequences, *attb*1 and *attb*2, at the ends of the pDONR207 vector containing attP1 and attP2 transposition sites was carried out using BP clonase. The clonase mix recognizes both the adapter sequences on the candidate gene and the transposition sites on the vector and conducts a transposition reaction that inserts the gene in the vector at the intended direction creating entry clone (Figure A.4).

Transposition of the candidate gene from the donor vector pDONR207 to the destination vectors, pMDC32 and pGWB412, was conducted with LR clonase mix. LR clonase recognizes the transposition sites in both donor and destination vectors and initiates another transposition reaction inserting the candidate gene into the destination vector in a direction specific manner creating expression clones (Figure A.3).

Both BP and LR reactions were performed with 10µl working solution according to manufacturer's instructions and after an hour incubation at 25°C, the reactions were halted with addition of proteinase-K and incubation at 37°C for 10 minutes. Basic Gateway® cloning diagram was given at Figure 4.2.

4.3.4. Agarose Gel Extraction

Agarose gel isolated products were used in both secondary PCR and BP cloning for specificity and efficiency. The desired bands on the agarose gel were excised with a scalpel under UV light. Gel-extraction was performed with Genejet Gel Extraction Kit (Thermo Scientific, USA) according to manufacturer's instructions. Concentrations and qualities of purified samples were measured with Nanodrop 1000.

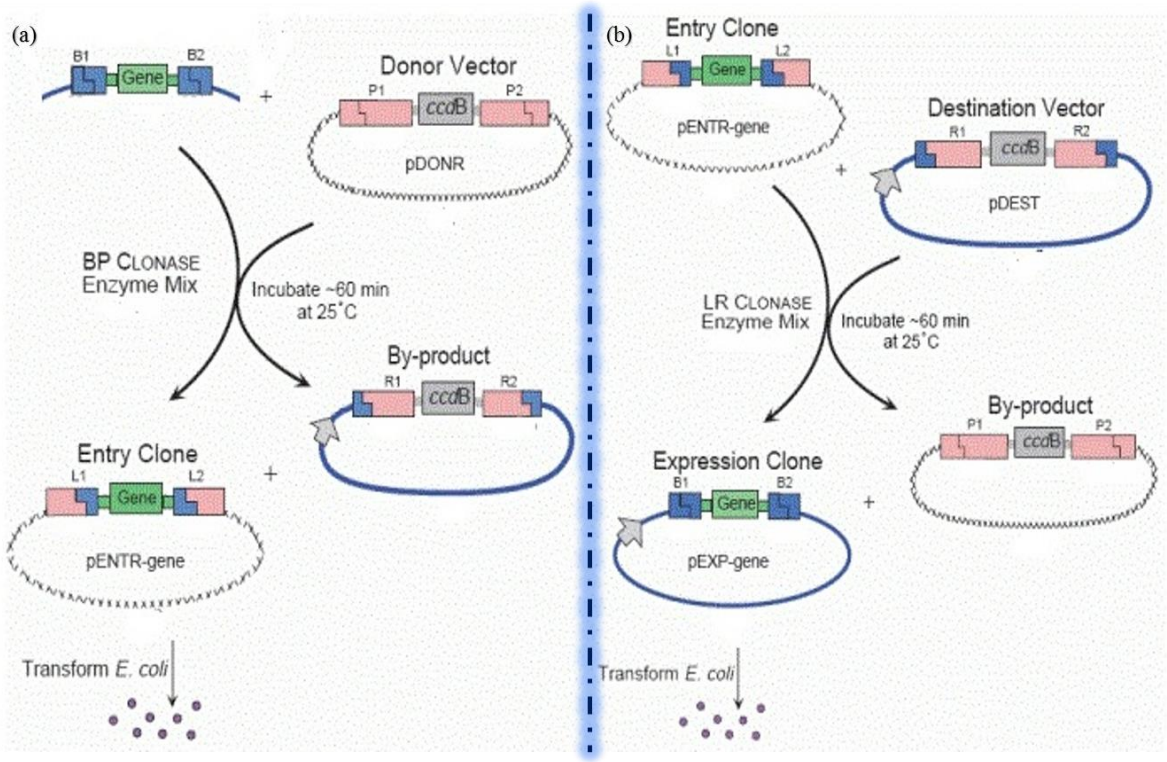

Figure 4.2. Gateway® cloning diagram. (a). BP reaction and Entry Clone production. (b). LR reaction and Expression Clone production. *ccdB* is a lethality gene for cells.

4.3.5. Bacterial Transformations

Chemically competent *E.coli* DH5α strain were prepared as described in [98] Chemically competent *Agrobacterium tumefaciens*, GV3101 strain were prepared as described in Holsters *et al*. [99]. Transformation of *E.coli* cells with generated recombinant vectors were carried out with 100µl aliquots of competent cells which were stored in -80°C before use and thawed in ice 5 min. prior to use. Purified plasmids (~100ng) were added on the cells and stirred with the pipette tip. Following 30 min. incubation on ice, the cells were exposed to 42°C for one min. for heat shock and returned to ice for further incubation of 5 min. Post ice incubation, the cells were left in 37°C for one hour in constant agitation after addition of 900 µl LB for healing and proliferation. Resulting bacterial cultures were spread on antibiotic (Table 4.5) containing LB-agar plates and incubated over-night at 37°C.

Table 4.5. Specific antibiotics and their concentrations for each vector

Vector / **Species**	***E.coli***	***A.tumefaciens***	***A.thaliana***
pGWB412	Spectinomycin (50µg/ml)	Spectinomycin (100µg/ml)	Kanamycin (75µg/ml)
pMDC32	Kanamycin (50µg/ml)	Kanamycin (50µg/ml)	Hygromycin (15µg/ml)
pDONR207	Kanamycin (50µg/ml)	-	-

Transformation of competent Agrobacterium cells has followed a similar approach. Frozen cells were thawed on ice for 30 min. before addition of 500ng of destination vector. The cells stirred with the tip of the pipette and flash frozen in liquid nitrogen for five min. before incubation at 37°C for 5 min. for heat shock. After the addition of 900 µl LB media, the cells were incubated on a 28°C rotating shaker for three to four hours. Resulting bacterial cultures were spread on antibiotic (Table 4.5) containing YEP agar medium for selection and incubated at 28°C for two days. Other than the vector specific selective antibiotic, all YEP mediums for Agrobacterium selection were prepared with 30µg/ml gentamycin and 100µg/ml rifampicin.

4.3.6. Colony PCR

Verification of transformation was performed by colony PCR. Selected colonies were transferred in 10µl of distilled water with the help of a pipette tip and 2µl of this solution was used as template in the PCR reaction. Primary *attb* primers were used in the reaction. Specifications of PCR cycle were given in Table 4.6. Following the PCR reactions, samples were run in 1% agarose gel, and positive colonies were detected by the presence of expected size products. Two of the colonies dissolved in distilled water that were proved to be positive were incubated in 1.5 ml LB at 37°C over-night and stored with half volume of glycerol at -80°C as stock for further use.

Table 4.6. Colony PCR cycle conditions

Temperature(°C)	Step	Time	Cycle
95	Initial Denaturation	7'	1
95	Denaturation	30"	30
52	Annealing	30"	
72	Extension	2'30"	
72	Final Extension	5'	1

4.3.7. Plasmid Isolation

Ten ml of bacterial culture containing the plasmid of interest were used for plasmid isolations by Nucleospin® Plasmid QuickPure kit, (Macherey-Nagel, Switzerland) according to manufacturer's instructions. The protocol for 'low-copy plasmid extraction' was followed to obtain higher yield.

4.3.8. Verification of Authenticity

The entry clone was verified for lack of mutations in the reading frame of insert sequences following the colony PCR amplification and purification of the insert. At least five colonies was used for colony PCR verifications and DNA sequence analysis of the insert was performed by Macrogen (Seoul, Republic of Korea) using gene specific sequencing primers (Table 3.10).

4.4. *In Planta* Transformation

Arabidopsis thaliana plants (Col-0 wild ecotype) were grown in Turf with twice a week watering regime using half strength Basal Nutrient Solution [94] at 22°C, 16/8 hours light-dark photoperiod and 50-60% humidity in JSPC-690 plant growth chamber. The

primary shoots were cut to accelerate multiple shoot growth. When most of the flower clusters were immature (inflorescence), the plants were considered ready for *in planta* transformation. (Figure 4.3a)

Floral dip method [100] was employed for *in planta* transformation of the Arabidopsis plants. Candidate gene inserted recombinant pMDC32 or pGWB412 vector carrying Agrobacterium primary cultures were grown overnight at 28°C shaker in 5ml YEP medium containing 30µg/ml gentamycin, 100µg/ml rifampicin and the vector specific antibiotic (Table 4.5). Main cultures were initiated with primary cultures and grown overnight at 28°C on 400ml of YEP media containing 100µg/ml rifampicin and the vector specific antibiotic. Following overnight growth, the cultures were centrifuged at 3000g for 10min and the supernatant was discarded. The pellet was resuspended in floral dip buffer containing 5% sucrose, 55nM benzylaminopurine (BAP), and 0.02% Silwet L-77. The inflorescences of plants were dipped in this buffer and kept for four min while gently agitating the solution (Figure 4.3b). Following the dipping procedure, the plants were covered using plastic wrap to protect the relative humidity level (Figure 4.3c) and positioned horizontally in a box that blocks the light for 24 hours. Following dark incubation of inflorescences with agrobacteria they were allowed to resume their normal growth until they reached to seed maturation stage for seed collection [100] (Figure 4.3d). Transformant seeds were selected in antibiotic containing MS medium [101]. Seedlings that were transformed with pGWB412 vector show a phenotype of green cotyledon formations in medium containing 75µg/ml kanamycin while non-transformant seedlings produce discolored (yellowish) cotyledons (Figure 4.3e). On the other hand pMDC32 vector carrying seeds germinated with longer hypocotyls in medium containing 15µg/ml hygromycin compared to non-transformed seedlings. (Figure 4.3f).

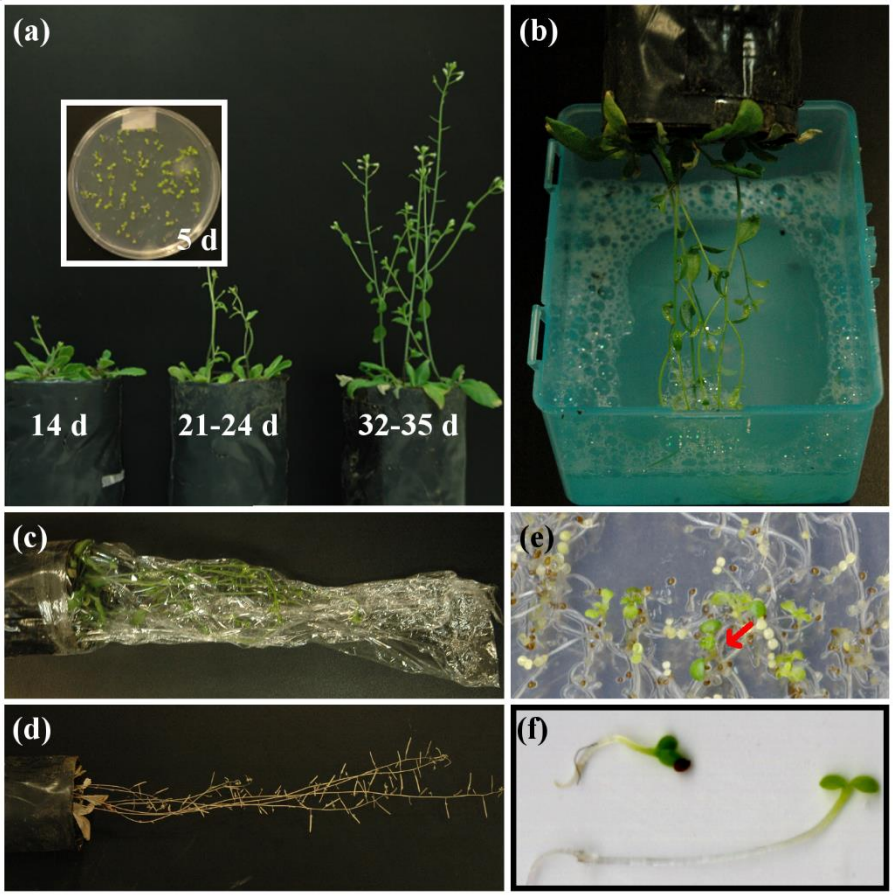

Figure 4.3. *In Planta* transformation and transformant phenotyping process. (a) Plant growth and preparation (b) Floral dip for transformation. (c) Humidity protection. (d) Plants ready for seed collection. (e-f) Antibiotic selection of transformant plants.

4.5. Verification of Transformation and Homozygosity of Arabidopsis Plants

Selective growth medium grown transformant plants were also verified by PCR analysis with Phire Plant Direct PCR Kit (Thermo, USA) according to manufacturer's instructions. Tips of the young, crisp leaves of transgenic plants were cut and collected in separate tubes containing 10µl dilution buffer, and grinded with the help of a sterile micropipette tip. Residual plant parts were precipitated by a brief centrifugation and the 2µl supernatant was used as template in each reaction. qPCR primers were used for amplification (Table 3.6) and these specific primers were also validated prior to their use by blasting their sequences to *Arabidopsis thaliana* genome to make sure that they do not amplify a sequence similar in amplicon length.

While first generation transgenic plants (T1) were expected to be heterozygous for the insert, second generation (T2) plants were expected to show 1/3 homozygosity as in a Mendelian inheritance fashion. To detect the homozygous lines between T2 generation plants, 50 separate lines were grown for T3 seed collection. These seeds, again separately were planted on antibiotic selection medium, and the lines that presented 100% transformation rate were approved as homozygous lines. Three homozygous lines for each expression clone were selected and utilized in physiological experiments.

4.6. Physiological Measurements

Plant growth for all physiological measurements were performed in 22°C with 16/8 hours light-dark photoperiod and 50-60% relative humidity in plant growth chamber. Three verified homozygous T3 Arabidopsis lines with both expression clones were selected and used for physiological measurements. Every measurement was constituted with Col-0 as control plant.

Salt treatment experiment for total weight, leaf area and leaf water potential comparisons initiated in small non-aerated hydroponics system and carried on with a large aerated hydroponics system up before the plants were sacrificed for analysis. Caps of the 0.5ml tubes with a single hole drilled on them (Figure 4.4.a) were used as GM agar holder for both large and small hydroponics systems. GM agar filled caps were placed on the sockets (Figure 4.4.b) of small hydroponics set up and individual seeds were planted by the help of a toothpick (Figure 4.4.c) on these caps through the holes on them (Figure 4.4.d). Germinations and initial growth were carried out with transparent covers over the hydroponics system for humidity protection (Figure 4.4.e-f). First trials implemented in small hydroponics system have proved that this system provides uniform plant growth up to 100mM NaCl treatment (Figure 4.4.g). After second week plants were transferred to aerated large hydroponics system for measurements (Figure 4.4.h-i). The mediums in boxes have gradually changed from GM to BNS medium after first week: First day one third, second day two thirds and third day all of the medium was replaced [94]. Mediums in the boxes were completed every day to make sure that agar and liquid mediums were in contact. All of the liquid mediums were replenished weekly.

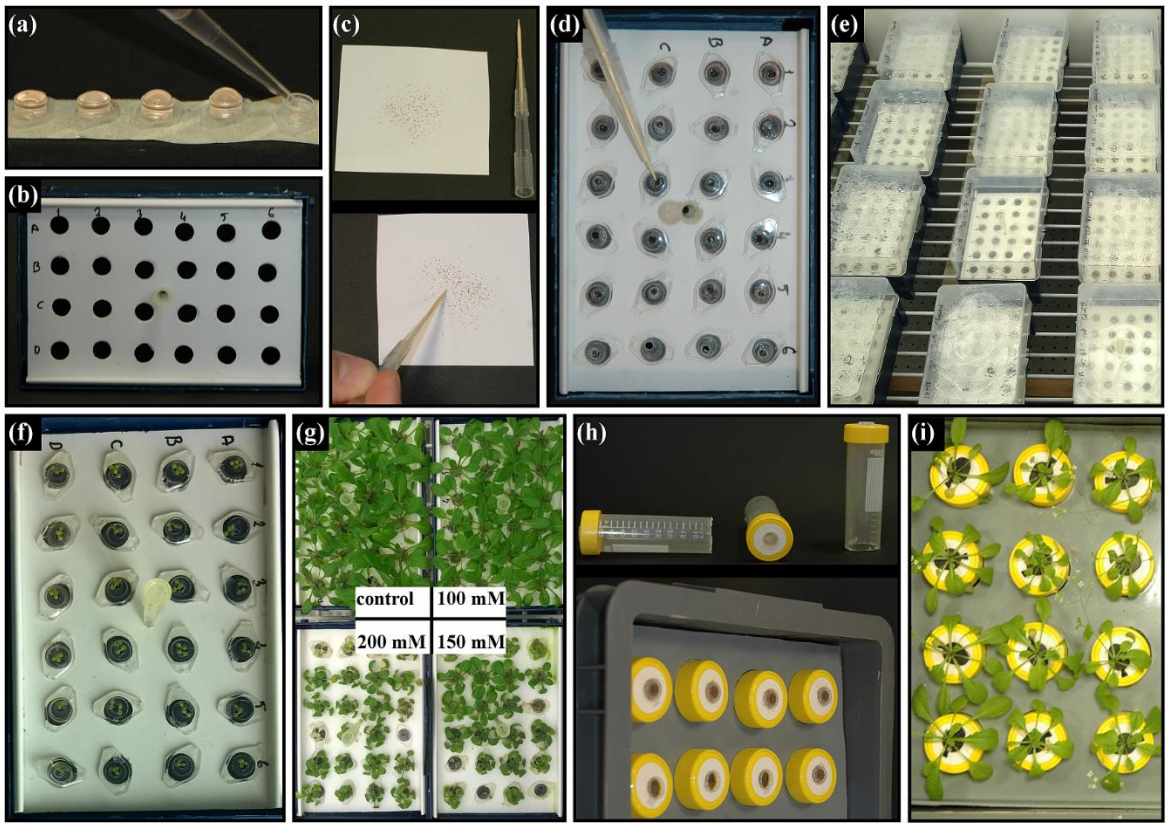

Figure 4.4. Hydroponics growth system for physiological measurements. (a) Germination medium preparation. (b) Small hydroponics set up. (c-d) Seed planting. (e-f) Seed germination in growth chamber. (g) Plant growth in different concentrations of salt treatment. (h-i) Aerated hydroponics system for growth and treatment.

4.6.1. Total Weight and Leaf Area Comparison

Sixteen T3 plants from each line and Col-0 were grown on large hydroponics system. After 14th day, half of the plants were gradually exposed to saline medium. Exposure started with 50mM NaCl on the first day, continued with 100mM and 150mM NaCl on the second and the third day respectively. The plants were left to grow in 150mM for five days and sacrificed for measurements of weight and leaf water potential.

For leaf area calculations, individual photos of canopy (green parts) from all plants were taken from 90° angle on a white background. For quantifications, a customized code developed for rosette size estimation in MATLAB® 2010b (Mathworks Inc., Natick, MA,

USA) [94] was employed and data collected from both control and salt treated plants were used for comparison.

Canopy and roots of the plants were fresh weighed and dry (after incubation at 80°C for two days) weighed separately. Both fresh and dry weights were recorded for individual plants.

4.6.2. Relative Water Content

Two intact rosette leaves from each plant grown in both control and treated conditions were cut and weighed. Leaves were kept in distilled water for 24 hours to gain their full turgor pressure, and weighed again. Dry weight measurement was performed after the leaves were dried in separate envelopes in 80°C oven for two days. The leaf water potentials were calculated using the formula; [(Fresh Weight – Dry Weight) / (Turgor Weight – Dry Weight)] * 100 [102].

4.6.3. Root Elongation

Root elongation measurements were performed using 20cm diameter plates containing BNS agar medium with 0mM and 125mM NaCl concentrations using 7 days old seedlings previously grown on MS medium. Five plants for each transgenic line (three transgenic line with the same vector insertion and Col-0 control in one plate; total of 20 seedlings) placed just about parallel to each other ensuring that their roots lain on a single line with no curves perpendicular to the line (Figure 4.5). Primary root tips were marked on the back of the plates with a marker pen and the plates were placed upright position in growth chamber after they were sealed with parafilm for protection of humidity. After 7 days growth incubation in BNS medium, the plates were collected for measurements. The pictures of the plates were scanned before and after the assay. Extended roots appendages and tips on the scanned pictures were marked and their total lengths were measured with ImageJ software [103] for comparison. Root length of each line measured after 7 days was normalized to its starting length to assess the overexpression related differences in primary and lateral root length growth in total.

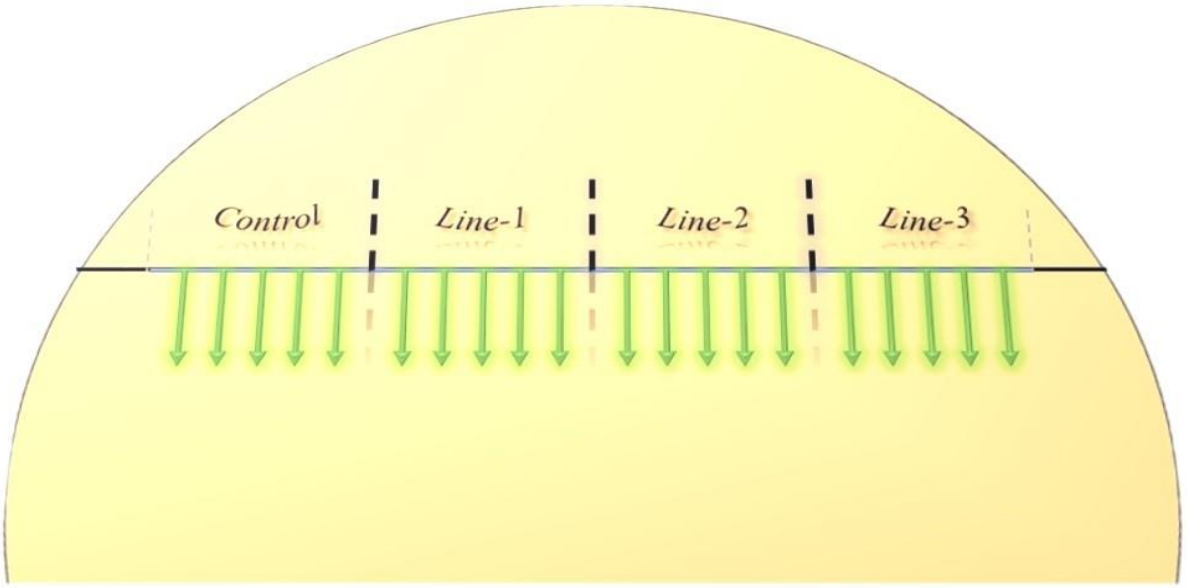

Figure 4.5. Set-up for root elongation measurements. Seven day old seedlings were placed as their roots track the direction of the arrows.

4.6.4. Germination Assay

Germination assays were performed as three replicate experiments. Seeds were planted on MS agar media plates containing 150mM, 125mM and 0mM NaCl concentrations. Three different transgenic lines and Col-0 seeds were germinated on single plate that was divided into four equal parts. Germination rates of individual lines on different concentration of NaCl were assessed by counting the number of germinated seeds (seedlings with cotyledon leaves) for a duration of five days. The count of germinating seeds was started after all the seeds were exposed to light for one day.

4.6.5. Statistical Analysis

Interquartile range method was employed for the removal of outliers from the collected data for total weight, leaf area, water potential and root elongation measurements [104]. Statistical analysis of weight, area and root elongation measurements were performed with two-way ANOVA employing the SPSS statistical package [105] with 95% confidence

interval level. P values for significance level were indicated with above asterisks: “*”: $p<0.05$, “**”: $p<0.01$, “***”: $p<0.001$, “****”: $p<0.0001$.

5. RESULTS

5.1. Selection of ChOMT as Candidate Gene for Overexpression Study

Generated upregulated gene pool (1237 transcripts) of root transcriptome analysis results obtained in our group [31] were the starting material for further bioinformatics analyses carried to select a potential candidate gene to analyze its functional role in salt tolerance response in this study.

In recent transcriptome studies with various plant species such as *Milletia pinnata* [106], *allium sativum* [107], *lenintula edodes* [108] and *Chorispora bungeana* [109] more than two-fold change ($\left|\log2\ (RPKM)\right| > 1$) in gene expression level with a false discovery rate below 10^{-3} was accepted as threshold to define differential expression of a gene. However there is no consensus strategy for the elimination of false positive genes which were defined as differentially expressed. Thus, the threshold points were elevated ($\log2\ (RPKM) \geq 2$, False Discovery Rate $< 10^{-4}$) to eliminate false positives as much as possible, because our study was focused on cloning and functional analysis of limited number of genes.

The number of raw reads mapped to a single transcript was also important because the expression level and the length of a transcript are proportional to the total number of raw reads mapped on it. In other words when we compare the transcripts with similar expression levels, the longer one would have more raw reads mapped on it. Since the sampling size increases the power of an experiment, differential expression of longer genes were detected with more accuracy compared to small ones [110]. Considering these, the transcripts which has less than 100 raw reads mapped on it were also eliminated from the list. We further filtered out the differentially ex pressed genes which were smaller than 750bp and which has a BLAST score less than 500, since the analysis pointed out that the percentage of annotated genes in the Nr protein database and their *E*-values with BLAST scores were correlated with gene length [31]. Although these filtering criteria might have led to elimination of some true positive data, it increased the reliability of selected candidates. These stringency cut-off point rearrangements have narrowed down the upregulated genes list from 1237 to 60 transcripts (Table B.1).

Transcripts that were annotated to be unknown, hypothetical or that were uncharacterized were eliminated from the list as they did not provide a reliable background information for their functions in the literature. After the removal of transcripts which encode extensively studied energy metabolism proteins such as cytochromeP450, ABC transporter members and transcription factors such as elongation factor alpha and TGF-beta together with repeated isoforms in the list, the number of transcripts have dropped to 43 (Table B.1– Grey colored transcripts were discarded; Bold-faced transcripts were selected as pre-candidate genes).

Five genes were further selected among the 43 transcripts to create a pre-candidate genes list (Table 4.1) for qPCR analysis. These genes were proposed to have prominent functions in the stress responses: "Vicilin-like antimicrobial peptides 2-3" was among the pre-candidate genes since it was defined as a member of antimicrobial peptides (AMPs) [111]. Although these proteins are known as member of plant innate immunity system they were reported to be induced under abiotic stress conditions like salinity, drought, heat and cold [32]. "Sulfate transporter" was also included for its proposed roles in abiotic stresses such as regulation of glutathione synthesis for ROS balancing and regulation of ABA hormone which is the major regulator of leaf stomatal conductance [112]. On the other hand, "BURP-domain containing protein" was selected for its plant specificity and reported expressional regulation in various stress treatments [113, 114]. "Ferric reductase" was implicated in reductive system [115] and proposed to have role in oxidative stress reduction [116 6]. Finally "isoliquiritigenin 2'-O-methyltransferase" (ChOMT) was added to the list since its substrate "isoliquiritigenin" is a plant secondary metabolite that was reported to be a very effective and pharmalogically valuable agent with antioxidant properties [60] and defined to be a potent inducer of nodulation in legumes [56]. All pre-candidate genes were cross-matched to NCBI Nr-database and previously released common bean transcriptome (phytozome.net) verified for the reliability of the annotations.

qPCR analyses were performed for both the verification of RNA-seq results and the observation of the expressional differences of the pre-candidate genes in tolerant and susceptible common bean root tissues. Results have produced one of the most important base for the elimination of pre-candidate genes: Ferric reductase gene was eliminated since it has presented a relative expression level (log_2) lower than 2 in both tolerant and susceptible common bean varieties according to the qPCR analysis results. Other four genes have shown

similar expressional levels for tolerant variety as in RNA-seq data. For the susceptible variety, three of the four genes (Vicilin-like antimicrobial peptides 2-3, BURP-domain containing protein and sulfate transporter) have also exhibited increase in expression level with similar fold-values as in tolerant variety. On the other hand 'isoliquritigenin 2' –O-methyltransferase' (ChOMT) gene has shown a substantial downregulation with a relative expression lower than -6 in the susceptible variety which points out a major expressional difference (fold-difference more than 2^9) in between the root tissues of tolerant and susceptible variety (Figure 5.1).

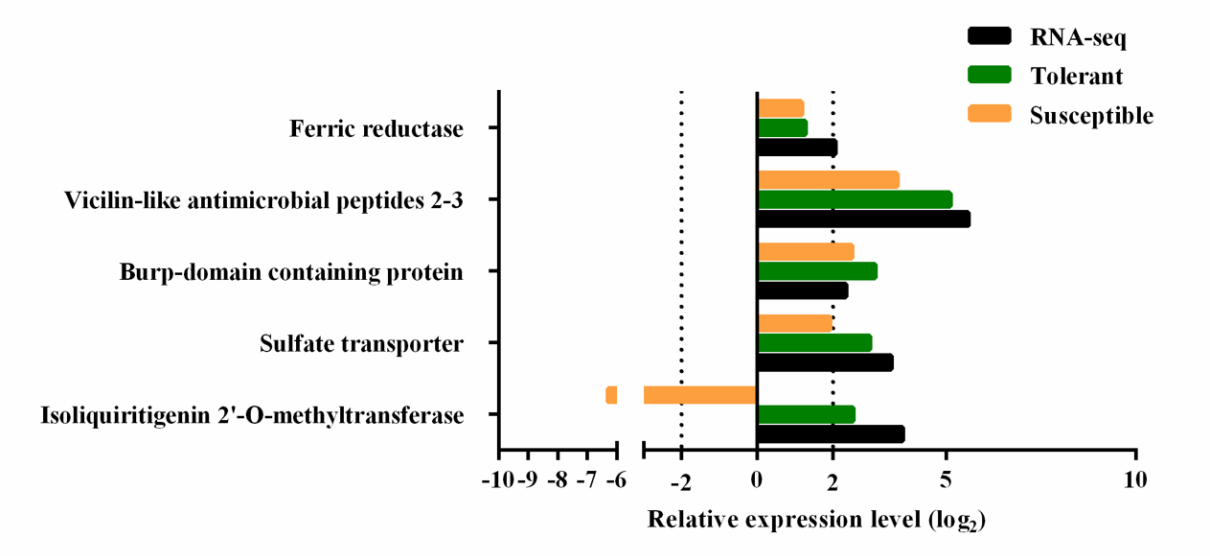

Figure 5.1. qPCR analysis results of pre-candidate genes.

Since the expressional difference of genes between two common bean varieties is the main reason for the variation of tolerance, this striking difference was the reason for the selection of this gene as the only potential candidate in this study.

Moreover KEGG pathway enrichment analysis of roots ($P < 0.05$) has revealed a major upregulation pattern on the biosynthesis of secondary metabolites which ChOMT is a part of: Nine out of 27 KEGG pathways that were found to be differentially regulated were related with plant secondary metabolism and eight of those indicated upregulated secondary metabolite biosynthesis (Figure 5.2) (such as ko00900, ko00940, ko00944) [31].

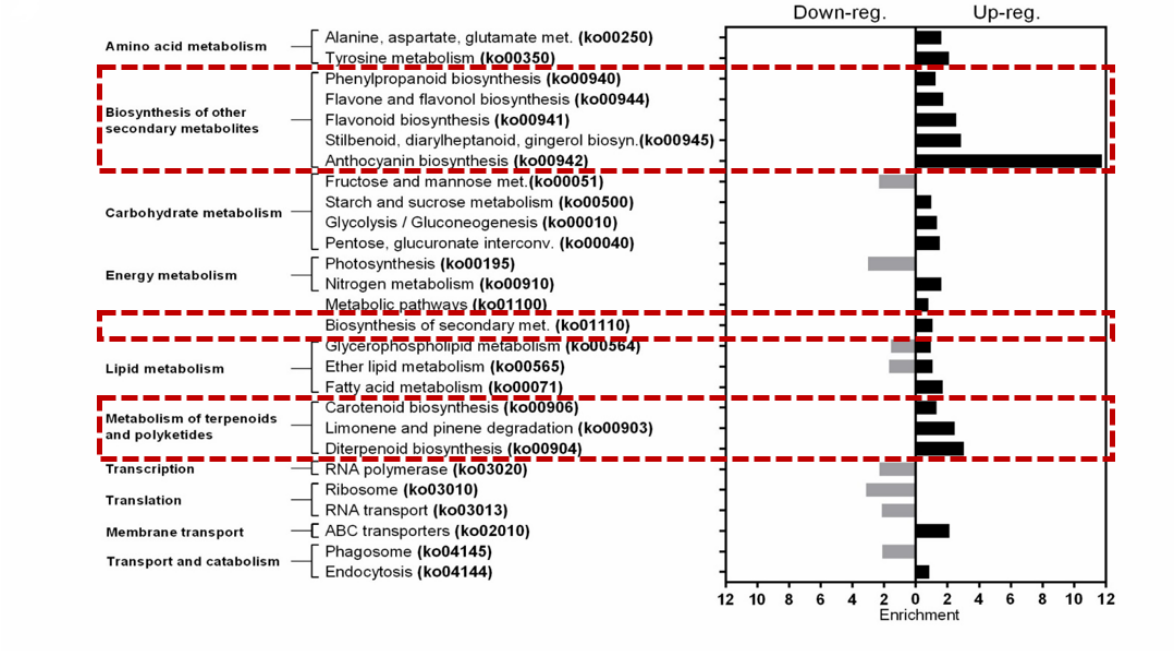

Figure 5.2. KEGG pathway enrichment analysis for salt-induced genes in roots of Ispir variety common bean. (Modified from Hiz *et al*., 2014) Sections in box indicate the upregulation patterns in secondary metabolism biosynthesis pathways.

Translated protein sequence of ChOMT gene was utilized to create an alignment tree employing UPGMA algorithm in CLC Genomics Workbench 5.5.1 together with the most similar proteins found in Nr-translated nucleotide database with tBlastx search in 36 different plant species (Figure 5.3).

Alignment tree has indicated the highest similarity to common bean ChOMT protein from *Glycine max, Glycyrrhiza echinata*, *Medicago sativa*, *Cicer arietnium*, *Medicago truncatula* and *Lotus japonicus* plants, which are also members of legume family. Conserved domain analysis, employing CLC Genomics Workbench 5.5.1 Pfam domain search tool, has detected a conserved "SAM-binding methyltransferase" region shared by those plants and common bean. When the detected conserved domain (SAM-binding methyltransferase domain) sequence of common bean was aligned with sequences from legume species and *Arabidopsis thaliana* (Figure 5.4) the results have demonstrated only 60% similarity in *Arabidopsis thaliana* while the homology to legume species ranged between 64%-86%. Such similarity most probably reflects selectivity to different substrates of the enzyme.

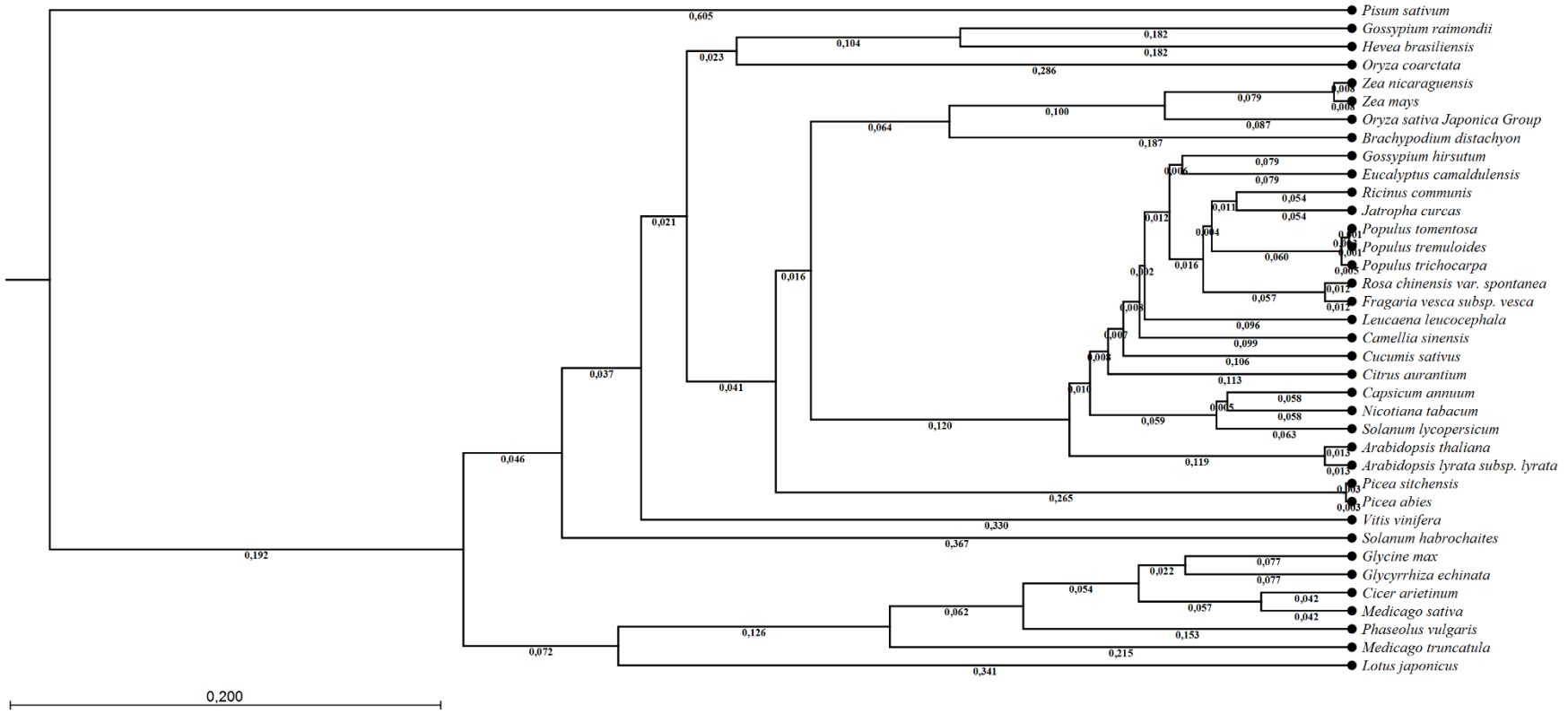

Figure 5.3. Alignment tree for common bean ChOMT protein with homolog proteins from 36 plant species. Values indicate the dissimilarity of sequences.

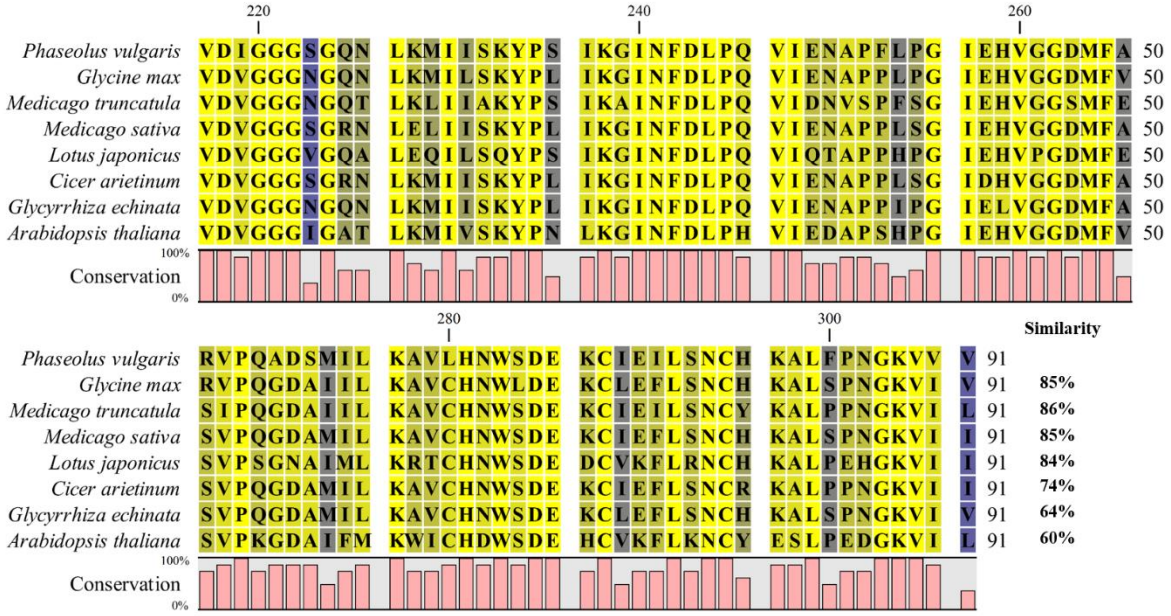

Figure 5.4. SAM-binding methyltransferase domain alignment for similarity analysis. (Change in the phase of colors from yellow to blue indicates site dissimilarity; Bars indicate the conservation status of each site among aligned domains)

5.2. Salt Treatment of Common Bean for Root Tissue RNA Extraction

RNA extraction for qRT-PCR reactions and cloning experiments were performed from the roots of eight seedlings for both tolerant (Ispir) and susceptible (TR43473) common bean (Figure 5.5) which were grown in control and saline conditions. Hydroponics system was preferred for homogenous and efficient growth [94] by allowing strict control on nutrient and NaCl concentrations in the treatment conditions. The "gradual step acclimation" method [93] was employed for NaCl treatments to minimize the risk of osmotic shock for the plants [117]. Treatments were started with 50mM NaCl and continued with 100mM NaCl next day. In the third day the NaCl concentration was increased to 125mM and treatment pursued for three more days.

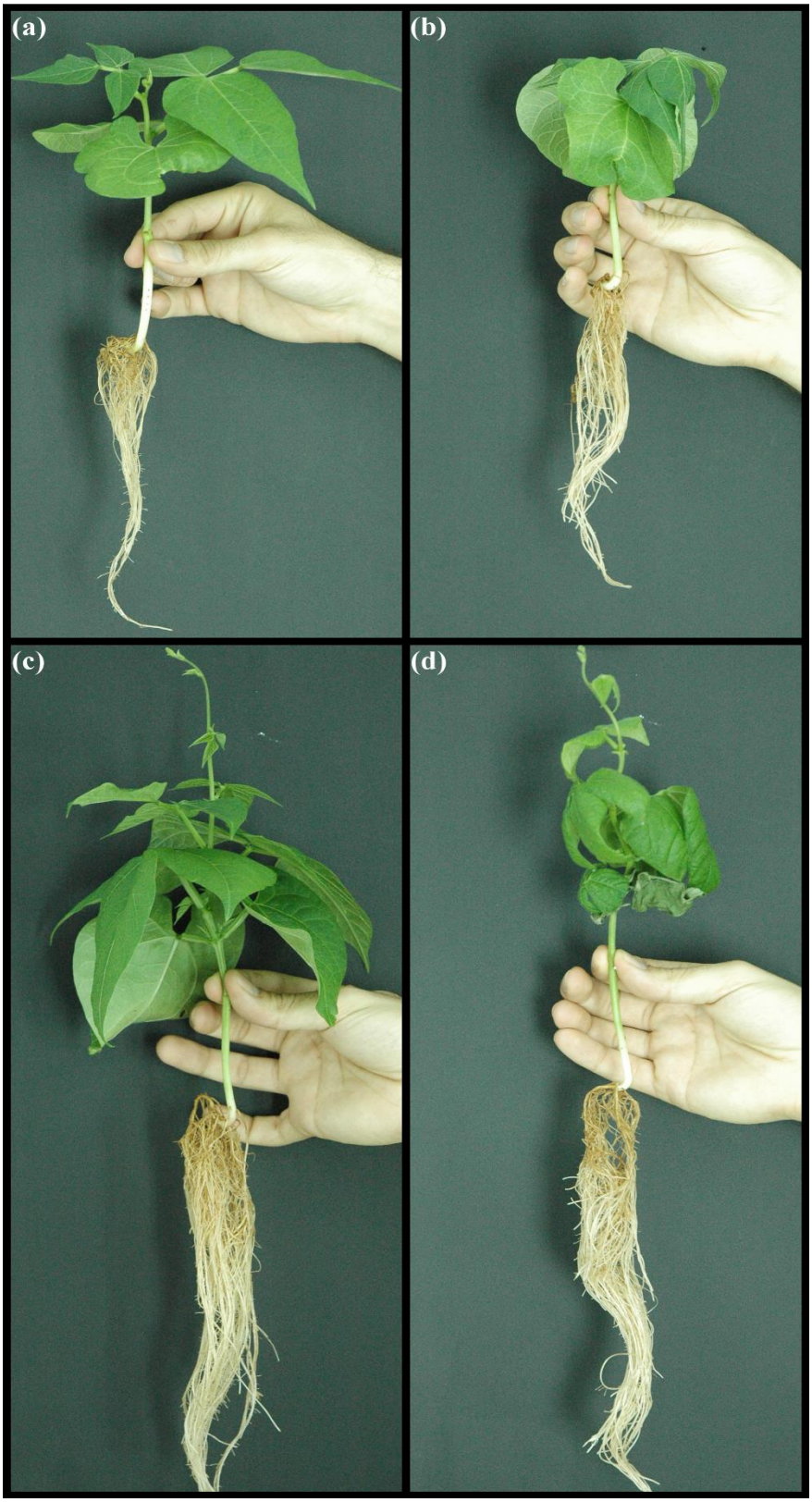

Figure 5.5. Salt-treatment of common bean plants for RNA extraction. Tolerant variety; Ispir grown in (a) control and (b) in 125mM NaCl conditions. Susceptible variety; TR43477 grown in (c) control and (d) in 125mM NaCl conditions.

5.3. Generation of Transgenic Arabidopsis Lines

5.3.1. Cloning of ChOMT gene to *Arabidopsis thaliana* via Gateway® technology

Gateway® cloning method was employed for the construction of ChOMT expression vectors since this system provides crucial advantages over restriction enzyme based cloning system.

In this method, the addition of flanking *attb* adaptor regions (30bp) to gene of interest requires two steps PCR reactions (primary and secondary) in which each PCR step allows addition of 15bp long adaptor sites. At the end of the two PCR steps, the BP reaction allows the recombination of adaptor containing gene into relevant recombination site of the donor vector. The generated entry clone contains the gene with 60bp extended length. The inserts in entry clones can be transferred to numerous destination vectors containing different tag and promoter type by LR reaction [118] to generate expression clones.

When the Gateway® system has been employed for the cloning of the ChOMT gene, the first step PCR reaction performed by primary *attb* primers generated the expected 1146bp (1116bpORF + 30bp adapter) fragment size (Figure 5.6a). The gel extracted and purified 1146bp fragment (83ng/µl) were used to perform the second step PCR reactions by secondary *attb* primers which resulted in the final length of the fragment to 1176bp (1116bp ORF + 60bp adapter) (Figure 5.7b). This fragment (127ng/µl) was transferred by recombination to the donor vector (pDONR207; Figure A.4) via BP clonase reaction and the obtained entry clone was transformed to *E.coli* bacteria. Confirmation of the transformation was performed with the colony PCR analysis of the randomly selected 5 positive colonies using secondary *attb* primers (Figure 5.7a). The insert authenticity in these entry clones were also verified by DNA sequence analysis (data not shown).

To generate the expression clones two different type of destination vectors both containing 35S constitutive promoter but one with no tag (pMDC32; Figure A.3b) and the other with an N-terminal FLAG-tag (pGWB412; Figure A.3a) [118] were utilized.

Again five randomly selected positive colonies were checked by colony PCR using secondary *attb* primers, which generated the expected 1176bp band on the agarose gel electrophoresis (Figure 5.7.b).

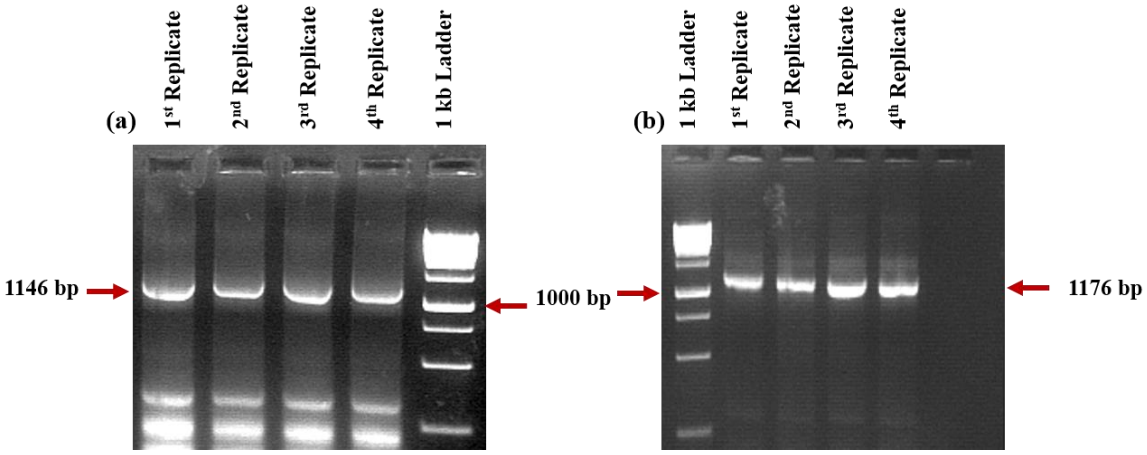

Figure 5.6. Agarose gel electrophoresis of two-step PCR samples for adapter addition and cloning. (a) Primary PCR results and (b) secondary PCR results.

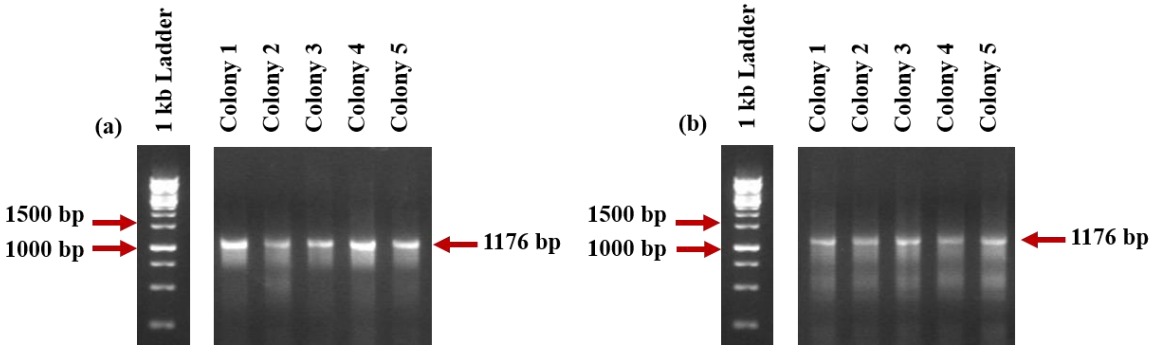

Figure 5.7. Colony PCR verifications for *E.coli* transformations. Agarose gel electrophoresis results for (a) entry clone and (b) pMDC32 expression clone.

After the Agrobacterium transformation of two different expression clones (pMDC32 and pGWB412 destination vectors), transformations were also confirmed by randomly selected 5 transformant colonies and colony PCR utilizing secondary *attb* primers (Figure 5.8). One agrobacterium colony for each expression clone was selected for floral dip method of *in planta* transformation of Arabidopsis Col-0 wild ecotypes.

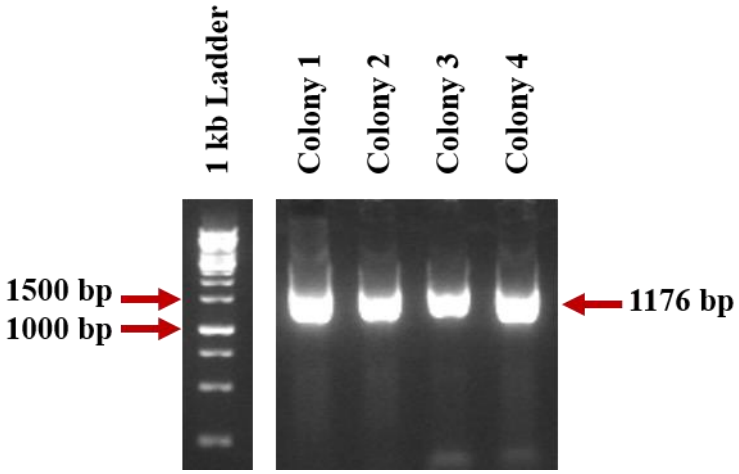

Figure 5.8. Colony PCR verification of expression clone in Agrobacterium.

5.3.2. *In Planta* Transformation of Arabidopsis Plants to Generate Transgenic Lines

The growth stages of the Arabidopsis Col-0 wild type were strictly monitored for *in planta* transformation since it has been shown that success rate of transformation in floral dip method [119-121] depend on the synchronization of inflorescence stages of the flower development [122]. To encourage the formation of high number of inflorescence formation per individual plants, the primary bolts formed at the 14th day of Col-0 plants (Figure 4.3a) were removed and the plants were allowed to generate the secondary bolts reaching to 10cm in length until 32-35th day of growth (Figure 4.3a). The plants at this stage were considered ready for floral dip. Floral dip method was employed simply by dipping the inflorescences of the 32-35 days old plants for 4 min into floral dip buffer solution prepared with main agrobacterium culture (Section 4.4) (Figure 4.3b). For both expression clones, three independent transgenic lines were verified to be homozygous for the ChOMT gene and utilized in physiological assays. Sample size of six independent lines provided reliability on the effects of the ChOMT overexpression which may have showed variability due to the site of insertion in the plant genome.

Transformant plants were cultivated up to T3 generation to achieve homozygosity. Five T1 plants for each construct have been verified to possess the recombinant ChOMT gene by qPCR primers (Table 3.6) which have generated 237bp bands (Figure A.1) as expected (Figure 5.9).

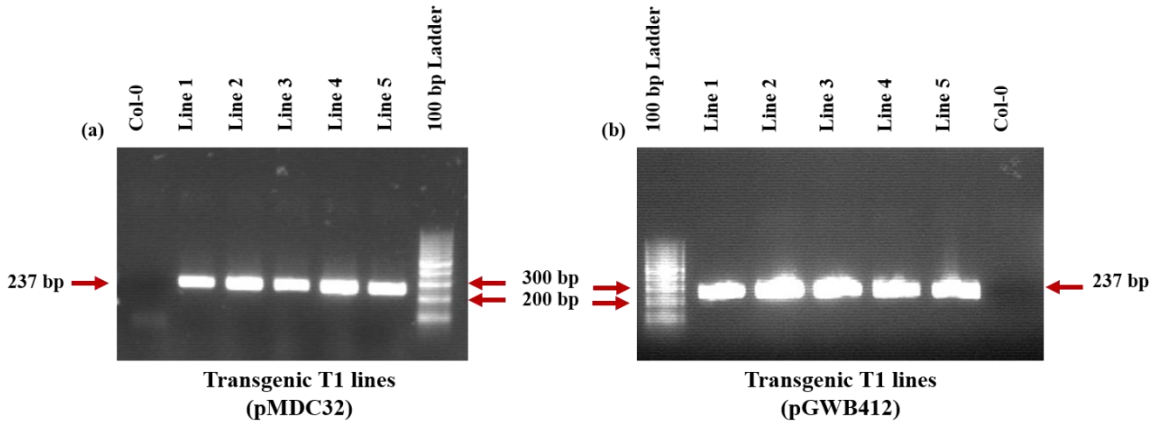

Figure 5.9. Verification of insertion in transgenic T1 lines (a) with pMDC32 and (b) with pGWB412 by PCR.

5.4. Physiological Analysis of the ChOMT Transgenic Arabidopsis Lines

5.4.1. Biomass, Leaf Area and Relative Water Content (RWC) Analysis

Analysis for biomass, leaf area and RWC parameters were performed after the removal of the outlier values. Initially the raw data sets of control and salt-treated transgenic lines for each type of parameters were analyzed with two-way Anova to assess the significance of variance between the tissue growth and RWC in saline and control conditions. All the statistical analyses were carried out within 95% confidence interval.

Secondly, the data obtained from each parameters for all lines were normalized separately by dividing the value of the difference between means to the mean of control condition to find the percentage of change in treatment condition.

Three different transgenic lines and a single Col-0 type plant for each type of expression clones (with pMDC32 and pGWB412) were utilized for the analysis of every physiological parameters tested. All the parameters were performed at 125mM NaCl concentration containing condition as they are indicated as salt treatment, and control conditions indicate the lack of excess salt in media. Designation of transgenic lines were done with destination vector number followed by “L” as abbreviation of line and finished with line number (eg. 412-L3 means Line3 with pGWB412).

Biomass analysis which covers the total fresh weight and total dry weight measurements at control and salt stress conditions were performed to understand the impact of stress on growth of Col-0 and transgenic lines.

Significant variation in total fresh weight of transgenic lines were observed with respect to Col-0 even at control condition. Except for the transgenic line 32-L1 in control condition and 32-L1 and 32-L2 lines under salt treatments, all the lines were shown significant increase in total fresh weight (Figure 5.11a). Normalized data indicated better stress response in transgenic lines 421-L1, 32-L1 and 32-L3 by 7-11% increase in total fresh weight under stress (Figure 5.10b).

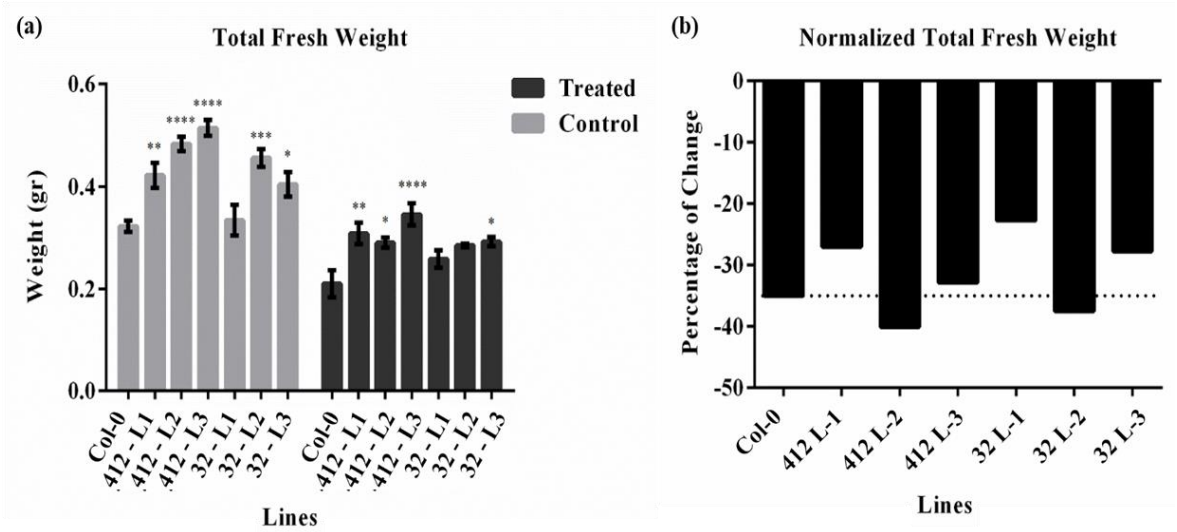

Figure 5.10. Total fresh weight evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized weight changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

As an indication of growth, total dry weight results were also shown similarity in pattern to the total fresh weight (Figure 5.11a and b). The ChOMT gene, mostly provided better growth in transgenic lines as it was observed in total fresh weight, canopy fresh weight (Figure 5.12a and b) and root weight results (Figure 5.13a and b) regardless of the presence of stress. Even during stress conditions, sustenance of a capacity to better water availability and conductance which brought down better mobilization of mineral and nutrients may bring

better growth performance in plants. Increase in the total biomass as well as canopy and root fresh weight reflect this capacity.

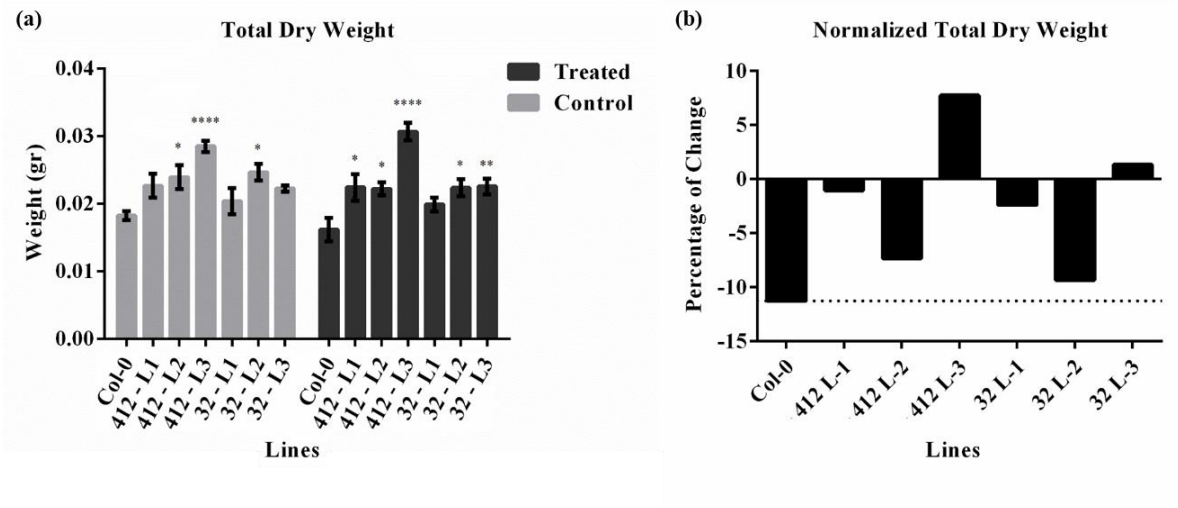

Figure 5.11. Total dry weight evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized weight changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

Except for the 412-L2 and 32-L2, (Figure 5.12b) the rest of the transgenic lines have shown 7%-15% better canopy development and except 412-L3 all transgenic lines have shown (28% to 3%) better root development (Figure 5.13b).

Leaf area size in plants correlates mainly with the change in the photosynthetically driven growth capacity and transpiration rate which both are controlled by cellular water maintenance. Under stress caused osmotic imbalance, better adjustment of turgor level increases endurance of plants to adverse conditions [123]. Leaf area comparisons revealed that four transgenic lines (412-L1, 412-L2, 412-L3, and 32-L2) have grown substantially larger leaves compared to Col-0 plants in control condition (Figure 5.14a). Although in treatment conditions, almost the same (412-L1, 412-L2 and 32-L2) transgenic lines performed significantly better than Col-0, this was most probably a reflection of the overshadowing effect of extensive growth of leaf area in control conditions in the first place (Figure 5.14b). But it was still possible to comment that most of the transgenic lines were

better in sustenance in leaf growth since they had lower change in the leaf area in treatment conditions.

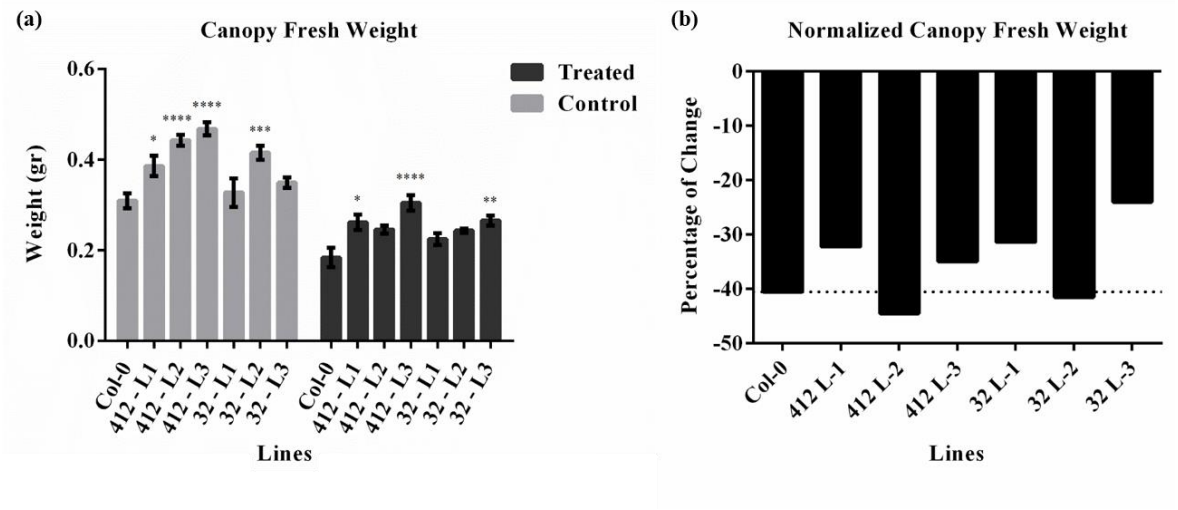

Figure 5.12. Canopy fresh weight evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized weight changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

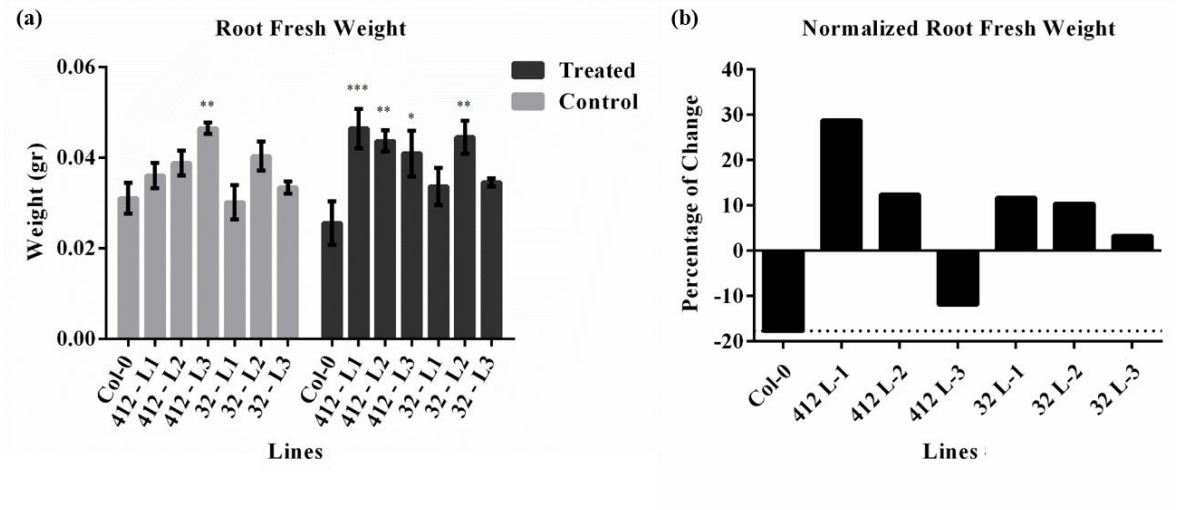

Figure 5.13. Root fresh weight evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized weight changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

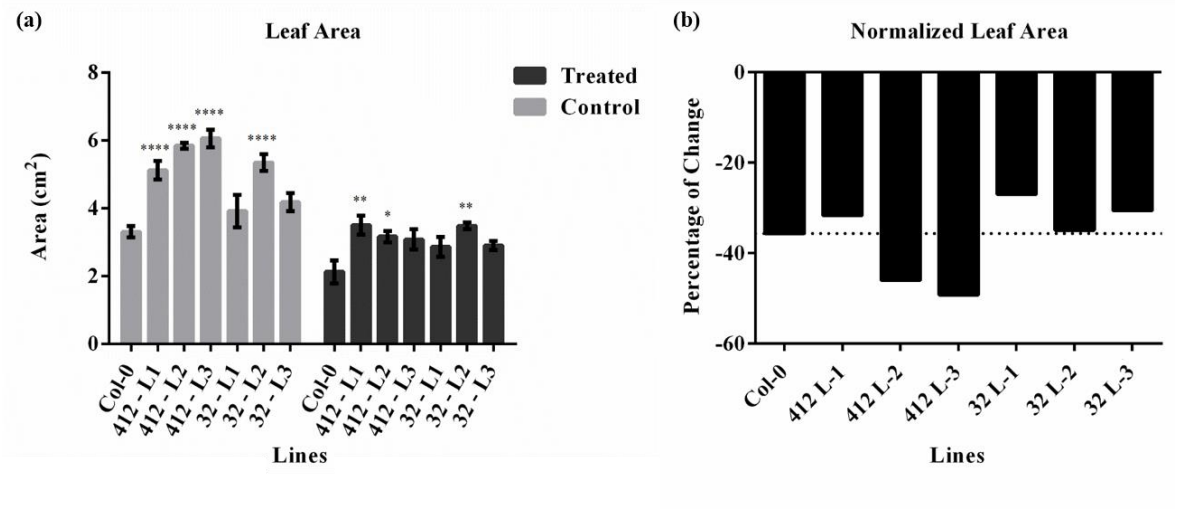

Figure 5.14. Leaf area evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized leaf area changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

RWC is accepted to be most appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit [102]. This parameter not only accounts for the leaf water potential of the plant but also demonstrates the osmotic adjustment for the conservation of cellular hydration in the leaves [124]. RWC measurements of the leaves of transgenic lines have reflected compelling evidence to improved capacity of maintaining cellular water content as a salt tolerance response by demonstrating a highly significant increase in RWC under treatment conditions (Figure 5.15a). While Col-0 plants were able to preserve less than 60% of their water content, all transgenic lines were shown much higher water retention capacity ranging from 80% to 92% (Figure 5.15b). This outcome have clearly demonstrated the improvement in the adjustment of water potential of ChOMT overexpressing transgenic lines to accommodate osmotic stress.

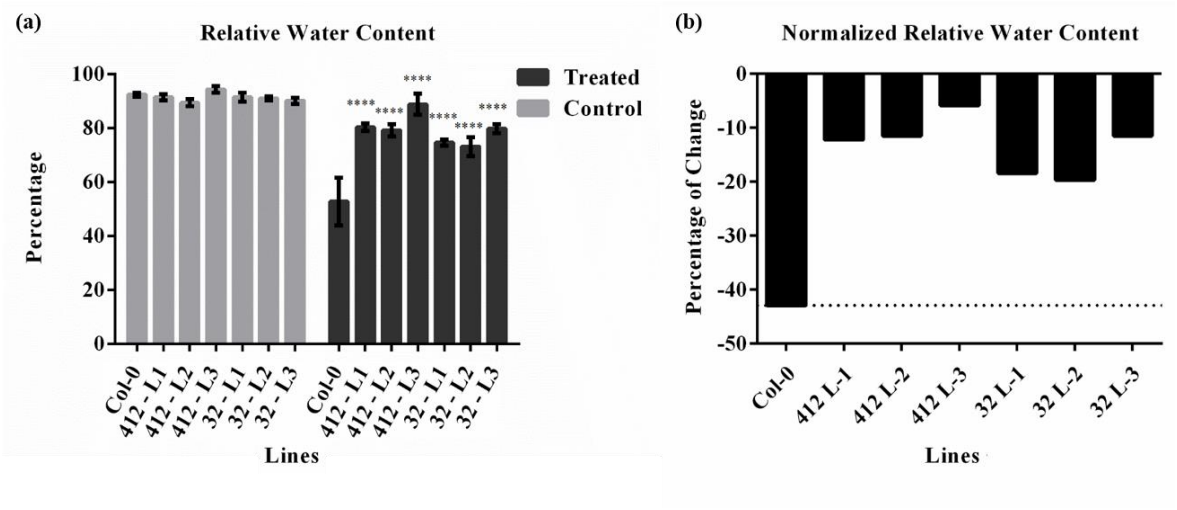

Figure 5.15. Relative water content evaluations. (a) Comparison for significance of variance in control and treated conditions. (b) Normalized water content changes. ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM)

5.4.2. Root Elongation Comparisons for Increased Growth Rate

Root systems can exhibit enormous plasticity on the level of biomass, morphology and/or physiology in response to different environmental parameters, like water availability [125, 126] or excess ions [127]. The shape and size of root systems is determined by the elongation of individual root tips, by the rate and location of lateral root development and by root longevity [128]. During salt stress the early onset of root growth inhibition has been attributed mostly to the toxic consequences of salt accumulation in the growing tissues, a reduction in water availability for cell expansion and cell wall hardening at the root tips with higher lignification thus limitation in cellular elongation [35, 36]. Despite of inhibition of linear growth, extensive lateral root development under the involvement of auxin redistribution to these lateral root tissues for salt tolerance has been also demonstrated in plants [39, 129-131]. Therefore root elongation and lateral root branching have been considered as good indicators of changes in root growth and architecture under salt stress conditions as tolerance response.

Root elongation measurements were carried out by outlining the initial and final lengths of the roots (Figure 5.16) with ImageJ software [103] to assess the relative elongation

obtained after 7 days in treatment and control conditions. Transgenic plants were shown significantly higher root elongation except 421-L3 line in control conditions in comparison to Col-0 (Figure 5.16) with increase in lateral root formation. However, under 125mM NaCl treatment conditions reduction in root growth were observed in both Col-0 and transgenic lines (Figure 5.16b).

These results signify that ChOMT overexpression caused a major improvement in root size. Despite of the expected root size reduction under 125mM NaCl conditions, to increase water availability to root tissue, induction of higher number of lateral growth reflected by lateral branching of roots was a distinctive phenotype of the transgenic lines (Figure 5.16b and Figure 5.17)

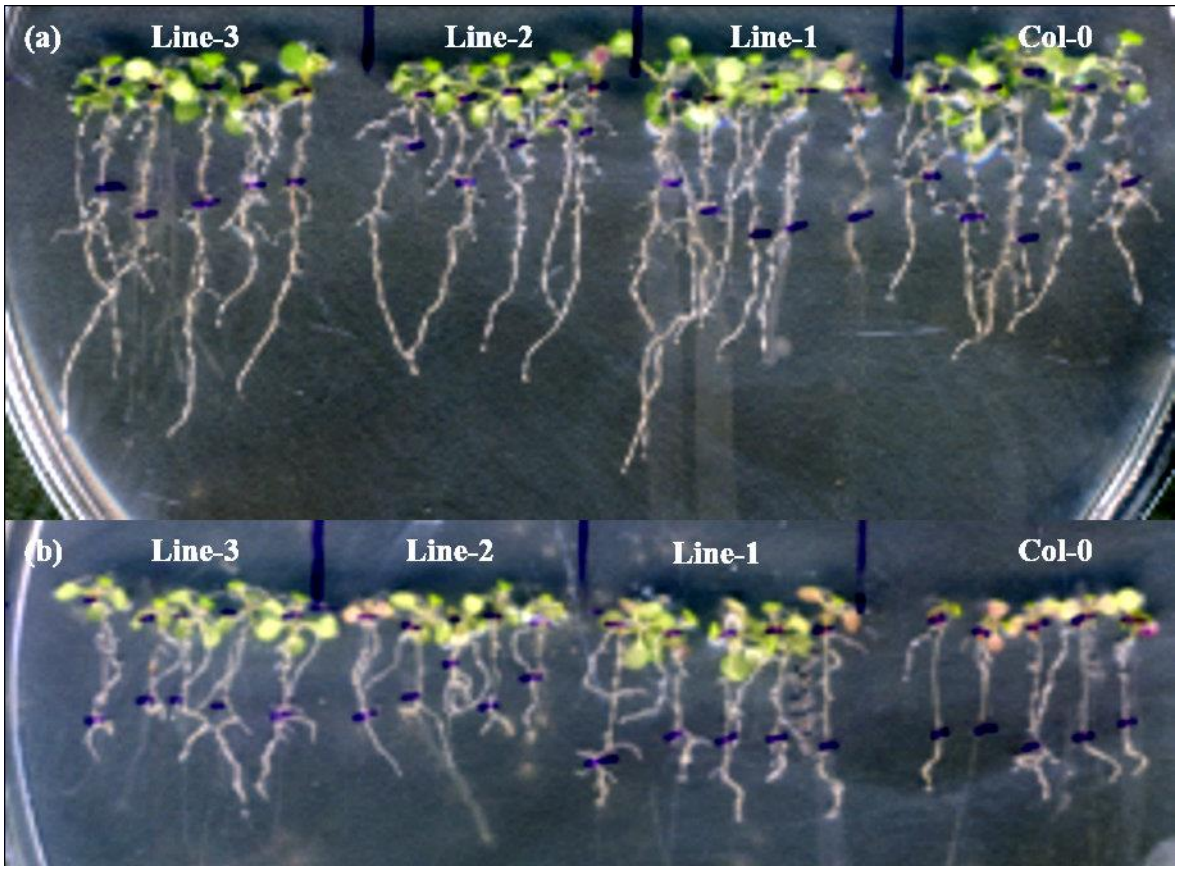

Figure 5.16. Root elongation assay example picture. Root lengths were measured in (a) control and (b) 125mM NaCl conditions. Blue marks on the roots indicate the initial root lengths.

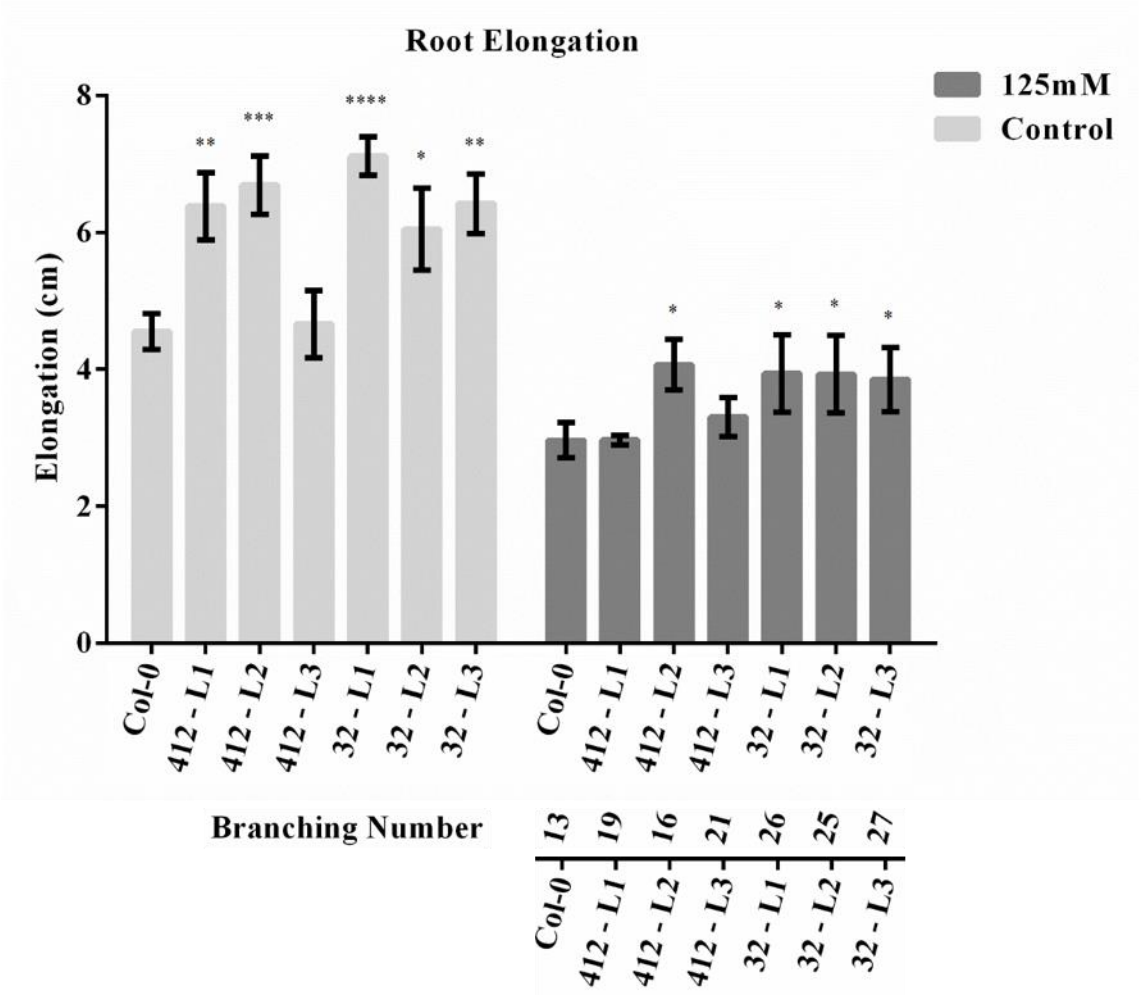

Figure 5.17. Root elongation variance in salt-treatment. Measurements were analyzed for control and 125 mM NaCl treatment conditions ("*": $p<0.05$, "**": $p<0.01$, "***": $p<0.001$, "****": $p<0.0001$) (Error bars indicate ± SEM). Number of emerged lateral roots (branching) in treatment conditions were indicated below each line.

5.4.3. Germination Assay

Seed germination and seedling emergence is the first step for successful production of most plants. The ability of seeds to germinate (often called seed viability) and to emerge (often represent vigor under stress) is the key for plant population existence in nature [132]. Therefore, ability of viable and vigor seeds production of a plant under stress conditions are good measures of stress tolerance response.

Percentages of seed germination out of 30 seeds for transgenic plants and out of 60 seeds for Col-0 were assessed for 5 days and recorded for two different NaCl treatment (125mM and 150mM) and control conditions (Figure 5.18).

In control conditions germination rate reached to 100% for all transgenic lines as well as Col-0 plants at the second day of seeding. (Figure 5.18a). Germination rates of Col-0 plants remained below 88% and 77% in 125mM and 150mM salt treatments respectively. (Figure 5.18b-c). However transgenic plants represented 95% (125mM NaCl) (Figure 5.18b) and 91% (150mM NaCl) (Figure 5.18c) germination and emergence under the same stress conditions, clearly indicating stress tolerance response in seed viability and seed vigor.

In summary, ChOMT overexpression had resulted in more than several improvements for *Arabidopsis thaliana* in salt stress conditions. While root and dry biomass results signified the enhanced growth potential, RWC results represented the protection of osmotic balance and water potential. Moreover increase in germination rates were the indicator of a better seed and seedling protection.

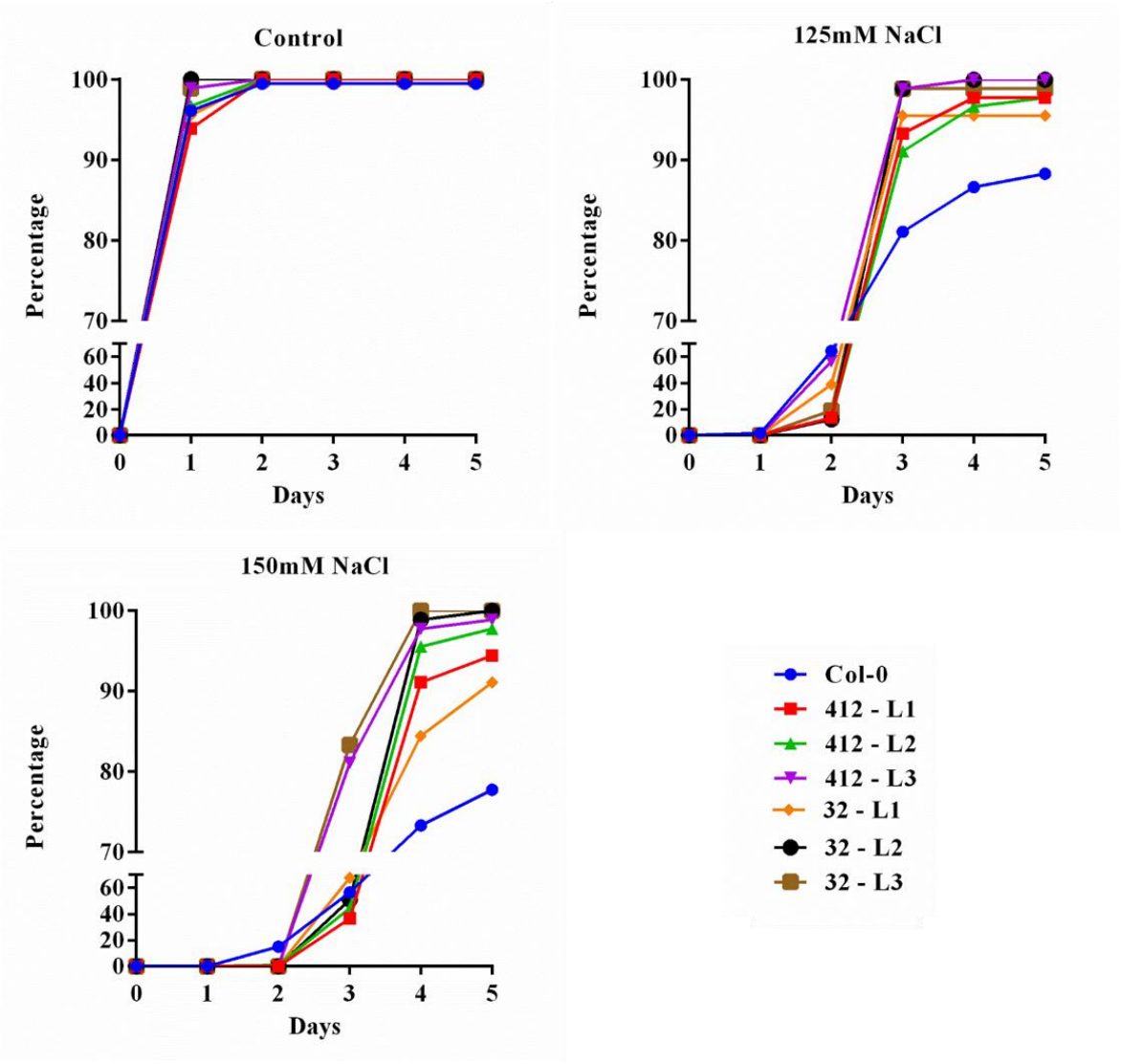

Figure 5.18. Germination rate graphics for (a) control, (b) 125mM NaCl and (c) 150mM NaCl conditions.

6. DISCUSSION & CONCLUSION

As salinity continues to be one of the major set-backs for agricultural prosperity all around the world, the need for a better comprehension of tolerance mechanisms to generate salt-tolerant crops increases every day. Recent transcriptome studies, provide great opportunities to gain insight on the plant response mechanisms to different environmental conditions as they are able to reflect the changes in gene expression of organisms.

The aim of this study was based on the data obtained by comprehensive *in silico* analysis of differentially expressed genes in common bean transcriptome under salt stress which was performed by our group [31]. The results have indicated the presence of a diverse differential expression in common bean even in different tissue types under salt stress and thus this project focused mainly on the data obtained from the root tissues as being the primary site exposed to salt stress. The vast amount of upregulated genes obtained from the root transcriptome results [31] has been the major starting gene pool in our study.

Evaluation of the upregulated genes to obtain a striking gene as a potential candidate to study the functional role on the salt tolerance using model Arabidopsis plant required application of several elimination criteria by bioinformatics tools on this gene pool. Firstly the criteria such as restriction of the defining conditions for relative upregulation by increasing the threshold points on the relative expression levels, narrowing down of the confidence interval for significance and false discovery rate value changes have been the primary steps to achieve a greater level of certitude on the data. Secondly, removal of the genes that were mapped with low number of raw fragments and those with low similarity scores in Nr-database annotations have eliminated the possible inconveniences such as uncertain ORF predictions, lack of reliable annotations and lack of functional differences for the queried genes.

Finally, the remaining potential candidate genes were investigated in detail via literature survey and within public databases for further elimination of those with already defined functional roles on salt and also drought stresses since tolerance mechanisms of both stresses share a common background in plants.

Eventually, the isoliquiritigenin 2'-O-methyltransferase gene (ChOMT) raised an attention as a candidate gene to pursue functional studies based on the prominent information for the potential roles of its substrate (isoliquiritigenin) which is a plant secondary metabolite proposed to be a very effective pharmacological agent exhibiting anticancer [57] and antioxidant [60] activity in human however not studied for similar functions in plant system. Studies on the common bean root transcriptome via KEGG pathway enrichment analysis by Hiz *et al*. (2014) has already indicated the significance of many upregulated genes in secondary metabolism pathways of plants and the ChOMT gene was also among them. A unique differential expression of this gene that confirmed by qPCR analysis between tolerant Ispir and susceptible TR45477 roots was also intriguing to pursue its possible role in salt tolerance via overexpressing in model organism under salt stress.

As a member of SAM-dependent OMT family gene, homology alignment analysis of the ChOMT has indicated a phylogenetic relationship with 36 plant species with similarity ranging from 26.7% to 87 % (Figure 5.3). When the conserved SAM-binding domain region was concerned, homologies among legume family were highest (Figure 5.4) whereas homology to Arabidopsis gene was only 60%, most probably due to different substrate specificity between legume versus Arabidopsis species.

Although, *Arabidopsis thaliana* were shown to produce the substrate molecule, isoliquiritigenin in their tissues [133], they do not possess an isoliquiritigenin methyltransferase gene therefore, transgenic Arabidopsis lines overexpressing common bean ChOMT has shown a significant biomass improvement in both control as well as salt-treatment conditions. Such improvement was reflected by the data obtained in total fresh weight, canopy weight and leaf area analysis of most transgenic lines (412-L1, 412-L3, 32-L1 and 32-L3 lines) in comparison to Col-0 ecotype (non-transgenic control) under salt stress. Root weight results also concurred biomass improvement in the same transgenic lines under stress, most probably by increase in dry matter through enhancement of solute content accumulation in root tissues which contribute to osmotic adjust. Accumulation of organic and inorganic solutes within tissues of tolerant plant species under osmotic stress have been well documented by several studies and considered to be one of the basic protection and survival strategy of tolerance mechanism in plants [30, 134]. Detected increase in the root dry weight in these transgenic lines were also contributed by the adventitious (i.e. lateral branching) root development (Figure 5.16b) observed as a distinctive phenotype during salt

stress. Studies on both salt and drought stress response in plants have indicated such lateral growth stimulation in roots [135] as a good example to plasticity in root growth regulation against environmental conditions during which increased root surface area empowers the plant to reach more water and nutrient reserves [136]. Interestingly a recent transcriptome study on the *cra*1 mutants of *Medicago truncatula* which exhibit compact root architecture, have demonstrated a significant down regulation in this gene which encodes a Caffeic Acid OMT [137]. This down regulation was well correlated with decrease in metabolites related with cell wall biosynthesis, differential accumulation of specific flavonoids groups and inhibited polar auxin hormone transport as being the major root growth regulator [137]. Therefore this study clearly supported the importance of OMTs in the legume root architecture via change in the expression of flavonoid substrate type under the stimulating effect of auxin hormone mobilization. Therefore it is possible to speculate that enhanced root lateral branching phenotype as well as decrease in the root elongation of ChOMT transgenic Arabidopsis lines could be the result of such functional role of ChOMT enzyme by use of specific flavonoid substrate to regulate cell wall architecture and polar auxin transport as tolerance mechanism.

Analysis of leaf relative water contents among ChOMT transgenic lines have demonstrated up to two to eight fold increase in the preservation of water content (Figure 5.15) in comparison to Col-0 control under salt stress. Osmotic potential difference between the canopy and root system in plants provides the driving force for the uptake of water and mineral by roots and efficient upward conduction to shoots and leaves. Osmotic stress generated by salinity around the root zone reduces the water availability to root cells and as a consequence decreases the water potential in shoots and leaves [138]. Therefore improvement in the leaf relative water content in transgenic lines could be the reflection of impact generated by efficient water absorbtion and conductance via changed root architectural system under salt response [139].

During plant development, seed germination and seedling formation represent the most vulnerable stages to any type of biotic or abiotic stress, thus, these stages are commonly regarded as predictive for plant responses to stress factors [140]. Observation of significant increase in the germination rate and emergence of seedlings in ChOMT transgenic lines up to 20% even at 150mM NaCl concentration when compared to control plants, could be evaluated as a strong indication of improved tolerance. In a study which examines the

changes in the proteome of pea seed germination under osmotic stress, similar indication of functional role to 6a-hydroxymaackian-3-O-methyltransferase enzyme were suggested when upon osmotic stress they observed drastic decline in the proteins of pea seed proteins function in in photosynthesis, glycolysis biosynthesis and cytoskeletal proteins of growth processes whereas a remarkable accumulation of proteins occurred which are involved in energy catabolism, signal transduction and cellular protection. Among the accumulated proteins, one was 6a-hydroxymaackian-3-O-methyltransferase which is involved in synthesis of a secondary metabolite, pisatin that takes role in seed germination recovery during osmotic stress [29]. In another study of chilling stress on the proteome of common bean seeds have drawn attention to the accumulated proteins which play key roles in secondary metabolism of common bean and particularly implicated the impact of homocysteine methyltransferase enzyme as a key player in the amino acids derived osmolyte biosynthesis during the post recovery rate of seed chilling response [28].

In recent years accumulated data from transcriptomics and proteomic studies on the mechanism of abiotic stress responses in plants accentuate the impact of regulation of osmotic imbalance through processes involving secondary metabolites, redirection of energy consumption and protective measures to ensure the integrity of cells. Therefore, a concerted effort must be shown to improve crop production under abiotic stress conditions via implementation of tolerant crop development. To do so, generation of crops expressing genes which play key roles in the above response mechanisms should be a priority.

In conclusion, our study has demonstrated the potential of common bean protein isoliquiritigenin 2'-O methyltransferase as a good candidate to improve salt tolerance in transgenic Arabidopsis lines possibly by creating an impact on;

- the accumulation of organic solute content and architectural change in root tissues to compensate adverse effect of osmotic stress and increase the chance to absorb and conduct water to ensure biomass and
- the protection of integrity of seed via increase in the germination potential and seedling emergence.

7. APPENDIX A

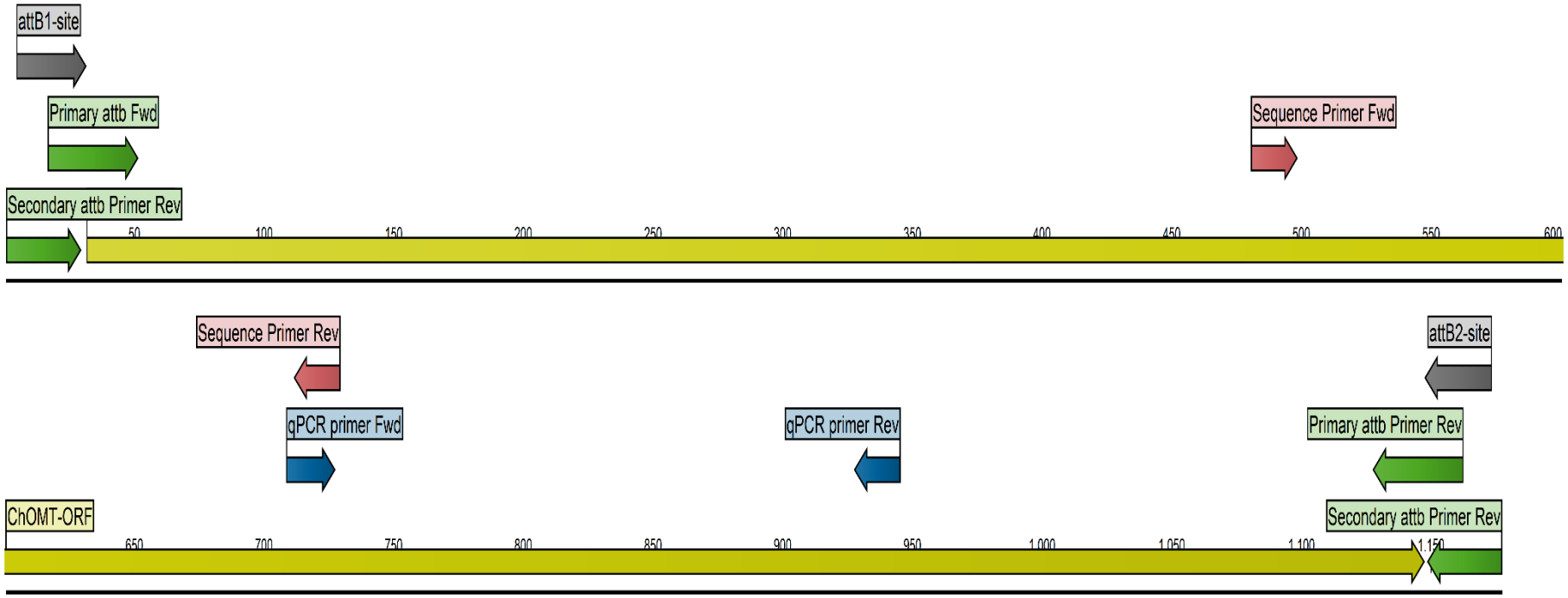

Figure A.1. ChOMT gene ORF sequence length and primer alignments.

Figure A.2. ORF sequence of ChOMT gene.

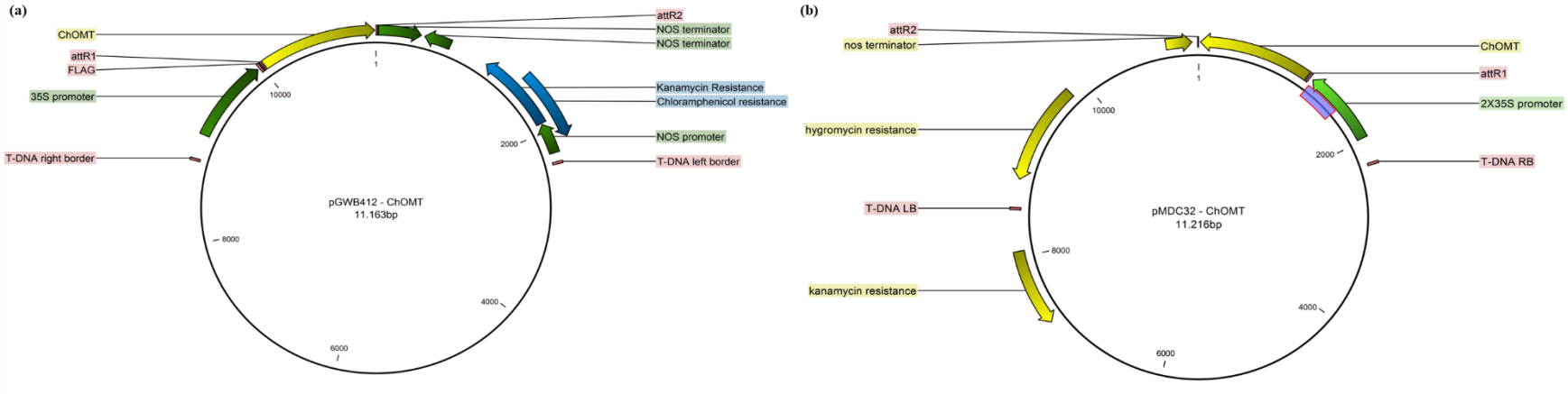

Figure A.3. Expression clones (a) with pGWB412 and (b) with pMDC32.

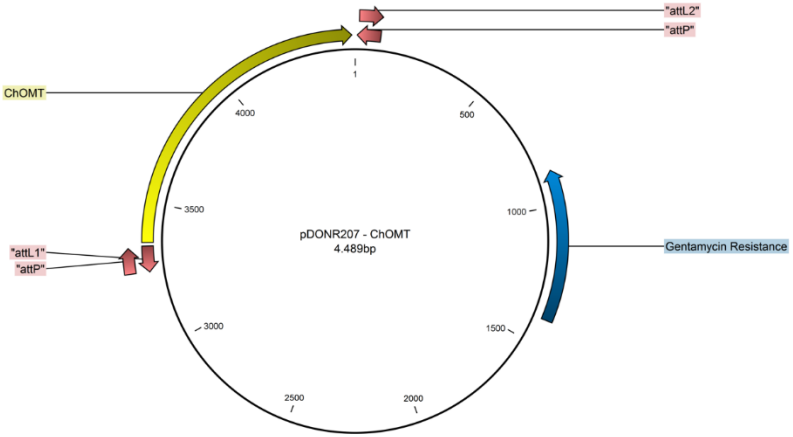

Figure A.4. Entry clone with pDONR207

8. APPENDIX B

Table B.1. Salt-induced candidate genes list generated by bioinformatics analysis of transcriptome data

Gene ID	Gene length	Raw read #	Fold diff. (log2)	FDR	GI number	Nr-score	Annotation	Organism
Unigene23432_All	**1232**	**2924**	**3.8402**	**4.38E-289**	**359806350**	**577**	**isoliquiritigenin 2'-O-methyltransferase-like**	***Glycine max***
Unigene4622_All	**1121**	**465**	**3.5426**	**2.611E-42**	**357489361**	**525**	**Sulfate transporter**	***Medicago truncatula***
Unigene5066_All	**1324**	**4243**	**2.3297**	**1.64E-227**	**188531129**	**684**	**BURP domain-containing protein**	***Phaseolus vulgaris***
Unigene623_All	**1457**	**436**	**5.567**	**2.935E-56**	**357483349**	**600**	**Vicilin-like antimicrobial peptides 2-3**	***Medicago truncatula***
Unigene8946_All	**795**	**1549**	**2.0566**	**4.892E-68**	**302633356**	**521**	**Ferric reductase**	***Phaseolus vulgaris***
CL1155.Contig2_All	2556	120	2.0611	9.0144E-06	357457573	801	Receptor-like protein kinase	*Medicago truncatula*
CL1181.Contig1_All	2241	4258	3.6053	0	3915037	1265	Sucrose-UDP glucosyltransferase 2	*Pisum sativum*
CL122.Contig5_All	1116	451	2.2662	1.5145E-23	342357374	570	beta-carotene hydroxylase	*Phaseolus vulgaris*
CL1421.Contig4_All	2689	689	3.9851	2.6879E-70	13161405	1381	VuP5CS	*Vigna unguiculata*
CL1705.Contig2_All	3270	459	2.151	9.1846E-22	357483115	1523	Guanine nucleotide-binding protein alpha-2 subunit	*Medicago truncatula*
CL3773.Contig2_All	1497	1199	2.1404	3.0758E-55	156739650	600	xyloglucan endotraglucosylase/hydrolase	*Vigna angularis*
CL4100.Contig1_All	4874	79	4.2823	2.491E-08	62177685	2934	NADH glutamate synthase precursor	*Phaseolus vulgaris*
CL237.Contig1_All	2292	279	2.549	3.8946E-17	357480003	1232	Heat-shock protein	*Medicago truncatula*
CL254.Contig1_All	1367	1421	2.6884	3.4653E-90	351727383	680	stearoyl-ACP desaturase	*Glycine max*

Table B.1. Salt-induced candidate genes list generated by bioinformatics analysis of transcriptome data (cont.)

Gene ID	Gene length	Raw read #	Fold diff. (log2)	FDR	GI number	Nr-score	Annotation	Organism
CL4211.Contig2_All	1850	3448	2.2547	2.7277E-174	13161397	768	CPRD2	*Vigna unguiculata*
CL3431.Contig2_All	959	103	6.6965	1.1873E-14	75708857	565	group 3 LEA protein	*Phaseolus vulgaris*
CL3588.Contig3_All	1887	11384	2.0974	0	452769	772	alcohol dehydrogenase-1F	*Phaseolus acutifolius*
CL364.Contig1_All	3684	2303	2.7142	8.7788E-152	156767195	1191	asparagine synthetase	*Phaseolus vulgaris*
CL4706.Contig1_All	2630	141	2.2867	1.9686E-07	322510094	912	G-type lectin S-receptor-like serine/threonine-protein kinase	*Arabidopsis thaliana*
CL5799.Contig1_All	1087	306	2.3193	5.5533E-16	357483497	658	Laccase-11	*Medicago truncatula*
CL2674.Contig3_All	2219	9600	2.129	0	351721519	1020	NRT2 protein	*Glycine max*
Unigene5227_All	1063	613	4.5024	1.4858E-68	116330	518	Acidic endochitinase	*Phaseolus angularis*
CL6565.Contig2_All	1272	56	10.5526	6.0656E-08	351727923	549	LEA protein precursor	*Glycine max*
CL7084.Contig4_All	1440	287	2.3201	7.3704E-64	357495245	564	Amino acid permease	*Medicago truncatula*
CL7116.Contig2_All	1561	272	3.2407	2.2979E-22	4336434	515	nodule-enhanced protein phosphatase type 2C	*Lotus japonicus*
CL7228.Contig2_All	1584	1564	3.4271	3.517E-133	20601	531	aspartate aminotransferase	*Panicum miliaceum*
CL7543.Contig1_All	892	551	2.2818	2.3601E-28	351722883	555	galactinol synthase	*Glycine max*
CL8395.Contig2_All	2973	1561	2.5726	1.1064E-95	351724503	1093	with no lysine kinase 12	*Glycine max*
CL936.Contig4_All	1826	505	2.206	2.2604E-25	357453013	751	Fatty acyl-CoA reductase	*Medicago truncatula*
CL959.Contig3_All	1570	372	3.141	2.1366E-29	357467067	654	Nucleoredoxin	*Medicago truncatula*
Unigene12807_All	975	178	12.6212	6.5809E-26	116395	502	Chalcone synthase	*Petroselinum crispum*
Unigene18985_All	1458	762	3.5869	7.5879E-70	357462847	597	O-glucosyltransferase	*Medicago truncatula*
Unigene19081_All	2085	1043	3.8999	2.3102E-103	357488469	984	Peptide transporter PTR	*Medicago truncatula*

Table B.1. Salt-induced candidate genes list generated by bioinformatics analysis of transcriptome data (cont.)

Gene ID	Gene length	Raw read #	Fold diff. (log2)	FDR	GI number	Nr-score	Annotation	Organism
Unigene21809_All	1435	779	2.4416	6.8887E-44	351726580	581	cysteine synthase	*Glycine max*
Unigene21694_All	1953	184	3.2872	1.1033E-15	357497661	614	Type I inositol-1,4,5-trisphosphate 5-phosphatase	*Medicago truncatula*
Unigene19805_All	1443	2606	3.0208	1.2531E-190	99032691	714	alcohol dehydrogenase	*Dimocarpus longan*
Unigene21795_All	2647	1934	2.6682	5.0346E-124	58825798	1322	sucrose-phosphate synthase 1	*Vitis vinifera*
Unigene22375_All	1032	988	2.5366	1.4939E-59	13161397	611	CPRD2	*Vigna unguiculata*
Unigene22480_All	1218	2520	3.5534	1.053E-227	351723211	590	endo-1,3-beta-glucanase	*Glycine max*
Unigene25160_All	2225	7845	2.2937	0	357482743	741	Isoflavone 2'-hydroxylase	*Medicago truncatula*
Unigene30293_All	1949	76	4.1848	4.6239E-08	30790423	835	polyphenol oxidase	*Medicago sativa*
Unigene4034_All	2456	175	2.1437	1.9152E-08	297822271	1152	ATPDR4/PDR4	*Arabidopsis lyrata*
Unigene4084_All	1825	776	2.1117	1.0427E-34	357485855	885	Polygalacturonase	*Medicago truncatula*
CL6505.Contig2_All	4055	9070	2.1062	0	357496247	1965	ABC transporter B family member	*Medicago truncatula*
Unigene32323_All	1122	202	2.0266	7.1122E-09	357479199	573	ABC transporter B family member	*Medicago truncatula*
Unigene528_All	1470	1059	2.3954	3.5278E-58	358346795	631	ABC transporter C family member	*Medicago truncatula*
Unigene21789_All	1899	283	2.4002	4.0843E-16	2739002	749	CYP83D1p	*Glycine max*
CL1457.Contig1_All	975	265	2.1645	8.4883E-13	59859756	508	elongation factor 1 alpha	*Cyclotella cryptica*
CL364.Contig2_All	2630	5480	3.027	0	156767195	1206	asparagine synthetase	*Phaseolus vulgaris*
CL364.Contig3_All	4462	1510	2.5185	1.2184E-89	156767195	1143	asparagine synthetase	*Phaseolus vulgaris*
CL364.Contig6_All	1441	12872	2.4651	0	156767195	634	asparagine synthetase	*Phaseolus vulgaris*
CL503.Contig4_All	1730	1802	2.5968	3.8813E-112	357483199	664	Cytochrome P450	*Medicago truncatula*

Table B.1. Salt-induced candidate genes list generated by bioinformatics analysis of transcriptome data (cont.)

Gene ID	Gene length	Raw read #	Fold diff. (log2)	FDR	GI number	Nr-score	Annotation	Organism
CL6246.Contig1_All	3746	1860	5.8813	1.9786E-246	10177143	915	retroelement pol polyprotein-like	*Arabidopsis thaliana*
CL7713.Contig2_All	1938	7774	4.9021	0	85001691	833	cytochrome P450 monooxygenase CYP76O2	*Glycine max*
CL791.Contig1_All	1787	6652	2.7087	0	5915845	711	Cytochrome P450 CP9	*Glycine max*
CL451.Contig2_All	1786	167	3.4741	1.3147E-14	357519287	565	Cytochrome P450	*Medicago truncatula*
Unigene11234_All	1945	207	2.1426	2.8012E-09	363814320	735	uncharacterized protein	*Glycine max*
Unigene15068_All	1427	3655	2.7247	4.1252E-242	18402564	631	uncharacterized protein	*Arabidopsis thaliana*
Unigene4000_All	1302	1234	2.3679	2.6558E-66	359807448	503	uncharacterized protein	*Glycine max*
CL4647.Contig5_All	1866	361	2.0667	6.3766E-16	87240677	946	TGF-beta receptor, type I/II extracellular region	*Medicago truncatula*

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