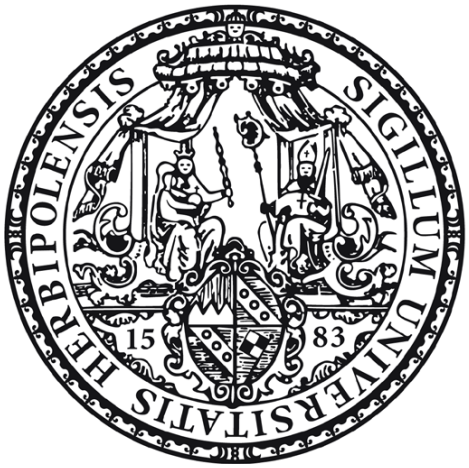

A Comparative Study on Guard Cell Function of the Glycophyte *Arabidopsis thaliana* and the Halophyte *Thellungiella salsuginea* Under Saline Growth Conditions

Eine vergleichende Studie zur Schließzellfunktion des Glycophyten *Arabidopsis thaliana* und des Halophyten *Thellungiella salsuginea* unter salinen Wachstumsbedingungen

Dissertation on the attainment of a doctorate in life sciences
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submitted by

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1. Introduction

Stomata are microscopic pores formed by a pair of guard cells and are located on the epidermal surface of the plants. Plants take environmental CO_2 from these pores and use it for carbon fixation via process called photosynthesis. During photosynthesis, plants produce oxygen as a by-product and release it to the environment via stomata, thus becoming a major source for environmental oxygen. Along gaseous exchange, plant water loss also takes place via stomata (Willmer and Fricker 1996). Hence, guard cells are vital not only for crop production but also for ecosystem. Guard cells functioning is affected under both abiotic and abiotic stress conditions. Among abiotic stress, soil salinity is one of the major threats to crop productivity. Soil salinity is a condition in which high concentration of soluble salts are found in soil. Soils having the Electric Conductivity (ECe) 4 dS/m or more at 25 ^{0}C (which is equal to 40 mM NaCl) are considered as saline (Brown Jr 2008; Munns and Tester 2008) and most of the plant species reduce their yield at this saline concentrations. Climate change and poor irrigation practices are one of the major sources of soil salinity. Sodium chloride (NaCl) is the most abundant salt found among all salts present in the agricultural lands. Increasing demands of food products can be met by perfecting good farming practices and improving plant traits by using conventional and modern plant breeding methods. For that, basic knowledge of single cell as well as their impact on whole plant physiology under saline growth conditions is required. Furthermore, cellular responses in various plant species is individually different and crucial for the respective stress tolerance (Zhu 2016). Therefore, comparative studies on plants cellular responses of salt sensitive and salt tolerant plant species could also pave the way for improving plant salt tolerance. Root cells have been extensively studies under saline growth conditions, however, knowledge about guard cells function on salinity is not fully understood. Therefore, comparative studies on guard cells of salt sensitive and salt tolerant plant species would enhance the knowledge of their functioning under salinity and hence would could lay the foundations for plant specific breeding programs.

1.1. Sensitivity of Plants to Salt Stress

Being a living organism, plants do sense and respond rapidly to environmental changes. Soil salinity poses two types of stress to the plants. The first phase of salinity is comparable to

drought, where increased levels of ions in the rooting zone results in low osmotic potential that reduces soil water potential. This in turn leads to low plant water uptake at the root zone, thus like drought stress, plant water balance is affected (Munns 2002). During the first phase of salt stress, plant growth rate is slowed down while in the second phase of stress accumulation of sodium and chloride leads to ion toxicity (Parihar et al. 2015). During osmotic stress, water status of the plants is imbalanced thus leading to lower transpiration rates, and loss of turgor. The osmotic stress indeed affects stomatal responses. Initially, responses are altered by water relations transiently and then modulated by Abscisic Acid (ABA) (Fricke et al. 2004). This is thought as a main reason for reduced plant growth (Munns and Tester 2008). Due to similar nature of drought and salt stress, many physiological and molecular responses of plants are closely related (Zhu 2002; Sah et al. 2016). In addition to osmotic stress, saline soils can also lead to ionic stress especially in prolonged growth on saline medium. Increased cellular ionic concentrations of salts causes abruption in enzymatic functioning, growth and development and leads to plant death in severe cases (Flowers et al. 2015; Munns and Tester 2008) . The tolerance of plants to salt stress is based on adjustments to the ion toxicity (Munns 2002; Flowers and Muscolo 2015). Plant salt tolerance is mediated by a variety of properties, such as adjusting transpiration via stomata, return of excess ions into the soil and the exclusion of toxic ions from the cytosol through the compartmentalization into the vacuoles and apoplast (Ismail et al. 2014; Flowers and Colmer 2015; Shabala 2013).

Plant responses to soil salinity depends on multiple factors including salt concentration, plant species, plant tissues, and cell types. Furthermore, it is also dependent on field conditions i.e. light intensity, soil pH, and soil type (Rejeb et al. 2014; Shrivastava and Kumar 2015). Contrasting responses have been observed in different plant species (Munns 2002). Plant species have been divided into two distinct classes based on their sensitivity to saline conditions. These include glycophytes and halophytes. Glycophytes are the group of plants that are sensitive to salt exposure. Plants belonging to this group exposed to certain saline conditions (i.e 100-200 mM NaCl) cause ion toxicity or even cell death. Whereas, halophytes are the group of plants that are tolerant to higher saline conditions and are able to survive where 99% of other plant species would be killed (Kazachkova et al. 2018). Halophytes complete their life cycle in salt concentration (mostly NaCl) of ≥200 mM (Flowers and Colmer 2008). The survival of the halophytes under an extreme saline condition is based

on its altered morphological, and anatomical structures (Flowers and Colmer 2015). These features can be at the tissue level (e.g. differences at epicuticular waxes, lipids, stomatal density, extra layer of palisade mesophyll cells etc.) that would help to modulate transpiration losses most effectively. Most of the halophytes have developed a second layer of endodermis and an extra layer of cortex in the roots that would resist ion transport from roots to shoots (Inan et al. 2004; Flowers et al. 1986; Kazachkova et al. 2018). Some halophytic plants have also evolved structures (called as salt glands) onto the epidermal surfaces to secret excessive ions (Dassanayake and Larkin 2017; Flowers and Colmer 2015; Santos et al. 2015).

1.2. Plant Responses to Salt Stress

Prolonged saline conditions often lead to increased cytosolic ionic concentrations which in turn causes death of the plant leaves. When accumulation of ions is faster than the formation of new leaves, adequate amounts of carbohydrate production is not achieved and hence plant growth rate is adversely affected (Munns and Tester 2008). However, halophytic plant species have developed specific set of traits/strategies over glycophytic plant species which help them to maintain cytosolic ionic concentrations under hostile saline conditions. Moreover, some halophytes can even tolerate elevated ionic concentrations in their cells (C Nikalje et al. 2017). Sodium chloride damage is associated with the accumulation of both ions in the leaves which results in necrosis initially at the leaf tips and margins and then working back to the leaf base (Tester 2003). In general, salt induced effects on plants include osmotic stress, ion toxicity, nutritional imbalance, oxidative stress, metabolic perturbance, membrane disorganization, genotoxicity, reduction of cell division and expansion (C Nikalje et al. 2017).

1.2.1. Ion Transport Mechanisms

1.2.1.1. *Potassium Transport*

Plants need essential nutrients for healthy growth. Among cations, potassium serves as an essential nutrient in the plant cell. It is required for proper enzymatic functioning, protein synthesis, osmoregulation, and plant growth (Tester 2003).Therefore, K^+ transport is critical for cytoplasmic and luminal pH, regulation of membrane potential and enzyme activity (Ragel et al. 2019). Plants acquire potassium from soil through potassium permeable protein

located on the root hairs. In classic potassium experiment, potassium absorption by roots was described as a high affinity and low affinity bi-phasic transport process (Epstein et al. 1963). It is considered that Low Affinity Transport System (LATS) in roots occur at the mM range, and activity of the system is independent of external K^+ concentrations (Maathuis and Sanders 1997; Chérel et al. 2013). Whereas, High Affinity Transport System (HATS) are quickly upregulated when the external K^+ concentration is decreased/scarce (Glass 1976; Glass and Dunlop 1978). Regarding the K^+ uptake, it was predicted that under most conditions K^+ transporters function as HATS, while K^+ permeable channels facilitate LATS (Maathuis and Sanders, 1997). Uptake and long-distance K^+ transport in plant cells is carried out by a variety of transporter proteins characterized into numerous families with varied structures and transport mechanisms. These comprise the channel families Shaker-like voltage-dependent, TPK (tandem-pore), and TPC (two-pore channels) (Hedrich 2012), the carrier-like families KT/HAK/KUP (K^+ transporter/high-affinity K^+ transporter/K^+ uptake permease) (Li et al. 2018; Nieves-Cordones et al. 2014), HKT (High affinity potassium transporter family) uniporters and symporters (Hamamoto et al. 2015), and CPA (cation-proton antiporters). The CPA family is the largest one and includes the NHX (Na^+/H^+ antiporter family) , CHX (Cation/H^+ exchanger), and KEA (Potassium efflux antiporters) transporters (Sze and Chanroj 2018). Out of the three families of K^+ transporters, CPA , HKT and KT/HAK/KUP, CPA was characterized as a K^+ (Na^+)/H^+ antiporters, HKT may co-transport Na^+ and K^+ or transport Na^+ only (Rubio et al. 1995; Uozumi et al. 2000), while KT/HAK/KUP were predicted to be H^+-coupled K^+ symporters (Mäser et al. 2001; Lebaudy et al. 2007).

At high concentration in the soil solution, potassium passes through the membrane mostly via channels. The channels simply give a passage for the ions allowing them to move down the electrochemical gradient. Plant voltage-gated K^+ channels are categorized into three sub-families based on their response to the membrane potential (Dreyer and Uozumi 2011): (1) Inward-rectifying channels (in Arabidopsis, these include AKT1, AKT6, KAT1, and KAT2); they open at hyperpolarized membrane potentials allowing the uptake of K^+. (2) Outward-rectifying channels that mediate K^+ release as they open at depolarized membrane potentials; this group is composed of SKOR and GORK channels. (3) Weakly rectifying channels that can mediate both K^+ uptake and release, (in Arabidopsis its representative is AKT2).

At low K^+ concentration, active transport systems are required to pull K^+ inside the cell against its electrochemical gradient. However, studies in several plant species have shown that channels may be involved in K^+ uptake in the high-affinity range of K^+ concentrations (Rubio et al. 2010) if the membrane is sufficiently hyperpolarized, i.e. highly electronegative inside (Hirsch et al. 1998; Rubio et al. 2010; Gierth and Mäser 2007). PM (Plasma membrane) localized potassium transport proteins involved in potassium uptake include AKT1, AKT2, KAT1, KAT2 and CHX20 (Wang and Wu 2013). AKT1 and HAK5, root-expressed K^+ transport proteins in *A. thaliana*, mediate K^+ uptake over a wide range of external K^+ concentrations (Yang et al. 2014). These two transport proteins facilitate almost all potassium uptake in *A. thaliana* from the external environment. HAK5 of *T. salsuginea* has also been reported in potassium uptake (Alemán et al. 2009). Similarly, TsHKT1 has been shown to have important role in potassium uptake in yeast cell lines (Ali et al. 2013). Arabidopsis mutant lacking HAK5 and AKT1 still took up K^+ and showed residual growth at external K^+ concentration above 1 mM, demonstrating the presence of other compensatory transport system(s) (Rubio et al. 2010; Pyo et al. 2010). As this low-affinity uptake is mainly sensitive to Ca^{+2} and other divalent metals therefore non-selective cyclic nucleotide-gated cation channels (CNGC) are likely to be the candidates for this alternative system (Caballero et al. 2012). AtCNGC3 and AtCNGC10 have been reported in K^+ uptake as their knock-out and knock down lines showed reduced K^+ contents (Kaplan et al. 2007). Moreover, AtCHX13, a plasma membrane cation/proton antiporter showed up-regulated under K^+ starvation conditions, thus suggesting its role in root K^+ uptake (Zhao et al. 2008). Similarly, member of HAK family, AtKUP7, is preferentially expressed in Arabidopsis roots, and therefore may also be instrumental in K^+ uptake and in K^+ loading into xylem sap, affecting K^+ translocation from roots to shoots (Han et al. 2016). Translocation of potassium to different organs is mediated by loading into the xylem stream by outward rectifying channels such as SKOR working at the root cortex cells (Wang and Wu 2013). Loading and unloading of potassium in the phloem is mediated by weakly rectifying K^+ channels e.g AKT2 (Lacombe et al. 2000; Gajdanowicz et al. 2011; Marten et al. 1999). After translocation of potassium to leaves, it can be taken up by the guard cells for turgor regulations and hence stomatal opening.

Under normal conditions, potassium concentration in the guard cells is 100 mM (Shabala et al. 2006). Both inward and outward K^+ channels were initially identified in stomatal

guard cells, mediating K^+ uptake and release, respectively (Schroeder et al. 1984; Hosoi et al. 1988; Schroeder et al. 1987). Further investigations showed that the inward K^+ channels KAT1 and KAT2 control the K^+ influx across the plasma membrane (Anderson et al. 1992; Kwak et al. 2001; Pilot et al. 2003; Szyroki et al. 2001) and the outward K^+ channel GORK is involved in K^+ efflux (Ache et al. 2000; Hosy et al. 2003). Plasma membrane located CHX member, CHX13, is also involved in potassium uptake in guard cells. Inside guard cells, the K^+ ions could be distributed and recycled between the cytoplasm and vacuole. The two major tonoplast-located NHX members, NHX1 and NHX2, mediate K^+ uptake into the vacuole, which in Arabidopsis is essential for K^+ pool creation and stomatal movement (Barragan et al. 2012; Bassil et al. 2011; Venema et al. 2002). Interruption in the guard cells of nhx1 nhx2 mutant plants resulted in more acidic vacuoles and the disappearance of the highly dynamic remodelling of vacuolar structure associated with stomatal movements (Andrés et al., 2014). The voltage independent K^+-selective channels were initially determined at the tonoplast of guard cells and were found crucial for vacuolar K^+ release during stomatal closure (Ward and Schroeder 1994). Further functional characterization revealed that the tonoplast-located TPK1 functions in K^+ transport (efflux) across the tonoplast and plays a role in intracellular K^+ homeostasis, which is important for stomatal closure and other several physiological activities (Gobert et al. 2007; Czempinski et al. 2002). Most of the reported potassium transporters have been characterized in Arabidopsis guard cells. In addition, characterization of potassium transporters of halophytic guard cells is very rare. As the guard cell K^+ channels (both inward and outward) in glycophytic plant species are selective for K^+ (Shabala et al. 2010; Hedrich 2012) thus, unless the properties of these channels are extremely changed in halophytes species, they are unlikely to mediate transport of other cations in and out of guard cells in halophytes (Véry et al. 1998).

1.2.1.2. Sodium Transport

Under saline environments, Na^+ uptake in plants is facilitated through pathways that generally function for K^+ intake. This is because both cations in hydrated forms have similar ionic radii thus making discrimination between the two ions difficult (Keisham et al. 2018). In lower levels Na^+ is harmless and uptake in the high affinity range may be a purely passive process (Garciadeblás et al. 2003; Haro et al. 2009). In the presence of ambient sodium, Na^+ uptake occurs via high affinity potassium transporters (e.g HKT1), Non-Selective Cation

Channels (NSCCs), and possibly potassium selective channels (e.g AKT1) (Zhang et al. 2010b; Amtmann and Sanders 1998; Maathuis 2014; Hedrich and Shabala 2018). Identification of NSCCs is still not fully understood (Zhang et al. 2010a; Demidchik and Tester 2002). Part of the electrophysiological measurements of NSCC-mediated currents may be due to the members of plant CNGCs (cyclic nucleotide gated channels). These NSCCs are activated by cGMP or cAMP and are permeable to both mono and di-valent cations including potassium and calcium (Zelman et al. 2012). Aquaporins have also been reported to take part in sodium uptake in plants (Byrt et al. 2017). During saline conditions, SOS (salt overly sensitive) pathway has been proposed to mediate sodium homeostasis in plant cells (Ji et al. 2013). It has also role in Na^+ loading into the xylem sap. However, its function may vary during severe stress conditions and include removal of Na^+ from the xylem stream (Maathuis 2014). HKT, NSCCs and CHX are involved in long distance translocation of Na^+ by loading into the xylem stream (Maathuis 2014). In a comparative study between *A. thaliana* and *T. salsuginea* a decrease in the potassium concentration of the roots of *T. salsuginea* was detected by increasing NaCl concentrations in the nutrient medium, but this was very low compared to *A. thaliana* (Aleman et al. 2009). It is also reported that *T. salsuginea* have higher potassium root/shoot ratios than *A. thaliana* under both non-saline and saline conditions (Aleman et al. 2009). Moreover, in halophytes, potassium transport proteins are highly selective for potassium (e.gHKT1) and can discriminate between sodium and potassium therefore the uptake of sodium is reduced under saline conditions (Volkov and Amtmann 2006; Wang et al. 2006). These features help halophytes to survive under saline conditions.

It is suggested that guard cells of both glycophytes and halophytes can use sodium for osmotic regulations (Hedrich and Shabala 2018). Energy Dispersive X-Ray Analysis (EDXA) showed accumulation of sodium in guard cells on the expense of potassium in glycophyte A. thaliana (Rienmuller et al. 2010). Sodium uptake in guard cells could be mediated by plasma membrane located HKT1 transporter (Hedrich and Shabala 2018). As potassium is vital for plant growth therefore potassium to sodium ratios are crucial for determining sodium toxicity in plants. Numerous studies reported decline in plant potassium content under saline conditions especially in glycophytes (Volkov et al. 2004; Cuin et al. 2008; Zhu et al. 1998). In contrast, salt stress in many halophytic species does not lead to a decrease in potassium content. Some of these species, such as *Atriplex nummularia* (Ramos et al. 2004) and

Chenopodium quinoa (Hariadi et al. 2010) have even higher potassium concentrations under moderate saline conditions

Lower levels of sodium in the cytosol are maintained by its sequestration either into the apoplast or into the vacuole. Sequestration of sodium is mostly achieved by Na^+/H^+ antiporters working at the plasma membrane and at the tonoplast (Kumar et al. 2018). One of the well-known mechanisms for sodium detoxification is Salt Overly Sensitive (SOS) pathway. The very first intracellular response of the increased ionic concentration is the elevation of calcium (Ca^{+2}) in the cytoplasm (Ji et al. 2013). It is well known that the Na^+ induced Ca^{+2} spikes in the root cells activate SOS signalling cascade that has a special role in maintaining and regulating ion homeostasis particularly Na^+ under saline stress (Golldack et al. 2014). Three components known as SOS1, SOS2 and SOS3 (salt overly1, 2 and 3 respectively) take part in SOS pathway to sequester cytosolic Na^+ into the apoplast. Elevated levels of Ca^{+2} are perceived by myristyolated calcium-binding protein SOS3. After binding with Ca^{+2}, SOS3 then interacts with SOS2 (a kinase also known as CIPK24 and SnRK3.11). This interaction leads SOS2 to plasma membrane where it activates SOS1 (a Na^+/H^+ antiporter) by phosphorylation, which in turn transport Na^+ into the apoplast (Ji et al. 2013). Constitutively higher expression of TsSOS1 in roots and shoots under non-saline and saline growth conditions respectively (Kant et al. 2006) suggests that Thellungiella has better mechanism in sodium loading and redistribution in the plants.

Along with SOS pathway sodium/proton exchangers (NHXs) play vital role in sodium compartmentation into the vacuoles (Deinlein et al. 2014; Maathuis 2014; Yamaguchi et al. 2013; Zhu 2003; Apse et al. 1999). . There are many reports suggesting that the larger ability of halophytes to sequester Na^+ in their vacuoles is related to both the constitutive expression of tonoplast located Na^+/ H^+ antiporters and the stimulation of their activity under saline growth conditions (Shabala 2013). Contrastingly, in glycophytes, these transporters are activated only under saline conditions. Moreover, it appears that such activation occurs only in salt tolerant glycophyte species, while in salt-sensitive plants, their expression levels are very low and not salt inducible (Apse et al. 1999; Zhang and Blumwald 2001). Both *A. thaliana* and *T. salsuginea* contain eight members of NHX transporters (Wu et al. 2012), among these four (NHX1-NHX4) are localized to tonoplast in *A. thaliana* (Bassil et al. 2019). Whereas

TsNHX1 is the only member that has been reported to be located on tonoplast (Wu et al. 2009), other members have yet to be analysed in this plant species.

1.2.1.3. Chloride Transport

Chloride (Cl^-) is a micronutrient that plays important role in photosynthesis, transpiration, nutrition, and growth. However, accumulation of Cl^- in the cytosol affects these key physiological processes by changing pH, membrane potential, osmoregulation, enzyme stability, volume control and turgor (White and Broadley 2001; Franco-Navarro et al. 2015). Its uptake from the medium is facilitated by anion transport proteins. Long distance translocation of Cl^- is achieved by its loading into the xylem by S and R-type anion channels and Nitrate transporters (NPFs). Among these SLAH1, SLAH2, ALMT9, ALMT12, NPF2.4 and NPF7.3 have been reported in Cl^- long distance translocation in *A. thaliana* (Li et al. 2017a; Baetz et al. 2016; De Angeli et al. 2013; Meyer et al. 2010).

Elevated levels of chloride can lead to leaf bleaching hence affecting photosynthesis and growth. Therefore, optimum chloride levels should be maintained. Like sodium, plants maintain cytosolic chloride levels by its sequestration into vacuoles or into apoplast under saline growth conditions. This happens in all major cells of the plants; however, root cortex seems to be the major site of Cl^- accumulation (Li et al. 2017a). This also affects root to shoot translocation of Cl^- in the plants. *T. salsuginea* contains an additional cortex layer in root zone that is useful in reduced chloride translocation to vegetative parts (Inan et al. 2004) In the shoot region, Cl^- is compartmentalized in the leaf epidermis to protect mesophyll cells hence protecting photosynthetic activity of the plants. As the newly emerged leaves tend to be more sensitive to ionic stress, therefore, they are protected from Cl^- accumulation by its translocation to the older leaves via phloem. The AtALMT9 is localized in the tonoplasts of guard cells, xylem parenchyma cells, and root stem cells. *A. thaliana* almt9 mutants have a lower chloride concentration in the shoot when treated with salt (Baetz et al. 2016). Also, some members of the family of the CLCs (Chloride Channel) seem to play a role in the transport and the accumulation of chloride in vacuoles. These chloride transport proteins are anion / proton antiporters that carry either chloride or nitrate through the proton gradient across the tonoplast. There are seven members of this family (i.e AtCLC-a to AtCLC-g) present in *A. thaliana* (Zifarelli and Pusch 2010). Among others, AtCLC-c and AtCLC-a participates in the Cl

loading into the guard cell vacuoles (Jossier et al. 2010a) whereas AtCLC-g works at mesophyll tonoplast. The other members of CLCs are known to be involved in NO_3^- homeostasis (De Angeli et al. 2006; Nguyen et al. 2015; Jossier et al. 2010b; von der Fecht-Bartenbach et al. 2010; Fecht-Bartenbach et al. 2007; Marmagne et al. 2007).

Guard cells of both glycophytic and halophytic plant species use chloride for osmotic regulations (Hedrich and Shabala 2018). It is suggested that the import of Cl^- into the guard cells takes place via chloride-proton symporters, however this has to be tested (Hedrich and Shabala 2018; Hedrich and Geiger 2017; Cubero-Font et al. 2016; Schäfer et al. 2018).

1.2.2. Plant Water Balance

Plant water loss through stomata generates force known as transpiration pull that helps plant roots in water uptake along with nutrients from soil. Plants use water in photosynthesis, therefore appropriate plant water status is essential for optimum plant growth. Decreased soil water potential under saline growth conditions reduces plant water uptake thus resulting in disturbed transpiration stream. This in turn affects plant development and almost all physiological processes (Yordanov et al. 2000). Both glycophytes and halophytes showed decrease in cell osmotic potential under saline growth conditions. However, glycophytes showed more changes in osmotic potential than glycophytes under similar saline growth conditions. Osmotic potential of *A. thaliana* leaves showed 400% decrease within twelve days upon treatment with 200 mM NaCl, followed soon after by death. In contrast, the osmotic potential of *T. salsuginea* leaves declined by only approximately 38% during the initial six days of exposure and did not decline further in the rest of the experiment (Inan et al. 2004).

An essential part of the adaptation to osmotic stress is the minimization of water loss over the surface. Therefore, drought resistant plants often show a certain amount of succulence and reduced leaf area (Males 2017). In addition, higher land plants have a cuticle and an epicuticular wax layer which prevents much of the evaporation of water from the plant tissue (Riederer 2006). The cuticle and the composition of the epicuticular wax layer varies from species to species and has a significant impact on the minimum amount of water that a plant loses to its environment (Schuster et al. 2016). Plants simultaneously transpire water and take CO_2 via stomata. Therefore, a reduction to water loss will prevent the diffusion of

atmospheric CO_2 into photosynthetically active tissue. In fact, much of the water, that plants lose to their environment is due to stomatal transpiration (Riederer and Schreiber 2001). Therefore, strict and efficient control of the stomata apparatus, especially under drought conditions, is critical to plant survival and development. Intrinsic Water Use Efficiency (WUE_i), i.e CO_2 assimilated per unit molecule of water, is a parameter that determines adaptability of plants under unfavorable conditions. WUE_i, has positive impact on energy production and hence growth and development. It has been reported that halophytes have higher WUEi than glycophytes (Shabala 2013).

Plants accumulate compatible osmolytes to balance osmotic differences (Hussain Wani et al. 2013) caused by salinity. Compatible solutes are organic, osmotically active compounds of low molar mass, which, even at high concentrations, have no negative effect on cell metabolism (Bohnert et al. 1995; Wang et al. 2003; Chen and Jiang 2010). These compatible solutes include sugars and sugar derivatives (e.g glycine betaine) and amino acids (e.g proline) (Chen and Jiang 2010). Accumulation of proline is associated with an increased salt tolerance. For example, NaCl-induced increase in the proline content of M. crystallinum has been demonstrated (Thomas and Bohnert 1993). Similarly, comparative study on *A. thaliana* and *T. salsuginea*, showed elevated levels of proline in both species under 150 mM NaCl treatment, however, the increase was significantly greater in the salt-tolerant *T. salsuginea* (Gong et al. 2005).

Osmotic stress induced proline synthesis in adult *A. thaliana* plants appears to be predominantly mediated by AtP5CS1 (Székely et al. 2008). Proline is synthesized in plants mainly by conversion of glutamate. Glutamate is converted by D1-pyrroline-5-carboxylate synthases (P5CS) into pyrroline-5-carboxylate (P5C), which is further reduced to proline by D1-pyrroline-5-carboxylate reductases (Szabados and Savoure 2010). The conversion of glutamate to P5C is the rate-limiting step. In the *A. thaliana* genome, two P5CS orthologues could be identified and their expression is induced by ABA and NaCl (Strizhov et al. 1997). Proline homeostasis in plant cells is additionally controlled by the degradation of proline by proline dehydrogenases (PDH) (Szabados and Savoure 2010). It was shown that In *A. thaliana* proline itself induces the expression of AtPDH in a negative feedback mechanism. Osmotic stress and ABA had a negative impact on PDH expression (Nakashima et al. 1998; Kiyosue et al. 1996). Salt tolerant T. salsuginea showed higher accumulation of proline along with

reduced activity of PDH enzyme than *A. thaliana* thus suggesting differential gene expression between glycophytes and halophytes contribute to salt tolerance in halophytes (Kant et al. 2006).

The increase in compatible solutes, such as proline, is often discussed as an adaptive response of plants to salt stress however, the accumulation in many species is too low to have a large impact on osmotic potential (Mansour and Ali 2017). For instance, salt induced proline increment in salt tolerant *Atriplex halimus* causes only about 3% of the osmotic adaptation of the leaf tissue (Bendaly et al. 2016). Several studies indicate that proline has an antioxidant effect and can reduce the oxidative stress under saline conditions. For instance, salt stress in *A. thaliana* p5cs1 loss mutants caused chlorosis due to chlorophyll degradation and increase in lipid peroxidation by free radicals (Székely et al. 2008). Conversely, by the addition of exogenous proline, salt induced ROS production in chloroplasts of mustard seedlings (Brassica juncea cv., Dira 367) was significantly reduced (Saradhi and Mohanty 1997). Accordingly, the role of proline in the response to salt stress conditions does not appear to lie solely in the osmotic adaptation, but to be multifaceted (Mansour and Ali 2017; KAVI KISHOR and Sreenivasulu 2014).

1.2.3. Reactive Oxygen Species (ROS) and Antioxidants

ROS are continuously produced in cell organelles (e.g. mitochondria, peroxisome, chloroplast) as unavoidable by-products of aerobic metabolism such as photosynthesis, respiration, and photorespiration (Apel and Hirt 2004; Abogadallah 2010). ROS accumulation in the cells causes damage to DNA, proteins, lipids, and carbohydrates. Therefore, strong regulation of ROS in the plant cells is highly required. Under normal conditions, plants use ROS as a signalling molecule to regulate several processes in the cell such as stomatal behaviour, programmed cell death and pathogen defence (Apel and Hirt 2004). However, under saline conditions and other biotic and abiotic stress conditions, the equilibrium between ROS production and its scavenging is disturbed which leads to oxidative burst of the cells (Apel and Hirt 2004).

ROS production during salt stress has been shown to take place via four processes. 1) Reduced transpiration during salt stress leads to the decreased internal CO_2 concentrations thus slowing down its reduction by Calvin cycle. This process leads to depletion of oxidized

NADP, a final electron acceptor in the PSI, and alternatively increases the leakage of electron to O_2 thus yielding superoxide (O_2^-). Moreover, ion toxicity caused by both sodium and chloride may disrupt the electron transport and provoke formation of O_2^- (Hsu and Kao 2003; Gossett et al. 1994; Ślesak et al. 2002). 2) Slowing down of Calvin cycle caused by lower internal levels of CO_2 induces photorespiration and leads to more production of H_2O_2 in peroxisomes (Leegood et al. 1995; Wingler et al. 2000; Ghannoum 2008). 3) Salt stress induced membrane bound NADPH oxidase and the apoplastic diamine oxidase contribute in the production of ROS (Hernández et al. 2001; Lin and Kao 2001; Mazel et al. 2004; Tsai et al. 2005). 4) Increased rates of respiration caused by salt stress leads to electron leakage to O_2^- (Fry et al. 1986; Moser et al. 1991; Jeanjean et al. 1993).

Equilibrium between ROS production and its detoxification is kept by enzymatic and non-enzymatic processes in plants. Enzymatic scavenging process of ROS in plants involves superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalyse (CAT). During salt stress, antioxidant enzymes mostly target O_2^- and H_2O_2. SODs act as first line of defence against ROS, where they convert O_2^- to H_2O_2 in peroxisomes, mitochondria, chloroplast, cytoplasm and apoplast. Further, detoxification of H_2O_2 is performed by APX, GPX and CAT (Abogadallah 2010; Bowler et al. 1992). Non-enzymatic detoxification of ROS is mediated by ascorbate and glutathione (GSH), as well as flavonoids, carotenoids, tocopherol, and alkaloids. It has been shown that mutants with decreased GSH and ascorbic acid content lead to hypersensitivity to stress (Huang et al. 2005). Tocopherol is found on the cell membranes and plays an important role in scavenging lipid peroxyl radicals (Munné-Bosch et al. 1999; Munne-Bosch 2005). Carotenoids have roles in quenching O_2^- and minimize its formation by accepting excessive energy from the chlorophylls (Siefermann-Harms 1987). Similarly, overproduction of antioxidant enzymes in different plants have shown tolerance to salt (Eltayeb et al. 2007; Prashanth et al. 2008).

Salt tolerant *T. salsuginea* has been reported with higher ROS production and better scavenging system under both stress and non-stress conditions (Pilarska et al. 2016; Wiciarz et al. 2015; Stepien and Johnson 2009). Pilarska et al. 2016 suggested that increased H_2O_2 production in *T. salsuginea* chloroplasts might be important for stress tolerance of this halophyte. It is suggested that the intrinsically higher SOD levels in halophytes are required for rapid induction of the H_2O_2 'signature', and to trigger a cascade of adaptive responses

(both genetic and physiological), while the role of other enzymatic antioxidants may be in decreasing the basal levels of H_2O_2, once the signalling has been processed (Bose et al. 2013).

1.2.4. Photosynthesis and Growth

Reduction in plant growth rate is one of the plant responses under saline conditions. Salt stress effects plant growth by combination of drought and ionic stress. Growth and development are largely dependent on long distance signature molecules such as phytohormones and their precursors (Fricke and Peters 2002; Munns et al. 2000). Changes in abundance of several enzymes involved in phytohormone metabolism such as jasmonic acid, gibberellin, ethylene, and ABA biosynthesis have been detected in salt-treated plants (Parihar et al. 2015). As phytohormones are vital for growth and development, therefore abruption in biosynthesis of these phytohormones would lead to drastic effects on plant growth and development. Candidate genes controlling growth are possibly involved in signalling pathways that start with a sensor and involve phytohormones, protein kinases, transcription factors, protein phosphatases, and other signalling molecules such as calmodulin binding proteins (Parihar et al. 2015). It is most likely that such genes are shared in abiotic stresses including drought and salt. Similarly, several proteins involved in signalling, ion transport, photosynthesis, ATP production, respiration, osmolytes, and carbohydrate metabolism are affected during saline growth conditions (Kosová et al. 2013).

Reduction in contents of photosynthetic pigments (chlorophyll and carotenoids) caused by sodium and chloride accumulation have been reported in many plant species including halophytes (Chutipaijit et al. 2011; Saha et al. 2010; Khan et al. 2013; Mane et al. 2010). Photosystem II is sensitive component of photosynthetic machinery in terms of salt stress. A considerable decrease in amount of Electron Transport Chain (ETC) and CO_2 assimilation have been found under saline conditions (Allakhverdiev et al. 2000; Stepien and Klobus 2005). Due to osmotic stress, there is enhanced stomatal closure and a reduced CO_2 availability. This has strong negative impact on CO_2 assimilation. Several glycophytic plants and crops exposed to salinity showed a decreased abundance of Rubisco large and small subunits (Aghaei et al. 2008; Pang et al. 2010; Sobhanian et al. 2010; Bandehagh et al. 2011; Chattopadhyay et al. 2011). These factors would limit the photosynthetic efficiency of plants and hence energy production which ultimately results in stunted plant growth phenotype. Salt

tolerant plants are better equipped in dealing with energy starvation during saline conditions. Rubisco activity of halophytic plants such as *T. salusuginea* have been reported higher than *A. thaliana* under both salt and non-saline conditions (Wiciarz et al. 2015). This could serve halophytic plants to cope with energy starvation.

1.3. Stomata Functions

Stomata are tiny pores on plant epidermal surface which are formed by a pair of guard cells. They have asymmetrically thickened, cell walls (Raschke 1975), which together with the cytoskeleton, predetermine the shape of these cells. Dicotyledonous plants (e.g *Arabidopsis thaliana and Thellungiella salsuginea*) possess kidney shaped guard cells whereas (fig. 1.1), monocotyledonous plants such as grasses (e.g *Hordeum vulgare*) often have dumbbell shaped guard cells accompanied by subsidiary cells (Tomlinson 1974; Rudall et al. 2017). By adjusting the size of the stomatal pore, plants can adapt transpiration and intake of CO_2 to external conditions. The width of the stomata is controlled by turgor changes in the guard cells (Roelfsema and Hedrich 2005). An increase in guard cell turgor leads to swelling of the cell (fig. 1.1 B and D). Cell walls and the cytoskeleton allowing only a longitudinal expansion of the guard cells, which in turn opens the pore thus allowing intake of environmental CO_2 along with plant water loss via transpiration. In contrast, decrease in guard cell turgor leads to stomatal closure (fig. 1.1 A and C). In general, stoma movements due to turgor changes in the guard cells are the result of intake or release of osmotically active ions, particularly potassium (K^+), chloride (Cl^-), nitrate (NO_3^-) and malate which in turn leads to influx or efflux of the water respectively via aquaporin, the water channels (Roelfsema and Hedrich 2005). Due to the charge of these ions, they can only pass through the plasma membrane of the guard cells via transport proteins that are permeable to them. To control the stomatal gas exchange and thus the transpiration, the activity of these transport proteins is strictly regulated in plants. Guard cels have not yet been fully characterized under saline growth conditions therefore salt induced molecular responses in these specialized cells is not fully understood.

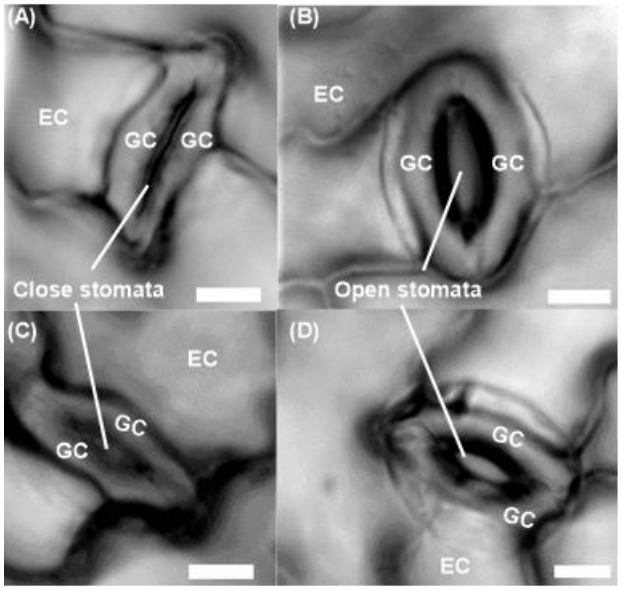

Figure 1.1. Light microscopic image of stomata: Lower epidermis of *A. thaliana* (A and B) and *T. salsuginea* (C and D) leaf showing pair of guard cells forming closed (A and C) and open (B and D) stomata from both plant species. Scale bar represents 10 µm in width. Where GC = guard cell and EC = epidermal cell.

1.3.1. Opening of Stomata

Light, high humidity and low CO_2 concentrations in the environment lead to stomatal opening. Both blue and red-light spectra can induce stomatal opening. Blue light induced opening response of stomata is enhanced by red light and are ubiquitous in all land plants except the fern species belonging to Polypodiopsida (Shimazaki et al. 2007; Doi et al. 2015). Blue light is perceived via blue-light photoreceptor protein kinases (phototropins, PHOT) which are activated upon blue light induced autophosphorylation. Via a cascade of further kinases, such as BLUS1 (Blue Light Signaling1) (Takemiya et al. 2013) and phosphatases, plasma-membrane-bound H^+ -ATPases are finally activated,. This leads to a proton efflux and thus hyperpolarization of the plasma membrane (Marten et al. 2010; Inoue and Kinoshita 2017). In total, there are eleven such plasma membrane H^+ ATPases in *A. thaliana* (AHA1-AHA11), of which mainly AHA1 and AHA2 are expressed in guard cells (Haruta et al. 2010). The hyperpolarization of the plasma membrane opens the voltage-dependent, inwardly rectifying potassium channels which leads to an influx of potassium (K^+). In *A. thaliana*, five such channels are known (KAT1 and KAT2, AKT1, AKT2 / 3, and KC1) and are thought to form functional homo- or heteromeric complexes (Lebaudy et al. 2008; Dreyer et al. 1997; Szyroki et al. 2001). Together with K^+, the concentration of counterions such as chloride (Cl^-) and malate in the guard cells are also increased (Raschke and Schnabl 1978). Influx of these anions are mediated by anion transport proteins located at the plasma membrane.

Guard cell response to red light seems to be indirectly induced compared to blue light (Roelfsema et al. 2001; Shimazaki et al. 2007). In contrast to blue light-dependent stomatal opening, mechanism for red light induced stomatal opening are a matter of debate (Roelfsema and Hedrich 2005; Shimazaki et al. 2007; Lawson et al. 2014; Vavasseur and Raghavendra 2005; Mott et al. 2014; Suetsugu et al. 2014). Red light causes membrane hyperpolarization in guard cells of *Vicia faba* (Serrano et al. 1988) and stomatal opening in the epidermal peels of Vicia and Commelina. Both responses to red light are suppressed by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), a photosynthetic electron transport inhibitor (Schwartz and Zeiger 1984). Red light induced stomatal opening is not evident in epidermal peels of Arabidopsis plants (Inoue et al. 2008; Takemiya et al. 2013), but opening is obvious in the intact leaves. In line with this observation, it is suggested that light diffusible substances are moved from mesophyll cells to guard cells to induce leaf stomatal opening (Mott et al. 2014; Fujita et al. 2013), but the substances have yet to be identified. Red light induced stomatal opening may be caused by a low intercellular concentration of CO_2 brought about by mesophyll photosynthesis because low CO_2 has been reported to cause stomatal opening (Roelfsema and Hedrich 2005; Horrer et al. 2016; Roelfsema et al. 2002). Unknown signal transduction pathway leads to an influx of osmotically active solutes into the guard cells at low intercellular CO_2 concentrations, which leads to the opening of the stomatal pore (Mott 2009; Roelfsema et al. 2001; Shimazaki et al. 2007).

Guard cells mainly accumulate K^+ salts during stomatal opening because this cation is available at concentrations of 2–10 mm in the guard cell apoplast under control conditions. These concentrations are consistent in the guard cells of both glycophytes and halophytes plant species (Speer and Kaiser 1991; Shabala et al. 2010; Shabala et al. 2013). However, stomatal opening is also supported by other monovalent cations such as Rb^+, Li^+, Cs^+ and Na^+ (Humble and Hsiao 1969). Except for Na^+ (under saline conditions), other cations do not accumulate in the apoplast at concentrations high enough to compete with K^+. As the halophytes use NaCl as a cheap osmoticum, therefore it is suggested that guard cells of these plant species can use sodium for osmotic adjustments in stomatal regulations (Hedrich and Shabala 2018).

1.3.2. Closing of Stomata

Most plants close their stomata to avoid unnecessary water loss (Willmer and Fricker 1996; Merilo et al. 2014). Like the mechanisms underlying stomatal opening and signal cascades, the signaling pathways and networks leading to stomatal closure are also known in some cases. Stomatal closure is an inversion of the opening movement in terms of osmolyte magnitude and flux directions. However, the associated transport systems differ. A switch from light to dark condition inactivates the H^+ pump and activates S-type anion channels of the SLAC1 (Slow Anion Channel 1) and SLAH3 (SLAC1 Homologue 3) type (Hedrich and Geiger 2017). This results in guard cells' anion release through these channels, the plasma membrane of the motor cell depolarises (Roelfsema and Hedrich 2005). This voltage drop across the plasma membrane activates voltage dependent K^+ channels of the GORK (Gated Outward Rectifying K^+ channel) type (Ache et al. 2000). Consequently, K^+ is released from guard cells together with the inorganic anions, (Ache et al. 2010) organic anions are decomposed and accompanying osmotic water efflux shrinks the guard cells. The plant hormone Abscisic acid (abbreviated as ABA) plays a key role in the stress-induced stomata closure especially in a drought condition.

1.3.3. ABA Biosynthesis and Signaling and its Role in Guard Cell function

The plant hormone ABA is also known as drought stress hormone and is ubiquitous in the plant kingdom (Finkelstein 2013). It is mainly formed in the vascular tissues (Finkelstein 2013), but also directly in guard cells (Bauer et al. 2013), and is involved in plant adaptation to unfavorable conditions. Higher land plants synthesize ABA from carotenoids (Endo et al. 2014). Genes belonging to ZEP (Zeaxanthin Epoxidase), AAO (Aldehyde Oxidase), NCED (9-Cis-Epoxycarotenoid Dioxygenase) gene families play an important role in ABA biosynthesis (Finkelstein 2013). *T. salsuginea* exhibits higher number of genes belonging to ZEP and AAO families than its wild relative *A. thaliana* (Wu et al. 2012). Conversion of carotenoids to ABA is a multi-step pathway which initiates in plastids and ends in cytosol. Different steps involved in conversion of ß-carotene to 9-cis-Violaxanthin take place in plastids which are mediated by ZEP and NCED gene families (Finkelstein 2013). Tan and co-workers showed that transformation of xanthophylls into xanthoxin by the enzyme NCED, is the rate-determining step (Tan et al. 2003). Xanthoxin is converted into ABA in further oxidation reactions which

occur in the cytosol. Conversion of xanthoxin to ABA occurs in two steps via ABA2 (ABA deficient 2), ABA3 (ABA deficient 3) and AAO3 (Abscisic Aldehyde Oxidase 3) genes. ABA can be stored in vacuoles and apoplast in an inactive form such as, ABA-GE (ABA- Glucose Ester). Arabidopsis operates two major pathways of ABA catabolism: (i) hydroxylation of ABA at the 8' position by P-450 type monooxygenases to give an unstable intermediate (8'-OH-ABA) that is isomerized to phaseic acid (PA), and (ii) by esterification of ABA to ABA-GE (Finkelstein 2013).. Thellungiella contains more genes than Arabidopsis belonging to CYP707A family. It has been shown that inactive form of ABA can also be reverted to active from via β-glucosidases (Finkelstein 2013; Lee et al. 2006). *Ataba-3* mutants, which show a clear wilting phenotype due to the lack of de novo synthesis of ABA, showed increased expression of the β-glucosidase AtBG1, indicating a compensatory function of this synthetic pathway for ABA de novo synthesis (Bauer et al. 2013). The reversible inactivation of ABA by conjugation with a glucose ester together with the guard cell autonomous de novo synthesis rapidly regulates ABA levels of the guard cells, thus ensuring an immediate response to environmental stimuli (Bauer et al. 2013; Munemasa et al. 2015).

Key components of the ABA signaling pathway in guard cells include ABA receptors, phosphatase, and kinases. In presence of ABA, ABA binds to the ABA receptors of the PYR (Pyrabactin Resistant) /PYL (PYR Like) family which together forms complex with phosphatases of the serine/threonine protein phosphatases of the PP2C (Protein Phosphatase C) family. Formation of this complex inhibits dephosphorylation activity of these phosphatases (Ma et al. 2009; Park et al. 2009). Expression analyzes showed that different PYR/PYL receptors are expressed in different tissues. In *A. thaliana* guard cells, mainly six (PYR1, PYL1, PYL2, PYL4, PYL5, and PYL8) of the 14 receptors are expressed, while PYL11-13 did not express strongly in any of the tissues examined (Gonzalez-Guzman et al. 2012). The simultaneous occurrence of several ABA receptors in the same tissue, as well as the differential expression of individual receptor, suggests a functional specification of the receptors (Gonzalez-Guzman et al. 2012). Despite the great homology, the ABA receptors have specific properties. For instance, AtPYR1 and AtPYL1-2 are homodimers in the cytosol, whereas PYL3-PYL10 are soluble monomers (Hao et al. 2011). Moreover, nucleus localization of PYR1 and PYL5 was also observed by (Santiago et al. 2009b; Park and Kim 2017). Interaction studies performed on ABA receptors with ABA and PP2C-A phosphatases showed different receptors have different affinity (Antoni et al.

2012; Tischer et al. 2017). The *A. thaliana* genome encodes 79 of these PP2C phosphatases, which can be classified into ten subgroups (A-J) based on sequence homology. At least six of the nine PP2Cs belonging to group A (ABI1, ABI2, HAB1 and HAB2, AHG1 and PP2CA / AHG3) are negative regulators in the ABA signaling pathway of guard cells (Schweighofer et al. 2004).

In the absence of ABA, the PP2CAs in turn dephosphorylate SnRKs (Sucrose non-fermenting-1-related protein kinases) family kinases thereby inhibiting them. SnRK2.6 kinase Open Stomata 1 (AtOST1) from *A. thaliana* was shown to be regulated by ABA and is involved in ABA induced stomatal closure (Mustilli et al. 2002). Furthermore, a physical interaction of OST1 and ABI1 was shown thus strengthening its role in ABA signaling (Yoshida et al. 2006). Complex formation of PP2CA phosphatases with the ABA receptors of the PYR / PYL family in the presence of ABA leads to a decrease in this interaction (Ma et al. 2009; Park et al. 2009) and thus a reduction in dephosphorylation of OST1 by the PP2Cs (Geiger et al. 2009; Vlad et al. 2009). This in turn increases the autophosphorylation activity of the kinases which eventually activates transport proteins in the guard cells, and hence modulates the stomata apertures. The activation of the SnRKs leads first to the activation of S-type anion channels such as SLAC1 (Slow Anion Channel-Associated 1). In 2009, a study in Xenopus oocytes demonstrated the activation of AtSLAC1 by AtOST1, which led to an efflux of both Cl^- and $NO3^-$ (Geiger et al. 2009). The S-type anion channel AtSLAH3 (SLAC1 Homologue 3) is also expressed in *A. thaliana* guard cells. However, in contrast to AtSLAC1, this has an increased conductivity for $NO3^-$ and unlike AtSLAC1, AtSLAH3 cannot be activated by AtOST1 but by NO_3^- itself (Geiger et al. 2011). Moreover, activation of both channels has been demonstrated by CPKs (Calcium-dependent Protein Kinases) (Geiger et al. 2011; Geiger et al. 2010). Other than SLAC / SLAHs, members of the ALMT family (Aluminum-activated Malate Transporters) also mediate an outward transportation of anions from guard cells. In addition to nitrate and malate, AtALMT12 which is expressed in *A. thaliana* guard cells, is also permeable to sulfate and, like AtSLAC1, can be activated in an ABA-dependent manner by OST1 (Imes et al. 2013). Aactivation of AtALMT12 via sulfate was also reported by (Malcheska et al. 2017). Finally, the outflow of anions leads to depolarization of the plasma membrane of guard cells, resulting in the opening of GORK (Gated Outward Rectifying K^+ channels), thus resulting in an immense efflux of K^+ (Ache et al. 2000). Water flows according to the water potential gradient, and the lower turgor of the guard cells causes them to collapse and hence stomatal closure.

Omics approaches have shown that *T. salsuginea* contains all key components for ABA signaling (Wu et al. 2012; Zhang et al. 2019). However, cell specific localization of ABA receptors and their interaction with phosphatases are lacking. In addition, signaling pathways for ABA induced responses are also scant in this halophytic plant species.

2. Aim of the Study

The aim of this study is to elucidate salt induced changes in salt sensitive and salt tolerant plant species under single and multiple times of salt exposures. These two salt conditions were opted to compare salt shock and salt stress induced changes in physiology of salt sensitive and salt tolerant plant species. For that, model plants from salt sensitive (*Arabidopsis thaliana* - Col-0) and salt tolerant (*Thellungiella. salsuginea* -Shandong) species were used. Selection of these cultivars were made based on their wild relatedness, where one has evolved as salt tolerant during evolutionary time while the other advanced as salt sensitive plant species. Along with salt induced changes in plant physiology, a special emphasis was given to observe salinity effects on the guard cell physiology of both plant species. Guard cells have been well characterized except under soil salinity. Moreover, guard cells of salt tolerant species have not been well documented. In the present study, we focus on guard cell responses under single and multiple times of salt exposures.

As guard cells are vital for environmental CO_2 uptake and hence photosynthesis and biomass production, therefore phenotypic and physiological parameters such as stomatal apertures, transpiration, CO_2 assimilation, guard cells photochemistry and plant water use efficiency were compared from both plant species. These characteristic of guard cells were further correlated with the whole plant phenotypic and physiological responses. Guard cell turgor regulations are largely dependent on potassium, nitrate, chloride and sugars. It has been proposed that guard cells can take up sodium and might use it as replacement of potassium for turgor regulations. This question was also addressed by measuring salt ions in the guard cells and the data were correlated with the transpiration rates. At molecular level, whole genome guard cells transcriptome analysis was performed to understand the molecular genetic mechanisms underlying membrane trafficking such as ion and water transport, compatible osmolyte and reactive oxygen species metabolism, photochemistry and energy related pathways in both plant species. Stress phytohormone, ABA, is vital for stomatal closure and stress adaptation. Salt induced ABA signalling in guard cells of both plant species was also investigated at physiological and molecular level to get insights into the possible roles of this phytohormone in salt tolerance. Finally, the comparisons between and within both plant

species at guard cell level have been made at molecular and physiological level to understand guard cell functioning under single and multiple times of salt exposure in both plant types.

3. Material and Methods

3.1. Plant Species and Growth Conditions

Wild type, *A. thaliana thaliana* plants of ecotype Columbia (Col-0) and its wild relative *T. salsuginea salsuginea* also known as *Eutrema salsugineum* (formerly known as *T. salsuginea halophila*) of Shandong ecotype, were used in the salt stress study. The whole experiment was performed on soil (from Einheitserde P, Germany). Plants were illuminated for 12 hours (10 A.M. to 10 P.M.) with a light intensity of 80-110 µmol s^{-1} m^{-2} using fluorescent lamps from Osram (L58W / 77 FLUORA; Munich, Germany) and Philips (TLD 58W / 840; Hamburg, Germany). The temperature was constantly held at 22°C and 16°C during the light and dark periods, respectively whereas relative air humidity was maintained at 60% all times.

Autoclaved soil was filled into the 100 ml pots having a dimeter of 7 cm. Seeds stored at 4^0C were sowed on soil and were grown under well-watered conditions for 2.5 weeks. Afterwards, seedlings were separated into single pots containing 91 ± 7 gm of autoclaved soil. Trays having the following dimensions of length=28 cm, width=18 cm and height=4.5 cm were used to keep the plant pots. Each tray could fit 11 plant pots. Plants were cultivated for 5-6 weeks (*A. thaliana*) and 7 weeks (*T. salsuginea*) under unstressed conditions prior to the start of the experiment.

3.2. Salt Experiment

Salt treatments were carried out under similar growth conditions as explained in section 3.1. Plants were exposed to short and long-time saline conditions which lasted for three and fifteen days respectively. Two approaches for long-time saline conditions were opted which were termed as “gradually increase saline conditions” and “constant high saline conditions”. In parallel, plants were cultivated on non-saline conditions throughout the experimental period as a control group. Four groups of treatments were carried out as follows:

a) **3x salt (gradually increase saline conditions):** For prolonged salt treatment 5-6 weeks old *A. thaliana,* and a 7 weeks old *T. salsuginea* plants were used. Being a glycophyte, *A. thaliana* encounter growth problems at salt conditions higher than 150 mM (Gong et al.

2005). Therefore, toxic saline conditions were obtained in steps to observe physiological changes. The tray containing 11 pots was at first filled with 1 L of 75 mM of NaCl solution on two successive days (day one and two of the experiment) for one hour each day. On the 3rd day, 90 mL of tap water per pot was poured on top of the soil to remove salts from the soil. This step was termed as 'washing' of the soil. Water was added slowly so that it had enough time to spread and run through the soil to be discarded. Till this point, the experiment was named as, 'First Phase' of the salt experiment (fig. 3.1). Next, the plants were grown from day 3 to 6 without any treatment. On day 7 and 8, the same plants were incubated in 150 mM NaCl for 1 hour each day (similarly as described before). On day 9, again soil 'washing' was performed like in phase one. This was termed as, 'Second Phase' of the salt experiment (fig. 3.1). After soil washing, plants were further grown without any treatment until day 12. On day 13 and 14, plants were incubated in 200 mM NaCl solution for one hour each day (similarly as described before). On day 15 samples were collected. Experimental time from day 13 to 15 was termed as 'Third Phase', of the salt experiment (fig. 3.1).

b) **3x salt (constant high saline conditions):** In this treatment, plants of the similar age (as in the previous section) were subjected to constantly high saline conditions for 15 days. In all three phases of the salt experiment, these group of plants were treated with 200 mM NaCl along with two washing steps (fig. 3.1). Salt and washing treatments were performed the same way as in the previous section.

c) **1x salt (single saline conditions):** Another group of salt treatment was performed under single saline conditions to distinguish the effects of multiple salt exposures to plants. For that plants of the similar age were irrigated in parallel to second and third phase of salt experiment with 150 mM and 200 mM NaCl respectively (fig. 3.1). At the end of each experiment phase samples were harvested.

d) **(-) salt (non-saline conditions):** Another group of plants experiencing no salt during their growth were grown in parallel to the salt treated groups. Irrigation and washing steps were performed with tap water on the same days as of the salt treated groups (fig. 3.1).

Other than transcriptomic analysis, all analyses were carried out on light adapted plants. For that samples were harvested after 4 hours of light period. Altogether, these saline conditions were opted to get insights into the short and long-term saline effects on plants

as a whole, and on guard cells as a specific. Characterization of both plant species was performed at physiological, molecular, chemical, biochemical and phenotypic levels.

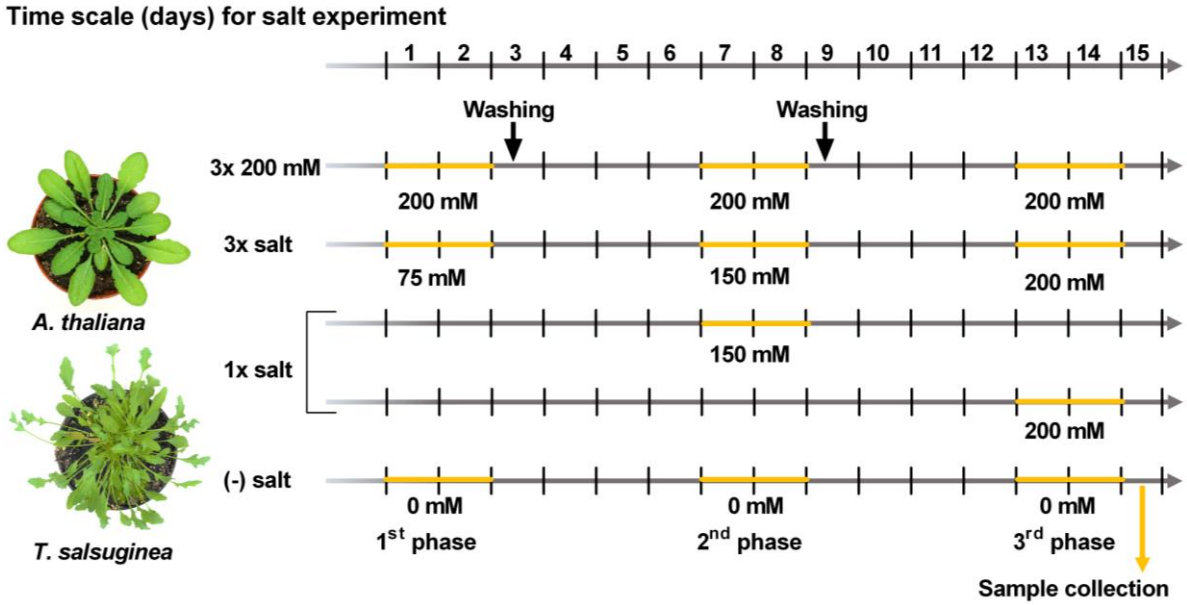

Figure 3.1. Salt treatment protocol. Both plant species subjected to various saline conditions are described here along with the time scale. Duration for each salt treatment experiment is defined in terms of days.

3.3. Guard Cell Extraction

Guard cell extraction was performed by a blending method (Bauer et al. 2013; Brosche and Mikael 2017). Mature leaves from two to three plants were used to get one guard cell enriched sample. Leaf blades were separated from petioles and midribs. The leaf blades were put in the mixer (MultiQuick 5, Braun, Germany) with approximately 300 mL of desalted water along with a hand full of crushed ice. The plant material was blended for one minute at the highest speed. The blended material was then poured through a nylon net having pore diameter of 210 µm . This step was performed slowly so all the material is collected at one place on the membrane. The collected material was transferred to the cup again by placing nylon net upside down on top of the cup followed by washing with desalted water (300 mL). A hand full of ice was added into the blender followed by blending. Altogether, blending was performed three times that finally yielded light green epidermal fractions onto the nylon net. Collection of these fractions from the nylon net was performed first by drying the excessive water with the paper towel and then scraping the sample with spatula. Samples were

collected in 1.5 mL tubes and were immediately frozen in liquid nitrogen for until further processing.

A part of the sample was used for microscopic analysis to observe guard cell enrichment. For that, different guard cell samples were stained with 0.01% neutral red having pH of 7.5 (AppliChem GbmH, Germany). After 30 minutes of incubation at room temperature, the samples were extensively washed with tap water to get rid of unbound staining dye from the samples. After washing, the samples were observed under microscope (BZ-Analyzer, Keynce, Germany) at 20x magnification for validation of intact guard cells.

Gurad Cells, Cell Walls and Cytoplasm Extraction in Presence of Exogeneous ABA : In a separate experiment, guard cells were isolated in presence of exogeneously applied ABA. For that a known concentration (10 ng/g FW) of ABA isotope $^{13}C_2$-ABA (S-(+)-ABA $^{13}C_2$, Qmx Laboratories Ltd, Essex, UK) was added to the ice water and guard cells from *A. thaliana* leaf samples were extracted by blending method. Ice water (washing solution) used for guard cells extration was also collecetd. It was further centrifuged at 4 ^{0}C at 3000 rpm in the centrifuge machine (J-20 XP, BECKMAN COULTER, USA) to segregate cell debris from the washing solution. Supernatant (washing solution without plant material) was transferred into the plastic bottles (soft polyethylene bottles- A. Hartenstein, Germany) and were stored at 4 ^{0}C until farther processing. Plant debris collected after centrifugation of guard cells washing solution was frozen in liquid nitrogen immediately and stored at -80 ^{0}C until further use.

Some of the guard cell samples isolated in presence of exogeneous ABA were additionally processed for separation of cell walls and cytoplasm. For that, frozen guard cell samples were placed into the pre-chilled metal cups along with metal balls provided by the manufacturer (Retsch, Germany). Cups were then fitted into the tissue lyzer (Vibration mill MM 2000, Retsch, Germany) and ground into fine powder by incubating at frequency of 30 Hz for three minutes. A volume of 15 mL deionized water was poured into the ground powder and the solution was transferred into 50 mL plastic centrifuge tubes (Eppendorf, Germany) followed by centrifugation at 4000 rcf at 4^{0}C in the centrifuge machine (5810 R, Eppendorf, Germany). Supernatant and cell debris were separated and stored at -80 ^{0}C untill further use. Confirmation of cell wall material was performed by using 0.01% neutral red staining solution.

3.4. Plant Phenotypic Parameters

3.4.1. Plant Survival Rate

Survival of the plants was defined by us in terms of successfully setting seeds hence transferring information to the successive generation. At the end of salt experiment, plants undergone saline conditions were further cultivated on non-saline conditions to observe seed setting. Survival percentage was calculated by using the following formula:

Survival percentage= (seed setting plants/total number of plants) * 100

3.4.2. Stomatal Density

Stomatal density of the plants was calculated by taking the imprints of the leaf stomata onto a thin film. Transparent gel nail polish (DW 150, Jade, France) was applied onto the upper and lower leaf surface followed by drying at room temperature for about 30 minutes. After drying, the layer of polish was carefully removed by using thin edged forceps. The imprints were inspected under the microscope (Axioskop 2, MOT Plus, ZEISS, Germany) and images were taken at 1000X magnification. Image J, a freely available image processing program (https://imagej.nih.gov/ij/download.html), was used for stomata count. Stomata were calculated for each picture and data were expressed in terms of stomata count per square millimeter.

3.4.3. Stomatal Apertures

Intact plants have been used in the present study to measure size of stomata. Leaves of the plants cultivated under different saline conditions were visualized upside down under the microscope (Axioskop 2 MOT Plus, ZEISS, Germany) at 1000 X. Keeping in mind that immature guard cells do not close their stomata, only mature guard cells were used for the calculation of stomatal apertures. Scale bar calibration was performed using 1 mm stage micrometer. Microscopic images were processed in ImageJ as described by the manufacturer for stomata size measurements.

3.4.4. Plant Surface Area

Surface area of the plants belonging to different groups of salt treatments were obtained by scanning. Leaves were separated from plant rosette and were scanned in scanner (MX-M362N- SHARP, UK). The scanning was performed in a way that none of the leaves overlap each other. Scanned images were processed as described by the manufacturer in ImageJ (https://imagej.nih.gov/ij/) to determine area.

3.5. Quantification of Chemical and Biochemical Parameters

3.5.1. Ions in Soil and Leaf Samples

Fully mature leaves taken from the plants were washed thoroughly in ultra-pure water to remove any dust/soil and were dried at 65 celcius (^{0}C) for 1 week. Leaf samples were ground into powder by using chrome–steel beads in the tissue lyser (Tissue lyzer II system, Qiagen, Germany) at frequency of 30 Hz for 1 minute. Samples were weighed and 0.5 M HNO_3 was added into each sample tube for extraction of ions. The samples ranging from 20-50 mg of dry weight (DW) were treated with 5 mL of 0.5 M HNO_3. The volume of the acid was increased accordingly to the increasing weight of the samples. The samples were incubated at room temperature overnight for the extraction of the ions followed by incubation at 90 ^{0}C for 3 hours on the following day. After acid digestion, the samples were centrifuged at 4000 relative centrifugal force (rcf) for 20 minutes in the centrifuge machine (5810 R, Eppendorf , Germany). Supernatant was transferred into the new tubes and diluted accordingly for the Na^+ and K^+ measurements in the flame photometer (PFP7, JENWAY, UK).

Soil samples collected at the end of the experiment were dried at 90 ^{0}C for 1 week. Ion extraction was performed with 1 M ammounium acetate as described by the manufacturer JENWAY (Scientific 2005) and (Simard 1993). For that, 40 mL of ammonium acetate was added into 2 g of dried soil and the samples were incubated overnight at room temperature. Addition of exceesive $NH4^+$ in the soil sample will displace the exchangeable alkali and alkali cations from the exchangeable sites of soil particles. After ammonium acetate treatment the slurry phase soil was separated by centrifuging at 4000 rcf for 20 minutes in centrifuge machine (5810 R, Eppendorf, Germany). Supernatant was transferred into new 50 mL clean tubes and diluted accordingly for ions determination using a flame photometer (PFP7, JENWAY, UK). Standard solutions (1 mM, 0.2 mM, 0.1 mM, 0.05 mM, 0.02 mM) for Na^+ and K^+ were prepared by using NaCl and KCl salts (provided by AppliChem GmbH, Germany). These solutions were

used to obtain standard curve that was then used for the calculation of respective ions' concentrations in the samples. After measuring of every five samples, new stanadrad curve was generated for accurate measurements as suggested by the manufacturer.

3.5.2. Ions in Guard Cells Samples

Elements (Na, K and Cl) were determined from open and close stomata of the plants from the salt stress experiments by using Energy Dispersive X-Ray Analysis (EDXA). For open and close stomata plants were exposed to light and dark-adapted conditions as explained in section 3.2. Leaf samples were collected, washed quickly in ultra-pure water, transferred to petri dishes filled with slush ethanol. Slush bath was prepared using ethanol as described in (Rondeau 1966). Leaf samples were further incubated for freeze drying in the lyophilizer (Alpha 1-2 LDplus, Martin Christ, Germany) for 48 hours at -48 ^{0}C and 0.014 mili bar (mbar) atmospheric pressure. Freeze dried samples were used in EDXA for quantification of elements (Na, K and Cl) as described by (Fromm et al. 1987).

3.5.3. Abscisic Acid

Phytohormone Abscisic acid (ABA) was quantified from guard cells, washing solution, cell debris, cell walls (see section 3.3) and leaves by using triple quadrupole Liquid Chromatography-Mass Spectrometry (LC-MS). Supernatant solution (collected during guard cell extraction and cell wall preparations) was first frozen at -80 ^{0}C followed by freeze-drying (Alpha 1-2 LDplus, Martin Christ, Germany) to avoid any material loss. After freeze-drying, bottles were washed thoroughly with 15-20 mL of organic solvent (methanol) to collect the phytohormone followed by vacuum drying at 50 ^{0}C in the speed vacuum (Type 2-18 CD- Christ, Osterode, Germany). Other samples i.e freeze dried guard cells, leaves, cell walls and plant debris were ground into fine powder and DW for each sample was recorded. Samples were further processed for the extraction of ABA. A volume of 950 µL of extraction solvent (ethyl acetate: formic acid (99:1 v/v)) solution was added into each sample and mixed vigorously until the complete suspension of sample in the solution. ABA internal standard solution ((D_4) ABA 1 ng/ µL dissolved in acetonitrile) was added (50 µL) to the samples as a control followed by mixing in the tissue lyzer (Tissue lyzer II system, Qiagen, Germany) for 3 minutes at frequency of 20 Hz. Samples were centrifuged afterwards for 10 minutes at room temperature

at 14000 rpm (miniSpin Plus, Eppendorf, Germany). Supernatant was transferred into new tubes followed by complete drying of the supernatant solution in speed vacuum (Type 2-18 CD- Christ, Osterode) at 40-50 ^{0}C temperature. After drying, the material was resuspended in 40 µL of acetonitrile: ultra-pure water (1:1 by volume) by vigorous mixing on vortex machine for 10 seconds followed by sonication for three minutes. This promotes a more efficient extraction by producing cavitation caused by ultrasonic waves that may facilitate transfer of compounds from the tissue into the solvent. Samples were then centrifuged for 10 minutes at room temperature at 1400 rpm (miniSpin Plus, Eppendorf, Germany) to collect the supernatant and stored at -20 ^{0}C until ABA quantification.

3.5.4. Plant Pigments

Plant pigments such as chlorophyll a, b, carotenoids and anthocyanins were quantified from the freeze-dried leaf samples. Leaf samples collected from the salt stress experiment plants were washed in water thoroughly to get rid of any dust/soil particles that may lead to false quantification of pigments. Samples were immediately frozen in liquid nitrogen followed by freeze drying as explained in section 3.4.2. The samples were ground using chrome–steel beads in the Tissue lyzer II system (Qiagen, Germany) at frequency of 30 Hz for 1 minute to get fine powder. Chlorophyll pigments were extracted from the protocol described in (Misyura et al. 2012). The samples were weighed and an 80% acetone:20% water solution was added to the samples and mixed vigorously on vortex mixer to re-suspend plant tissue. Chlorophylls are hydrophobic in nature and therefore can be extracted from plant material by using organic solvents which will take up water and hence allowing these pigments to be extracted (Lichtenthaler and Buschmann 2001). The extraction was performed with 1.5 mL of acetone solution () for 2-3 times or until the extraction solution was transparent (not green anymore). Extracted (80%) solution was centrifuged at room temperature at 14000 rpm (miniSpin Plus, Eppendorf, Germany) for 10 minutes. Supernatant was transferred into new tubes and a subsample of 250 µL of the extract was transferred to 10 mm light path glass cuvette (QS-104-0S, HELLMA, Germany) along with 750 µL of fresh acetone solution (80%) for pigments quantification by using UV-VIS spectrophotometer (U-1500, HITACHI, Japan). The absorbance values were taken at 480, 645, and 663 nm wavelengths. A volume of 1 mL of 80% acetone solution was used as blank for each wavelength. The absorbance values were put into the following formulae to calculate the desired pigment concentrations.

Chlorophyll a (µg/mL) = (12.25 * A663.2) – (2.58 * A646.8)

Chlorophyll b (µg/mL) = (21.50 * A646.8) – (5.10 * A663.2)

Carotenoids (µg/mL) = (100 * A470 -1.82* C_a – 85.02* C_b)/198

Where 'A' stands for the absorbance value at given wavelengths and C_a and C_b represent values for chlorophyll a and b respectively. Data represent in mg/g DW.

Anthocyanin extraction was performed by using 1% acidic methanol as described in (Feinbaum and Ausubel 1988; Heijde et al. 2013). Sample collection and drying was performed similar as described earlier for chlorophyll and carotenoids. Anthocyanins are soluble in organic solvents and water. Therefore, in the present study acidic methanol (1% acidic) was used and 300 µL of this solution was added to each sample followed by vigorous mixing to suspend plant material in the solution. Mild acidic conditions are helpful for the stability of anthocyanins. Samples were kept overnight in dark at 4 ^{0}C for anthocyanin extraction. After incubation, 200 µL ultra-pure H_20 along with 500 µL of chloroform was added into each sample followed by centrifugation for 5 minutes at 14000 rpm (miniSpin Plus, Eppendorf, Germany). Addition of chloroform is helpful for separating anthocyanins from chlorophylls. Supernatant was transferred into new tubes and equal amount of 60% acidic methanol (prepared in ultra-pure water) was added into each tube. 320 µL of this solution was mixed in 480 µL of 1% acidic methanol (final volume 800 µL) and the absorbance at 530 nm and 657 nm was taken by using UV-VIS spectrophotometer (U 1500, HITACHI, Japan). A mixture of 480 µL of acidic methanol and 320 µL of ultra-pure water was used as blank. Anthocyanin was quantified from the equation $A_{530} - 0.33*A_{657}$ (where A= absorption value) as described in (MacLeod et al. 2015). Subtracting absorption values of 657 nm wavelength from the values at 530 nm would decrease the chance of any possible contamination of chlorophylls.

3.6. Determination of Physiological Parameters

3.6.1. Total Plant Water Content

Total plant water content was quantified by obtaining fresh and dry weights of the plants. Plant rosette was harvested and immediately weighed on the electric balance (FX-40, A&D Electronic, USA) to obtain the fresh weight (FW). The rosette was then incubated at 37^{0}C

for 2 weeks for complete drying of the plant material. Samples were weighed in between for precautionary measures to obtain constant dry weight. This ensures complete evaporation of the water from sample. Dry weights (DW) were recorded by using the electric weighing balance (FX-40, A&D Electronic, USA). Following equation was used from (Turner 1981) to get the total plant water content (in %).

$$\text{Plant water content \%} = (FW\text{-}DW/FW) * 100$$

3.6.2. Osmotic Potential

Salt and non-salt treated plants were analysed for osmotic potential. Two different approaches were performed to determine the osmotic potential i) use of dry weight and ii) use of cell sap. Both approaches were compared to have most reliable results. In the first approach, leaf samples from the salt stress experiment were collected, washed completely in ultra-pure water and were freeze-dried under similar conditions as described in section 3.4.2. Freeze drying was preferred over heat drying to avoid any possible degradation of the osmolytes. Plant material was ground to powder (as explained in section 3.4.3) and equal dry weight was taken for each sample. The samples were then suspended in equal volumes of ultra-pure water followed by centrifugation at 14000 rpm (miniSpin Plus, Eppendorf, Germany) for 10 minutes. Supernatant was collected and 10 µL of this solution was placed in the osmometer (WESCOR-VAPOR-5520) and values for the osmolytes were recorded for each sample.

In the second approach, leaves collected from the plants were first cleaned by rinsing in ultra-pure water followed by drying using paper towel. These steps were performed as fast as possible to avoid any stress. Leaves were placed in the 5 mL syringe (PRIMO) and the sample was pressed to get the cell sap. Collection was performed in 1.5 mL tubes. 10 µL of the sap was placed in the osmometer (VAPRO-5520, WESCOR, USA) and osmolarity was recorded for each sample. Osmometer was first calibrated with the known osmolarity solutions provided by the manufacturer before measuring the samples. These values of osmolarity were converted to osmotic potential as described in (Harris-Valle et al. 2018) by using van't-Hoff equation as follows:

$$\Psi s = CiRT$$

Where Ψs stands for osmotic potential, C_i refers to osmolarity, R for gas constant and T for temperature in kelvin (K). The calculations were determined in Mega-Pascal (MPa).

3.6.3. Transpiration Rates and Photosynthesis Parameters

Gas exchange: Infra-Red-Gas-Analysers (IRGA) (LI 7000; Li-Cor, Lincoln, NE, USA) were used to detect the amount of water vapours in the air stream. Soil transpiration was avoided by covering the soil surface with water tight plastic foil. Gas composition (1 L/min) was adjusted and controlled by mass flow meters from red-y-smart series (ww.voegtlin.com). Relative air humidity was set to 50%, environmental temperature to 20 ^{0}C and CO_2 concentration to 400 ppm for each cuvette. Illumination was provided by white LEDs (Cree Xlamp CXA2520 LED) and the light beam with photon flux density of 125 µE was directed to the cuvette by two fibre-optics (Fiber Illuminator FL-460; www.walz.com). Illumination was provided in the gas exchange machine at the same time as the plants used to have during experimental period (i.e. 10 am). Before exposing to light, the base line for each plant was obtained by providing dark condition to reach stable values. After achieving the base line, the plants were exposed to light conditions (125 µE) for the next 1.5 hours. In the following, again base line was obtained by providing the dark condition. To observe light response curves, plants were subjected to different light intensities i.e. 100, 200, 400, 600, 800, 1000 and 1250 µE. Values were recorded at each light intensity for 30 minutes.

Intrinsic Water Use Efficiency (WUE_i): At the end of the experiment, plants were scanned, surface area was calculated as described in section 3.4.4. Transpiration and CO_2 assimilation rates were normalized to surface area. Plant intrinsic water use efficiency (WUEi) was calculated by using transpiration and CO_2 assimilation rates as described in (Polley 2002; Tambussi et al. 2007).

3.6.4. Pulse-Amplitude-Modulation (PAM) Fluorometry of Guard Cells

Chlorophyll a fluorescence excitation of PSII of guard cells of salt and non-salt treated plants was analyzed using microscope-based Pulse Amplitude Modulation (PAM). This fluorescence-based method is useful to analyse photochemical and non-photochemical quenching of the photosynthetic cells. Maximum/optimal quantum yield is the efficiency of PSII when all the reaction centres are opened. This is a sensitive indicator for measuring the

photosynthetic performance of plants. Isolated guard cells (see section 3.3.1) were resuspended in about 100 µL of 10 mM MES/Tris pH 6.1, 10 mM KCl, 2.5 mM NaHCO3 buffer as mentioned in (Goh et al. 1999) followed by dark incubation for 20 minutes. Guard cells resuspended solution was then transferred to the glass slide using pipette. A 40-fold water objective lens of the microscope (Axioskop 2 MOT, Zeiss GmbH) was used for focusing a guard cell pair isolated from other cell components such as trichomes or adhering chloroplasts. The essential components of the microscopy–PAM fluorometer were the PAM control unit (Heinz Walz GmbH, Effeltrich, Germany), a blue light-emitting diode (LED; type NSBG 500, Nichia, Tokyo, Japan) serving as a pulse-modulated measuring light source, and a miniature photomultiplier (type PC/PM-MC,Walz). The modulated blue LED substitutes for the usual xenon arc excitation lamp of the epifluorescence microscope. Its light is filtered through a blue filter (BG 39, Schott, Mainz, Germany). It was focused via the collimator optics and the objective lens of the microscope on the sample plane. The photomultiplier was mounted on top of the photo-adaptor of the microscope where an ocular lens (10 x) and an iris diaphragm were installed. The excitation light derived from a blue light-emitting diode (LED) was reflected by a dichroic beam splitter into the objective lens and focused on the sample plane. The red chlorophyll fluorescence collected by the same objective lens was directed via the ocular lens towards the photomultiplier detector. It passed a second dichroic filter and an iris diaphragm in the focal plane of the ocular as well as a filter (RG-645) in front of the photomultiplier. The pre-amplified signal was further processed by the pulse amplitude modulation (PAM) control unit which also controls the pulse program of the blue excitation beam. The system was operated, and data were acquired/analysed under control of a Pentium personal computer and the wincontrol software. By means of diaphragm, the guard cell pair was focused to a blue light emitting diode. Before the beginning of the exposure according to the PAM protocol (as described in Goh et al., 1999), an extra 10 minutes dark adaptation took place. The normal chlorophyll fluorescence (F) and the maximum achievable fluorescence (Fm or Fm') on saturation pulses were monitored over a span of 04:40 minutes. The light intensity of the blue light beam was determined using the Universal Light Meter (ULM-500, Walz, Gcermany) and an LS-C sensor with a diameter of 3 µm.

The first recorded value of a typical PAM protocol is the maximum quantum yield or optimal quantum yield (Fv/Fm). This is obtained at the start of the experiment (0 minutes),

with a saturating blue light pulse ((SAT of 225 µE intensity) of 0.8 second to a pre-dark-adapted pair of guard cells and maximum florescence (Fm) was recorded. Variable florescence (Fv) was obtained using formula i.e Fv = Fm-F0. Where, F0 is the basic fluorescence in the dark before starting the measurement. The ratio between maximum and variable florescence will give rise to quantum yield. After 40 seconds actinic light having an intensity of 65 µE was switched on permanently and a SAT pulse was given after every 20 seconds during the experiment. From this time point on, all of the measured fluorescence values of each saturation pulse representing the maximum fluorescence in light (Fm ') and the fluorescence emission of the actinic light (F) were calculated as Effective Quantum Yield according to the formula Y = (Fm'-F) / Fm'. Furthermore, Non-Photochemical Quenching (NPQ), was also calculated to determine the non-photochemical quenching of fluorescence by heat release as follows.

$$NPQ = (Fm-Fm') / Fm \ .$$

3.7. Gene Expression Analyses

3.7.1. Isolation of RNA

Frozen plant material was first ground into very fine powder by steel milling balls using the Tissue lyzer II system (Qiagen, Germany) at a frequency of 30 Hz for 2 minutes. RNA kit from NucleoSpin was used and the manufacturer instructions with minor modifications were followed to extract the RNA. 10 µL of tris(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) an RNA stabilizer was added in 1 mL of RA1 lysis buffer. 700 µL of this mixture was added to each ground sample and was vortexed vigorously to suspend the sample in the buffer. Samples were then centrifuged at 11000 x g for 1 minute. Supernatant was transferred into NucleoSpin column (Violet ring) and was centrifuged at 11000 x g for 1 minute. Flow through was transferred into a clean 1.5 mL tube and equal volume of 70% ethanol was added followed by vigorous vortexing for 20 seconds. This mixture was then transferred into the RNA binding column (NucleoSpin RNA Plant Column (light blue ring) followed by centrifugation at 11,000 x g for 30 seconds. Flow-through was discarded. Afterwards washing was performed onto the binding columns by using two types of buffers provided by the company. 200 µL of buffer RAW2 was added into the binding column and the column was centrifuged for 30 seconds at 11,000 x g. Flow through was discarded. Then buffer RA3 was used for next two washing, first

with 600 µL and then with 250 µL. Each time centrifugation was done for 30 seconds at 11,000 x g followed by discarding the flow through. Columns were dried first by centrifuging at 11,000 x g for 1 minute and then incubating with open lids at 37 ^{0}C for about 15 minutes. 25-35 µL of RNAse free water (provided by the manufacturer) was added into the dried RNA binding column and placed at room temperature for 1 minute. Afterwards, RNA was collected into the clean 1.5 mL tubes (provided with the kit) by centrifuging at 11,000 x g for 1 minute. RNA concentration was detected by using nanodrop (2000 c- UV-VIS spectrophotometer, Thermo Scientific, USA). A quality score of ~2.0 was used for optical density at 260/280 and 260/230 absorbance ratios. For each treatment 8-10 samples were collected for RNA extraction.

3.7.2. Reverse Transcription

cDNA (complementary DNA) synthesis from mRNA was performed according to the protocol described in (Biemelt et al. 2004). Prior to cDNA synthesis, DNase digestion was performed to get pure RNA thus avoiding possible contamination of the genomic DNA. For that, 2.5 µg of the RNA was used in combination with 1 µL DNAse 1 (10 units/ µL), 0.5 µL RNAse inhibitor (40 units/ µL) and 2 µL of 10X DNAse buffer (all ingredients provided by Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and final volume of 20 µL was achieved by adding DEPC water. Final reaction volume of 20 µL was prepared and incubated at 37 ^{0}C for 30 minutes in the incubator provided by Eppendorf (Thermomixer 5436). After DNAse digestion, cleaning of RNA was performed by using isopropanol and ethanol. In the first place, isopropanol (70 µL) (AppliChem GmbH, Germany), 10 µL glycogen (20mg/mL) (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and 10 µL of 5 mM ammonium acetate was added in each sample. The samples were centrifuged at 14000 rpm (5810 R, Eppendorf, Germany) and at 4 ^{0}C for 20 minutes. After centrifugation, the supernatant was removed carefully without disturbing the pellet. A volume of 500 µL of 70% ethanol was added into each sample and centrifuged again at 14000 rpm and at 4 ^{0}C for 15 minutes. Without disturbing the pellet, supernatant was discarded followed by pellet drying at 37 ^{0}C for next 15-20 minutes in the incubator. A volume of 10 µL of RNAse free water (provided along the kit) was added into each sample to dissolve the pellet.

cDNA synthesis was performed with 2 µg of RNA template for 10 µL reaction volume. Volume of 0.4 µl Oligo-dT-Primer (100 µM), 0.5 µl dNTPs (10 mM), 2 µl RT-Puffer (5x, Promega,

Germany), 0.5 µl RNAase inhibitor). The mixture was then incubated for 2 minutes at 70°C followed by cooling on ice. In the following, 0.4 µl MMLV-RT reverse transcriptase (100 U/µl, *Maloney Murine Leukaemia Virus Reverse Transcriptase*, Promega, Mannheim, Germany) was added to the sample, which was then incubated for 1 hour at 42 °C. After mRNA denaturation the reverse transcriptase MMLV-RT generated a single-strand cDNA which was used for either qRT-PCR reaction or stored at -80°C.

3.7.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

All qPCR measurements were performed with the Mastercycler RealPlex (Eppendorf, Hamburg, Germany) in a reaction volume of 20 µl. For each reaction, 10 µl SYBR-Green (ABsolute QPCR SYBR green capillary mix, Thermo Scientific) and 2 µl cDNA template were used. Volume of 8 µl of HPLC-H2O dissolved with forward and reverse primer (final concentration 0.3 µM for each primer) was added to the reaction mixture. The PCR program initially included a denaturation step of 15 min and a melting curve measurement at the end. The standard 3-step PCR program was extended by one step by measuring the fluorescence at 79 °C to denature any primer dimers and small non-specific low melting point PCR products. Finally, by gradually increasing the temperature from 79 ° C to 95 °C, the melting temperature of the PCR product was determined (Table 1.1).

Transcripts of a gene of interest were determined by using standard curve for the gene of interest. The starting materials for the standard series were PCR products purified according to the manufacturer's instructions using the DNA purification kit (Quiagen, Hilden, Germany). The concentration of the purified product was determined spectrophotometrically anodrop 200c, Thermo Fisher Scientific). The template DNA was diluted from the highest concentration of 10fg / µl in a 1:10 dilution series to the lowest concentration of 0.01 fg / µl with tRNA-H2O. To avoid binding of the cDNA to the walls of measuring vessels and to adjust its concentration to the measuring buffers, the synthesized cDNA was diluted twenty-fold with H_20 tRNA, i.e. HPLC H_2O (Carl Roth GmbH, Karlsruhe, Germany) was supplemented with 10 ng/µl tRNA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The standards and samples were measured simultaneously in the same 96 well plate for each gene of interest. By means of the standards the Ct of the measurements could be converted into fg DNA. Since the fragment length of the amplicons and the weight of DNA were known (Giulietti et al. 2001)

1 µg of 1000 bp DNA = 9.1x 10^{11} molecules.

The initial number of molecules could be calculated in a PCR reaction. Since the cDNA concentration varies from sample to sample, all results were related to 10,000 molecules of the respective housekeeping gene. Two actins (ACT2/8) from *A. thaliana* were used as housekeeping gene: *AtActin2* (AT3g18780)/ and *AtActin8* (AT1g49240), whereas *TsActin2* (Thlg0010987) of the *T. salsuginea* was used as housekeeping gene. As the transcripts of gene of interests were normalized to housekeeping gene therefore equal volumes of the serial dilutions of the housekeeping genes were used along with the samples and standard curves were generated which was then used for the quantification of transcripts.

Table 1.1: Program used in the present study for qPCR analysis

Temperature	Duration	Function	Number of cycles
95 °C	15 min	Denaturation	01
95 °C	15 sec.	Denaturation	
Tm	20 sec.	Primer-annealing	
72°C	20 sec.	Amplification	40
79°C		Fluorescence measurement	
95	5 sec.	Denaturation	01
79-95°C	0.3 °C/sec.	Fluorescence measurement	01
40°C	30 sec.	cooling	01

3.7.4. Primer Design

All primers used for *A. thalaina* gene expression were designed using the Oligo7 Primer Analysis Software (Rychlik 2007) and synthesized from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). Primers used for *T. salsuginea* were designed at NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity and optimal annealing temperature of newly designed primers was tested by temperature gradient PCR (reaction mixture as explained in section 3.6.3) (annealing temperature range of 48 °C to 65 °C) and the PCR products by melting point analysis and DNA agarose gel electrophoresis (see 3.7.5). In

addition, the amplicon was sequenced according to Sanger (GATC Biotech AG, Konstanz, Germany).

3.7.5. DNA Gel Electrophoresis

PCR products were examined by gel electrophoresis to finding annealing temperatures for designed primers. In most cases PCR product was smaller than 400 bp, therefore 2% agarose gel (prepared in W/V ratios of of agarose and 1x TAE buffer solution) containing 0.5 µl / 100 ml of the fluorescent dye GelGreen (GelGreen Nucleic Acid Stain, Biotium, Hayward, California, USA) was used. DNA bands could be made visible by the excitation with UV light (254-366nm) using the gel imager IX 20 (Intas, Göttingen, Germany). The marker used was a 100 bp provided by Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA.

3.7.6. Statistical Analysis

Statistical analysis was performed in OriginPro 2017. Variance in the mean values of all populations belonging to different salt treatments were found by Analysis of Variance (ANOVA). For that, one-ways or two-way ANOVA was uses (depending on the variables). Significance differences between the variance of different population groups were found out using multiple comparison analysis by Bonferroni test. All data shown are mean ± SEM. Different letters show significance (P-value ≤ 0.05) between values as determined by Bonferroni test.

3.8. Transcriptomic Microarray Analysis

For whole genome transcriptomic analysis microarray hybridization was used. *T. salsuginea* guard cells microarray analysis was performed at Oak labs (https://www.oak-labs.com/) where 60-mer oligonucleotide probe arrays (44 K) from Agilent was used (Lee et al. 2013). *A. thaliana* guard cells transcriptomic analysis was performed at the Department of Biochemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg, where GeneChip® arrays (Agilent) was used for the analysis.

Guard cell samples were collected from light and dark-adapted plants from the third phase of the salt experiment (see section 3.2). Guard cell samples were collected from (-) salt, 1x salt (only 200 mM) and 3x salt treated group of plants of both species. For light adapted

conditions, plants were kept in light (similar conditions as explained in section 3.1) at the beginning of light period for four hours (from 10 am to 2 pm). Whereas, dark-adapted conditions were achieved by initially exposing plants to light for two hours (from 10 am to 12 noon) followed by two hours of complete darkness (from 12 noon to 2 pm). All conditions were supplied in the growth cabinet where plants were grown during the whole experiment (section 3.1). Four guard cell samples were collected from each treatment under each condition (i.e light and dark), hence total 24 samples were harvested from each plant for the microarray analysis.

Data pre-processing was performed using the Bioconductor software (Huber et al. 2015) with the statistical programming environment R (R Development Core Team 2011). Normalization has been performed using negative control probes and quantile normalization using negative and positive control probes as implemented in the neqc function (Shi et al. 2010) of the Limma package (Ritchie et al. 2015). Differential gene expression for all stimuli was calculated using the moderated t-statistic approach as implemented in the R-package Limma, which has been specifically developed for the analysis of small sample size experiments. The P-values of all results were corrected for multiple testing by using the false discovery rate (FDR) (Benjamini and Hochberg 1995). qPCR was carried out using gene specific primers of the selected genes (nine from each plant species) to validate the microarray results. Pearson's correlation coefficient of qPCR and microarray results was calculated to determine the association between both data sets. Analysis was performed using R version 3.5.3 (Team 2019).

PCA (Principal Component Analysis): It is a statistical technique that uses an orthogonal transformation to convert a set of observations of possibly correlated variables (e.g. gene expression profiles) into a set of linearly uncorrelated variables called principal components. So, all 24 microarray data sets collected from each plant species were put to PCA to find out the main components that accounted for the variation in transcriptomes. Here we use a variant of PCA called correspondence analysis as implemented in the vegan package (Oksanen et al. 2019). Differentially expressed genes were put to gene enrichment analysis as described in (Usadel et al. 2006). For that, Over Representation Analysis (ORA) was performed in Pagemean with Fisher's exact test at threshold value of log2 fold change 1. Furthermore, multiple hypothesis testing correction was performed using Benjamini-Hochberg method

which at once tests hundreds of possibilities and presents all the altered Mapman functional categories. For different molecular pathways analysis, genes annotated according to Ath-AGI-LOCUS Tair 10 (for *A. thaliana*) and Thels-Agilent44K (for *T. salsuginea*) were chosen for each pathway using MapMan 3.5.1R2 software (https://mapman.gabipd.org/download).

4. Results

Plant of both plant species subjected to diverse saline growth conditions were analysed at phenotypic, physiological and molecular level. Emphasis was given on the salt induced guard cell specific responses (both at physiological and molecular level) which were then correlated with the whole plant behaviour under similar growth conditions.

4.1. Establishing the Salt Application Protocol

Most of the salt stress studies have been conducted on plants grown on artificial growth mediums (e.g hydroponic and agar) which may not be directly comparable to the soil conditions as the interactions between soil solution and soil matrix can affect responses to soil salinity. For example, different salt induced responses have been reported under hydroponic and soil grown conditions in barley cultivars (Tavakkoli et al. 2010). Therefore, the present study was designed to observe salt induced responses in plants grown on natural growth medium, i.e soil. For that, two approaches of salt application were chosen to observe single (synonym short-time) and multiple (synonym long-time) salt application effects on plants as explained in section 3.2. Single salt (1x salt) treatment lasted for about three days whereas, multiple saline (3x salt) conditions were applied for fifteen days. Designing of the salt application protocol was to (i) increase soil salinity in each phase and (ii) reduce soil salinity in between the phases of the experiment. Therefore, before proceeding to the characterization of plants cultivated under saline conditions, soil ion content was analysed. For that, samples were taken from 3x salt, 1x salt and non-saline conditions and were tested for sodium and potassium content using flame photometer. Among the long-time saline conditions, two approaches were designed where plants were grown on (i) gradually increased saline conditions (3x salt) and (ii) constantly high saline conditions (3x 200mM).

4.1.1. Single and Multiple Saline Treatments Exhibited Similar Soil Na^+/K^+ Ratios within Each Phase of the Experiment

In each phase of the salt experiment concentration for single-time salt condition was increased which was also reflected in the soil sodium content (Fig. 4.1A). Washing steps performed in first and second phase of the experiment led to the reduction of sodium ions in

the soil compared to their respective controls. After first washing step, sodium levels showed similar content as in non-saline soils (Fig. 4.1A 1st phase). First washing step was performed after 75 mM NaCl application. After this washing step, the content tended to decrease, but the differences were not significant when compared to 75 mM salt application. Second washing was performed after 2x salt (i.e 75+150 mM) and caused significant reduction in sodium levels compared to before washing (i.e 2x salt) (Fig. 4.1A 2nd phase). However, this could not remove sodium ions completely (as sodium levels are significantly higher than non-saline soils) which is probably because of sodium bindings to the soil particles as leaching can only remove readily available ions in the soil. Nevertheless, soil sodium measurements after washing indicates indeed washing step was useful to decrease soil salinity. Purpose of multiple salt application was to steadily increase soil salinity with the progress of experimental period (i.e from 1st to second and then to 3rd phase). Sodium levels in the 2x and 3x salt application showed significant increase compared to their respective predecessor conditions (Fig. 4.1A).

Soil potassium content within each phase of the salt experiment showed non-significant changes in all conditions (Fig. 4.1B). However, with the progress of the experimental time, the levels tended to decrease compared to the first phase of the experiment which is most probably due to its uptake by plants as potassium is the main component for plant growth. Sodium to potassium ratios are one of the factors that determine soil salinity. Indeed, ratios increased significantly from phase to phase during the saline conditions. However, ratios between single and multiple times salt application did not show significant differences within the same experimental phase which is most probably due to the washing applications (Fig. 4.1C). Altogether, soil cation measurements indicate that the purpose of washing and multiple salt application was fulfilled.

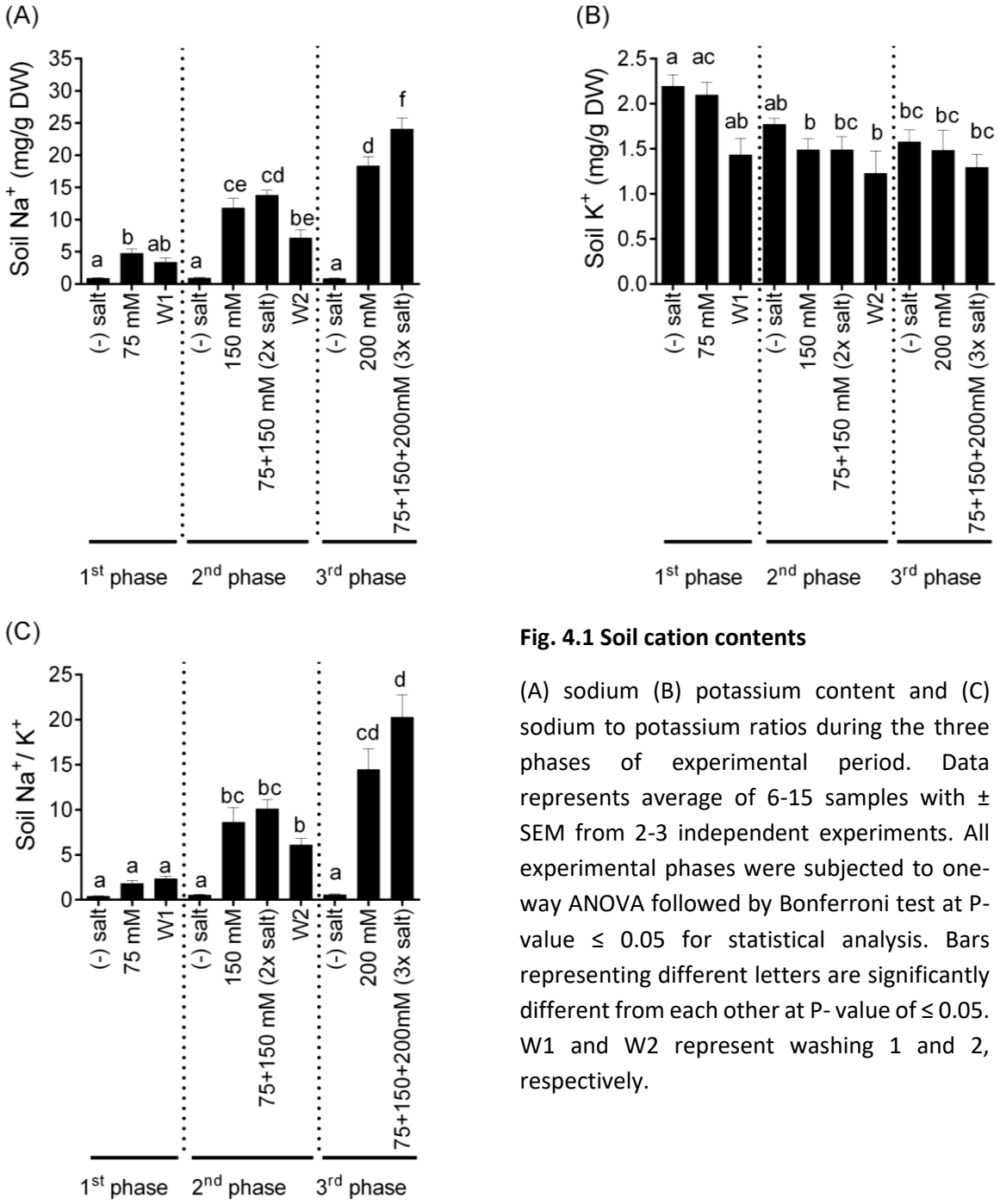

Fig. 4.1 Soil cation contents

(A) sodium (B) potassium content and (C) sodium to potassium ratios during the three phases of experimental period. Data represents average of 6-15 samples with ± SEM from 2-3 independent experiments. All experimental phases were subjected to one-way ANOVA followed by Bonferroni test at P-value ≤ 0.05 for statistical analysis. Bars representing different letters are significantly different from each other at P- value of ≤ 0.05. W1 and W2 represent washing 1 and 2, respectively.

4.2. Selection of Long-Term Saline Treatments Based on Plant Phenotypes

After establishing the salt application protocol, next step was to select one of the long-term salt application conditions i.e. gradually increased saline conditions (3x salt) and constantly high saline conditions (3x 200 mM) for characterization of both plant species. Purpose of cultivating plants on long-time saline conditions was to observe whether plants

can cope with multiple salt exposures, therefore plants from both saline groups were grown for reproduction after end of the salt exposures. This parameter of survival (reproduction) was set because of salt tolerance as described by (Munns and Termaat 1986).

4.2.1. Arabidopsis did not Reproduce on 3x 200 mM Salt Treatment

Plant of both plant species could successfully survive at the end of experiment under all growth conditions (Fig. 4.2A). However, under 3x 200 mM salt growth, Arabidopsis plants exhibited smaller rosette size and darker leaf colour compared to other salt grown plants (Fig. 4.2A). *A. thaliana* plants cultivated on both long-time saline conditions showed change in leaf colour. But salt induced coloration was more profound for plants grown on 3x 200 mM saline soils. Interestingly, plants were able to survive during and after the salt periods (i.e. two weeks) under both long-term saline conditions. *A. thaliana* plants facing 1x salt did not show any visual phenotype compared to the plants facing non-saline conditions (Fig. 4.2A). Similarly, *T. salsuginea* plants grown on 1x salt conditions did not show any phenotype. Likewise, no visible differences were observed in *T. saluginea* plants under both long-time saline growth environments. However, comparing plants from 3x 200 mM growth conditions with that of non-saline conditions, minute differences in phenotype were observed. Overall, *T. salsuginea* did not encounter severe growth problems under any saline conditions (Fig. 4.2A).

After the end of salt periods, plants from all salt treatments were further cultivated for seed setting. Both plant species could successfully set seeds under all growth conditions except of *A. thaliana* cultivated on 3x 200 mM saline soils (Fig. 4.2B). These plants could not survive and hence were unable to reproduce. We hypothesized that it was due to increased sodium and chloride levels in leaves as proposed in previous reports (Volkov et al. 2004; Lugan et al. 2010). Hypothesis was tested by measuring sodium, potassium and chloride content in the leaves of both plant species.

A. thaliana plants exposed to constant high saline conditions (3x 200mM) accumulated much more sodium and chloride than rest of the salt treated groups (Fig. 4.3A). Under 3x 200 mM saline conditions, *A. thaliana* plants accumulated 4.8 and 1.5 times more sodium and chloride respectively in leaves than 3x salt treated plants However, plants cultivated under gradually increasing saline conditions (3x salt) exhibited similar sodium levels as of short-time (1x) salt treated plants. Yet, leaf chloride levels in these plants (3x salt) were significantly

higher than 1x salt grown plants. Salt tolerant, *T. salsuginea* plants exposed to both long-time saline conditions showed similar levels of sodium and chloride in their leaves (Fig. 4.3A and B). *T. salsuginea* plants cultivated on 3x salt conditions displayed significantly higher levels of sodium in leaves than 1x salt treated plants. However, chloride levels remain the same under both saline conditions. *A. thaliana* plants accumulated 2.2 and 4.5 times more sodium than *T. salsuginea* under 1x and 3x 200 mM salt conditions respectively. Both plant species cultivated on 3x salt accumulated similar sodium in their leaves. Leaf chloride levels were 1.5, 1.9 and 2.3 times higher in *A. thaliana* than *T. salsuginea* under 1x, 3x and 3x 200 mM salt conditions respectively. Sodium and chloride measurements suggest that *A. thaliana* is accumulating more salt ions than *T. salsuginea* especially under 3x 200 mM growth medium.

Potassium, a major cation required for plant growth, decreased drastically under all saline conditions in *A. thaliana* compared to non-salt grown plants (Fig. 4.3C). Levels in leaves were strongly reduced on 1x salt medium; however, it did not decline further under any of the long- time saline growth conditions. Leaf potassium content in *T. salsuginea* only diminished at 3x 200 mM growth conditions when compared to control plants (Fig. 4.3C). Moreover, *T. salsuginea* leaves exhibited 1.5, 3.1, and 2.6-times higher potassium levels in leaves than *A. thaliana* at (-), 1x and 3x saline growth conditions respectively. Potassium content of *T. salsuginea* leaves depreciated to the basal levels of *A. thaliana* (i.e control plants) only under long-time saline environments (Fig. 4.3C). Decrease in potassium and increase in sodium levels led to elevated leaf Na^+/K^+ levels especially in *A. thaliana* (Fig. 4.3D). Salt tolerant, *T. salsuginea* showed escalation in Na^+/K^+ only on long-time saline conditions. Moreover, the ratios were higher in *A. thaliana* than *T. salsuginea* under the provided soil salinity. Elevated Na^+/K^+ and Cl^- levels in *A. thaliana* leaves led to the death of plants on 3x 200 mM salt.

These data reveal that salt sensitive plants can grow better and can also transmit their genetic information to the next generation under higher saline conditions if exposed earlier to lower saline conditions. Based on these observations gradually increased saline conditions (3x salt) were chosen for further experiments. The data were collected at the end of the third phase of the salt application (i.e on day 15).

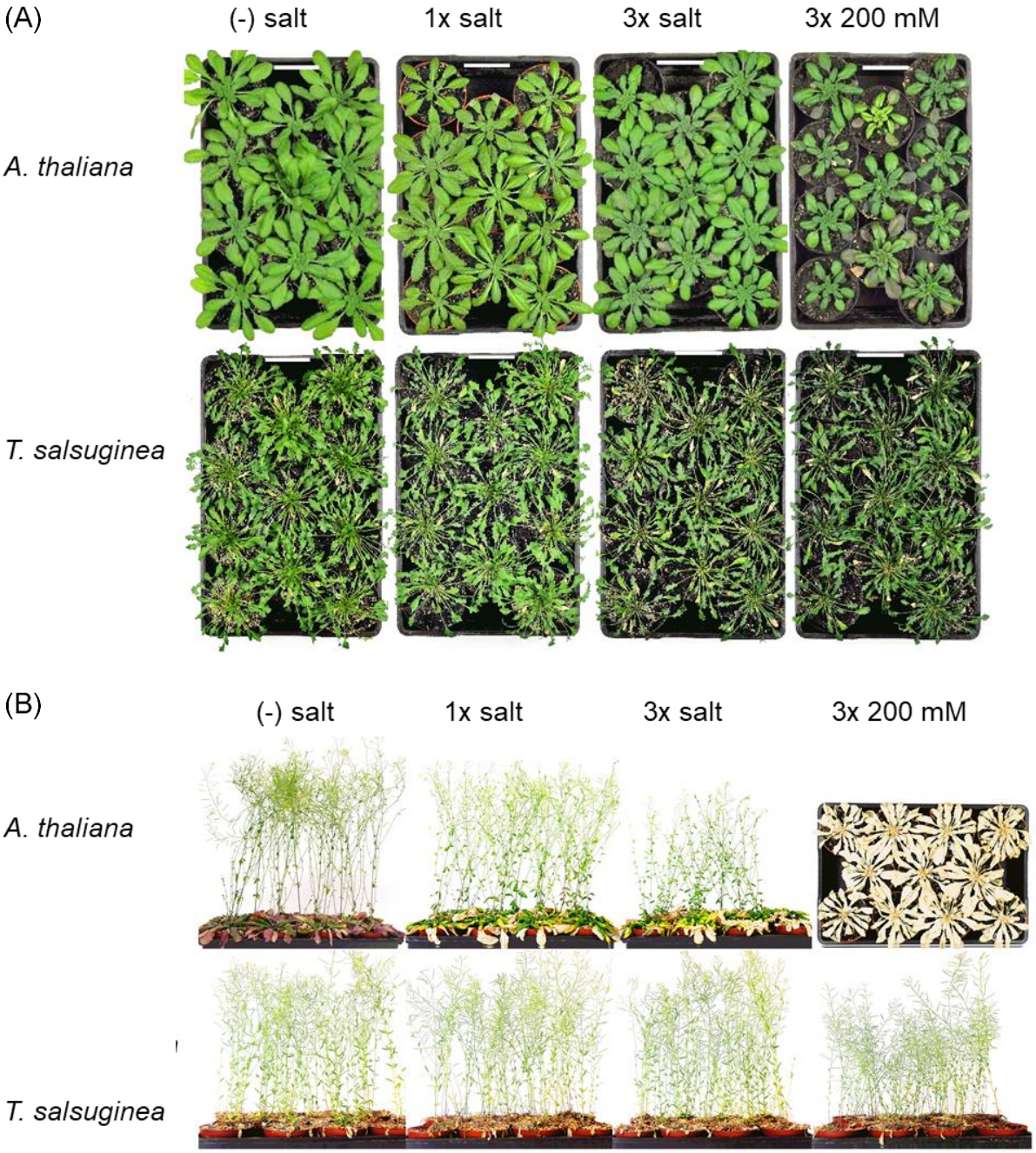

Fig. 4.2 *A. thaliana* and *T. salsuginea* phenotypes under different saline conditions

(A) plants undergone non, single and multiple times saline conditions. Pictures were taken at the end of salt experiment (15 days). Scale bars represent 5 cm in length. (B) Different salt treated plants of both species at the time of seed setting. Pictures were taken at the time of siliques formation after saline treatments.

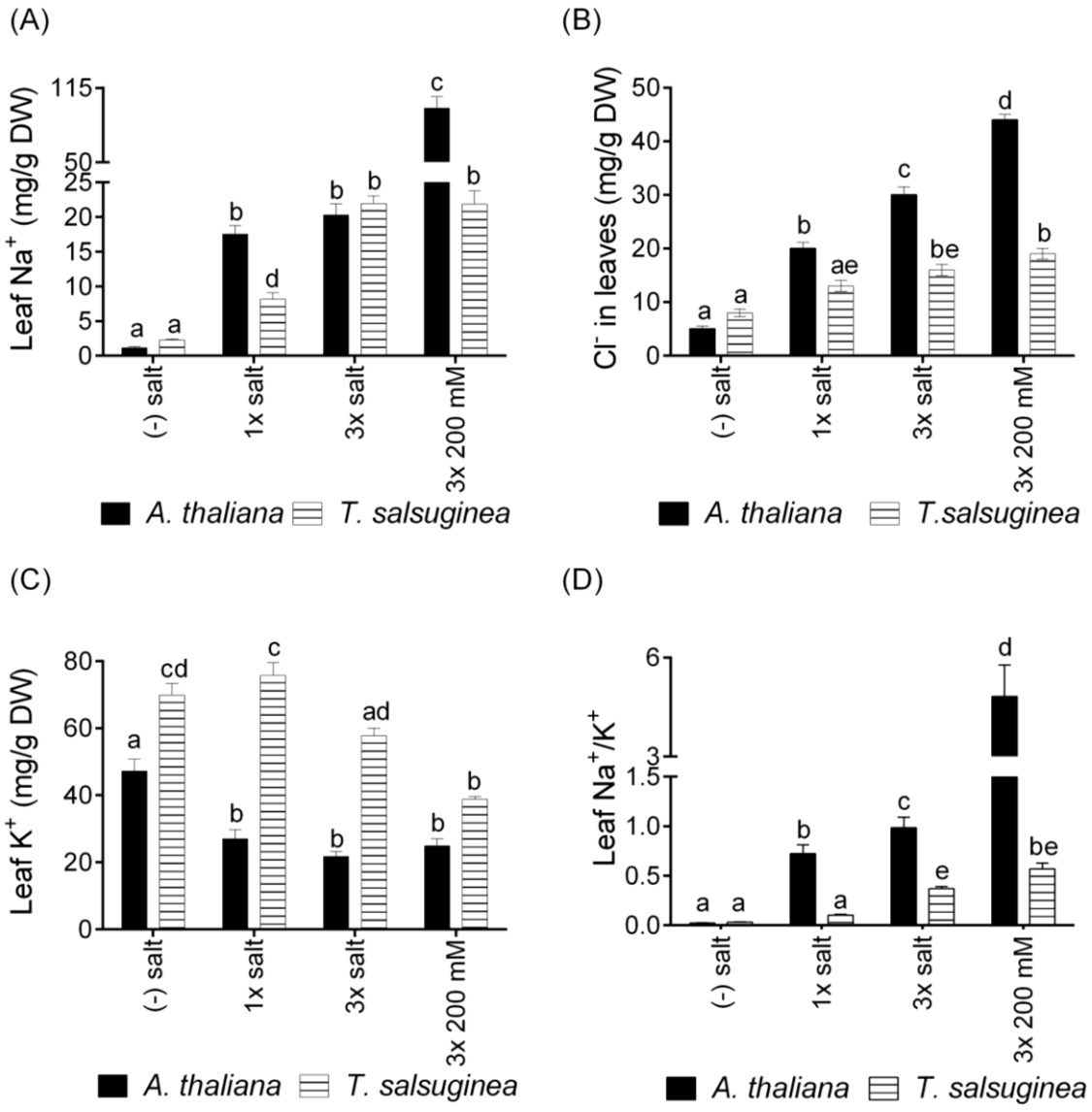

Fig. 4.3 Leaf ionic content in both plant types under different saline conditions

(A) Sodium (B) Chloride (C) Potassium and (D) Sodium to potassium ratios in leaves of both plant species after 15 days of various degrees of saline treatments. Data represents the means ± SEM of 14-18 samples collected from 4-8 individual experiments. Two-way ANOVA followed by Bonferroni post-hoc test for mutiple test corrections was performed for statistical analysis. Bars with different letters show significant differences at P-value ≤ 0.05 from each other.

4.3. Responses of *A. thaliana* and *T. salsuginea* Rosette Leaves to Soil Salinity

After selection of the salt application conditions, further experiments were conducted to study responses of rosette leaves of both plant species. As the responses of the plants vary under different conditions, therefore before observing guard cell functioning of both plant

species whole plant responses were observed. Later, these observations were correlated with the guard cell behaviour under provided growth environments. For that, physiological and phenotypic observation of both plant species were studied.

4.3.1. Salinity Reduces Pigment Content of *A. thaliana*

Among the plant pigments (chlorophyll A, B, carotenoids and anthocyanin) quantified from both plant species, 3x salt grown *A. thaliana* plants showed decrease in chlorophyll A and carotenoids (Fig. 4.4A and C). However, chlorophyll B levels remain unchanged in all saline conditions (Fig. 4.4B). Salt tolerant, *T. salsuginea* did not represent any change in the leaf pigments (Fig. 4.4A, B and C). Both plants showed similar amounts of chlorophylls and carotenoids under control conditions. Anthocyanin is known as stress pigment whose levels in the plants are increased especially under abiotic stress conditions (Kazachkova et al. 2013). Its levels were only significantly elevated in salt sensitive *A. thaliana* plants under both short and long-time saline conditions (Fig. 4.4D).

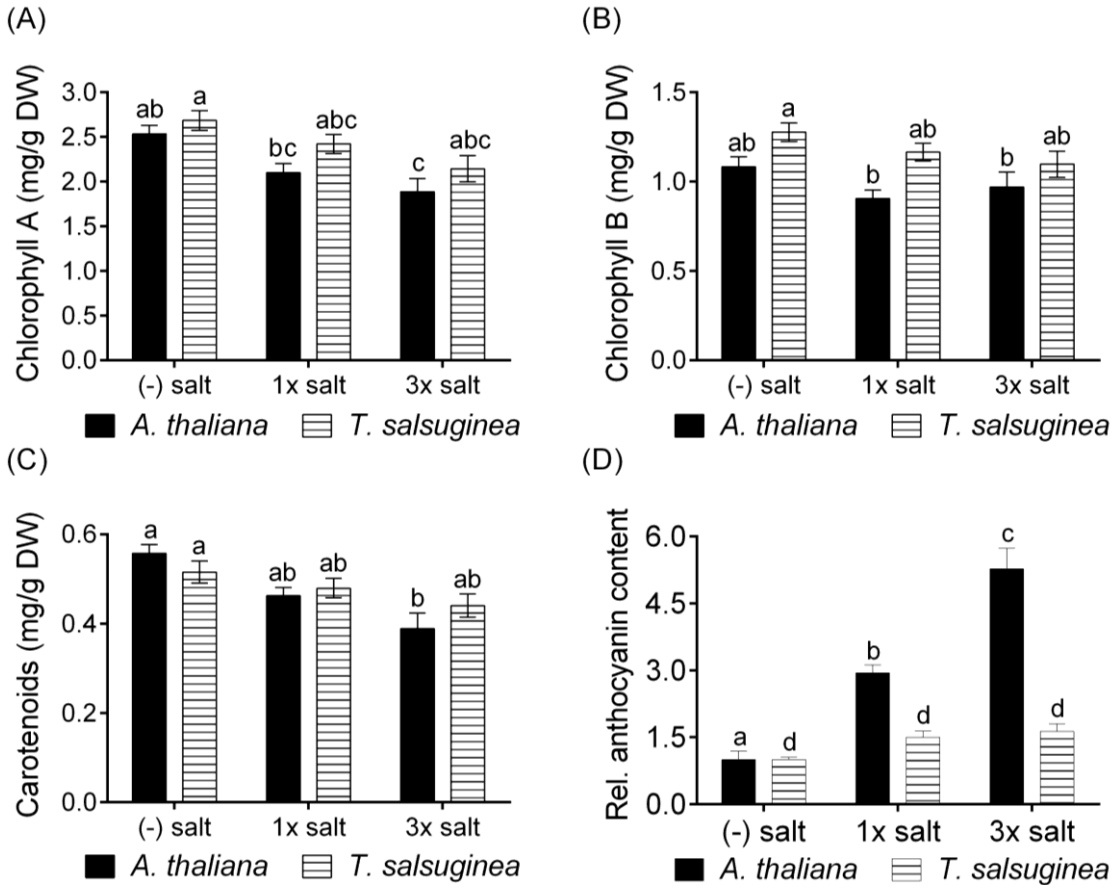

Fig. 4.4 Leaf pigments under saline growth conditions

(A) Chlorophyll A (B) Chlorophyll B (C) carotenoids and (D) Anthocyanin content of both plant species under various saline treatments. Data represents the means ± SEM of 14-18 samples collected from 4-8 individual experiments conducted on *T. salsuginea* and *A. thaliana* respectively. Two-way ANOVA followed by Bonferroni post hoc test for mutiple test corrections was performed for statistics. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.3.2. Salininity Reduces Plant Surface Area of *A. thaliana*

Surface area measurements of both plant species was performed as a growth parameter. *A. thaliana* plants showed only reduction (35%) in their growth when subjected to 3x saline conditions (Fig. 4.5). Under 1x saline conditions, *A. thaliana* plants decreased about 22 % in their growth, however, overall growth rate remained the same when compared to the non-salt treated plants. *T. salsuginea* did not show any depreciation in growth under any of the saline conditions. However, *T. salsuginea* exhibited 73%, 70% and 55% of *A. thaliana* surface area under non, 1x and 3x saline conditions respectively (Fig. 4.5).

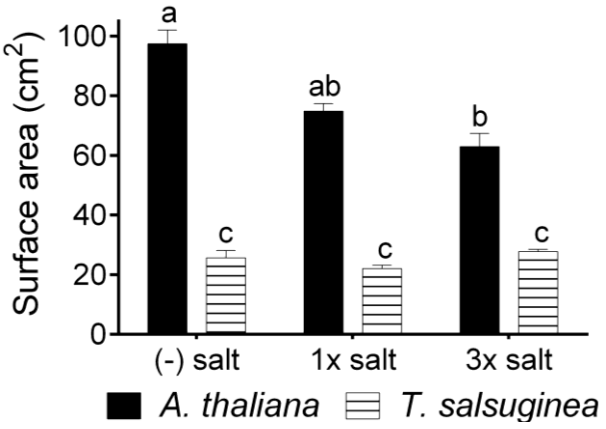

Fig. 4.5 Plant rosette surface area

Surface area of *A. thaliana* and *T. salsuginea* grown under diiferent saline conditions. Data represents the means ± SE of 9-19 samples collected from 3 individual experiments. Two-way ANOVA followed by Bonferroni post-hoc test for mutiple test corrections was performed for statistics. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.3.3. Salinity Reduces Plant Water Content and Osmotic Potential in Both Plant Species

Salt stress is always accompanied by drought stress which in turn causes changes in the plant water status and osmotic potential. Both plant species exhibited similar behaviour in terms of their water levels under saline conditions irrespective of its duration. The content reduced slightly but significantly in both plant species under 1x salt growth and kept on decreasing even stronger under 3x salt conditions compared to their respective controls (Fig. 4.6A). Overall, *T. salsuginea* showed lower water content than *A. thaliana* under all conditions.

Osmotic potential is corelated with the solute concentration in the cell. Increase in leaf ionic content on one hand and decrease in water content on the other hand in *A. thaliana* led to the reduction of osmotic potential (Fig. 4.6B). The osmotic potential decreased 1-fold under 3x salt conditions as compared to the control plants which is consistent with the water and ionic levels in leaves. Whereas, *T. salsuginea* osmotic potential was only affected (around 0.5-fold) after 3x saline conditions (Fig. 4.6B). Though the ionic status of *T. salsuginea* increased under 1x salt conditions but it did not cause changes in osmotic potential. Moreover, it was observed that provided saline conditions caused higher changes in osmotic potential in *A. thaliana* than *T. salsuginea* which is in correlation with the increased sodium and chloride levels (Fig. 4.3A and B) of *A. thaliana* leaves.

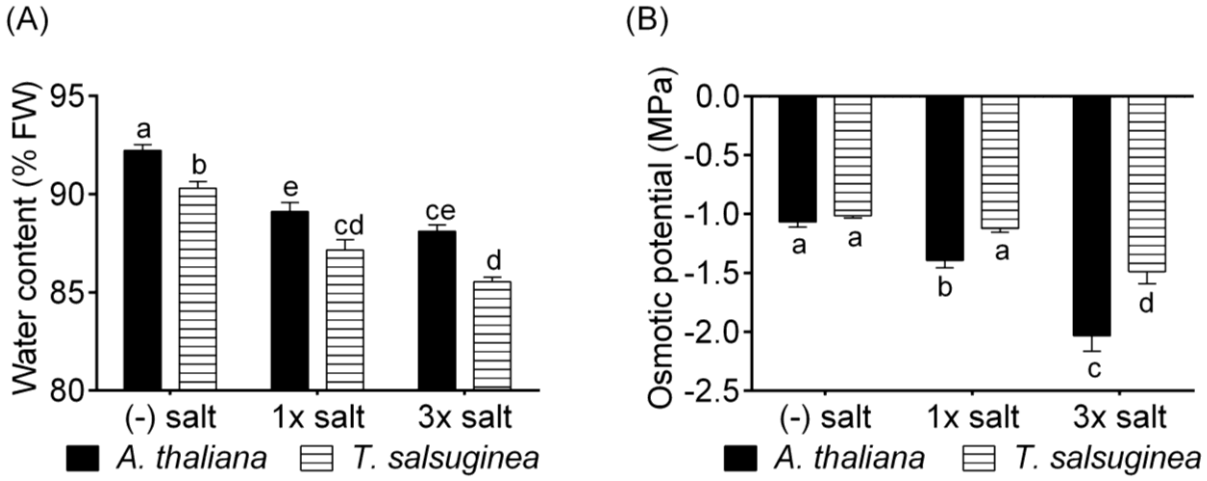

Fig. 4.6 Water status of both plant species

(A) Plant water content and (B) Leaf osmotic potential of both plant species under different saline conditions. Data represents the means ± SEM of 13-19 samples collected from 3 individual experiments. Two-way ANOVA followed by Bonferroni post-hoc test for mutiple test corrections was performed for statistical anaylsis. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.3.4. Salinity Causes Transient ABA Accumulation in Both Leaf Types

Phytohormone ABA is known as stress hormone and plays vital role especially under abiotic stress (Fernando and Schroeder 2016). Therefore, the quantification of this phytohormone was performed to get insights into its role of salt stress adaptation under saline conditions. Initially, the content was quantified in the leaves of both plant species that have undergone different saline treatments. During non-saline conditions, basal ABA content of both plant species did not show any significant differences from each other (Fig. 4.7). ABA

levels increased significantly when both plant species were subjected to 1x salt conditions. However, increase was more profound in *A. thaliana* leaves than *T. salsuginea* compared to their respective controls (non-salt), where former showed 4.7 times and later showed 2 times increase respectively. Moreover, under similar conditions (i.e 1x salt), ABA content of *A. thaliana* leaves were significantly higher than *T. salsuginea* (Fig. 4.7). Interestingly, on 3x salt medium, ABA levels did not increase further in both plant species but instead decreased compare to 1x salt growth conditions. On 3x salt medium, salt tolerant, *T. salsuginea* set back their ABA content to the basal levels (i.e non-salt growth conditions) but salt sensitive, *A. thaliana* still had more ABA content than non- salt treated plants.

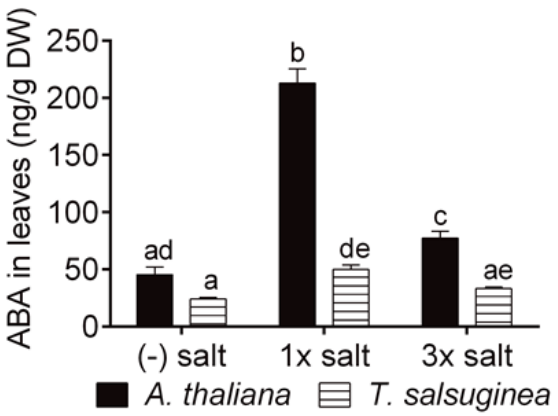

Fig. 4.7 ABA in leaves

A. thaliana and *T. salsuginea* ABA content in leaves under saline conditions. Data represent the average ±SEM of 9-19 samples collected from 3 individual experiments. Two-way ANOVA followed by Bonferroni post hoc test for mutiple test corrections was performed for statistics. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.4. Responses of *A. thaliana* and *T. salsuginea* Guard Cells to Soil Salinity

After analysing salt induced changes in leaves, guard cells of both plant species were characterized at physiological, biochemical and molecular level to get better insights into their functioning under saline growth conditions. Guard cell responses were correlated to the whole plant performance and hence comparisons between both plant species were made.

4.4.1. Arabidopsis Guard Cells Accumulated Both Salt Ions and Thellungiella Only Sodium

Guard cells use ions for osmotic adjustments and hence modulate stomatal apertures. Both plant species were investigated for salt ions in guard cells to get insights into their

possible roles in guard cell physiology under saline conditions. Energy Dispersive X-Ray Analysis (EDXA) of steady open state of the stomata demonstrated indeed both guard cell types accumulate sodium during opening of the stomata on saline environments (Fig. 4.8A). Moreover, similar amounts of sodium were found in both guard cell types under comparable conditions. Major differences were observed in the chloride levels where Arabidopsis guard cells progressively accumulated chloride under both salt conditions whereas Thellungiella guard cells kept similar levels under all environments (Fig. 4.8B). Similarly, potassium amount was decreased in both guard cell types under both salt conditions however, decrease was stronger in Arabidopsis guard cells compared to Thellungiella guard cells. Moreover, Thellungiella guard cells maintained higher potassium levels than Arabidopsis under both saline environments (Fig. 4.8C).

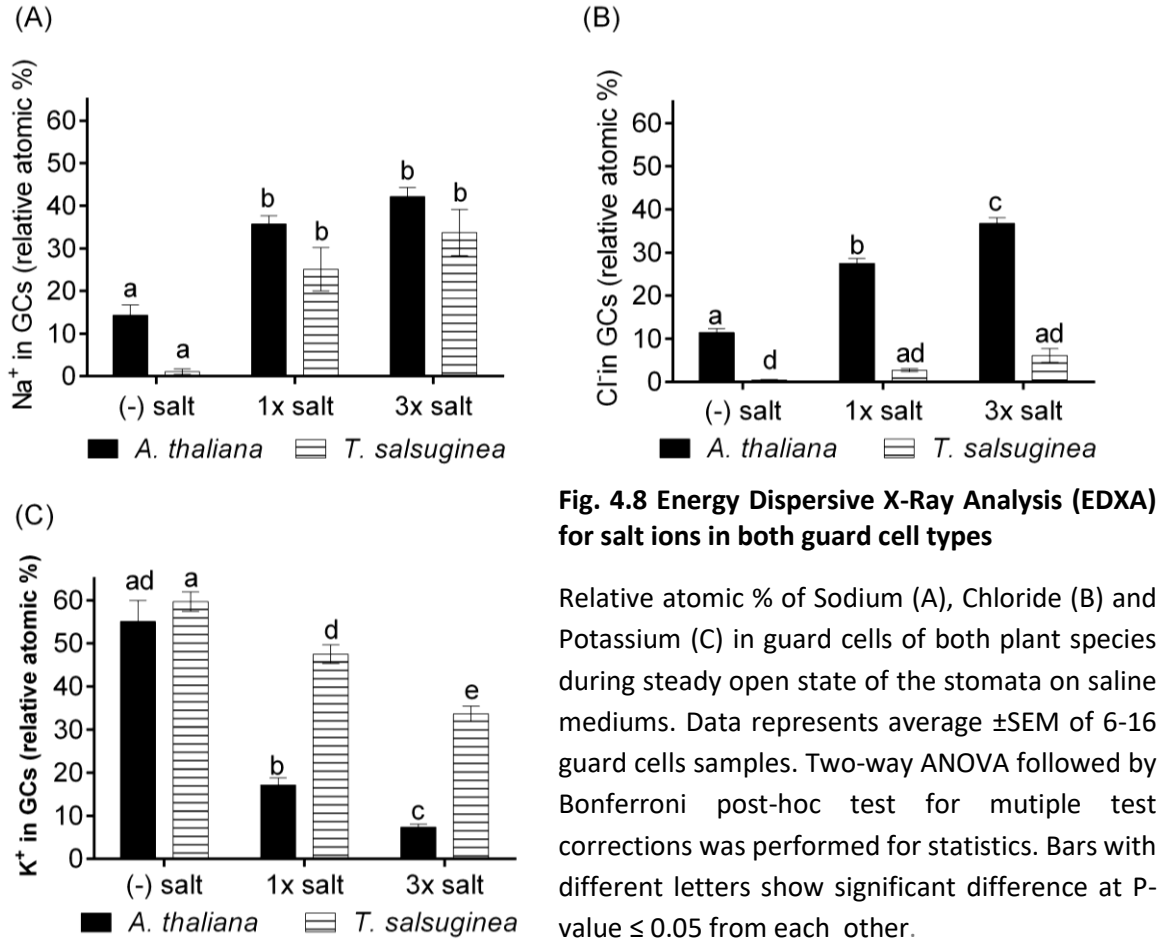

Fig. 4.8 Energy Dispersive X-Ray Analysis (EDXA) for salt ions in both guard cell types

Relative atomic % of Sodium (A), Chloride (B) and Potassium (C) in guard cells of both plant species during steady open state of the stomata on saline mediums. Data represents average ±SEM of 6-16 guard cells samples. Two-way ANOVA followed by Bonferroni post-hoc test for mutiple test corrections was performed for statistics. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.4.2. Short-time Salt Treatment Led to Increase in Guard Cell ABA in Both Plant Species

It has been proposed earlier that guard cells of *A. thaliana* are equipped with autonomous ABA production (Bauer et al. 2013). Present study demonstrated that both plant species increase their leaf ABA levels when cultivated under saline conditions (Fig. 4.7). Therefore, ABA was quantified in the guard cells of both plant species cultivated under different saline conditions. Guard cells are abundantly located on the epidermis of leaves and can be isolated by blending method (see section 3.3). This method gives enriched guard cell samples along with the cell wall components. Being a lipophilic molecule, ABA may attach to cell wall components which might be carried over in the guard cell preparations. Therefore, verification of the measured ABA from guard cells was necessary. During the guard cell extraction process, the ABA isotope $^{13}C_2$-ABA (S-(+)-ABA $^{13}C_2$, Qmx Laboratories Ltd, Essex, UK) was added to the ice water and guard cells from *A. thaliana* leaf samples were extracted. If ABA binds to the cell walls this externally added ABA would be recorded in the cell walls fractions. After blending, the fractions of each step were collected (i.e ice water solution (WS), cell debris (CD i.e destroyed guard cells) and intact guard cells (GC)) followed by $^{13}C_2$-ABA quantification. Neutral red staining assays confirmed the enrichment of intact guard cells (Fig. 4.9A) or the destruction of the guard cells (Fig. 4.9B). Quantification of externally added $^{13}C_2$-ABA isotope to the guard cell preparation showed its presence exclusively in the washing solution (WS) and not in the guard cell preparations (Fig. 4.9C). This analysis demonstrated that the guard cell preparation did not carry over ABA with cell wall debris.

Under similar growth conditions, guard cells of both plant species showed similar levels of ABA. On 1x salt growth conditions, ABA levels were significantly increased in *A. thaliana* guard cells whereas *T. salsuginea* did not show significant increase compared to their respective control plants (i.e (-) salt) (Fig. 4.9D). On 3x salt medium, both plant species did not show further increase in ABA but instead set the content to the basal levels (Fig. 4.9D). ABA biosynthesis sites are not only restricted to guard cells. Therefore, further validation was performed to confirm induction in guard cell ABA is solely by its bioproduction in the guard cells and not by transportation from other biosynthesis sites (such as roots and mesophylls). For that, *A. thaliana* plants deficient in ABA production (*aba3-1*) and, its compliment line with

guard cells specific promoter, p*MYB60::ABA3* plants capable of synthesizing ABA in guard cells only (Bauer et al. 2013) were used. Both plant lines were grown on 1x salt conditions followed by collection of leaves and isolation of guard cells.

Selection of this saline condition (i.e 1xsalt) was opted based on the maximum increase in leaf ABA levels compared to other saline conditions (Fig. 4.7). Leaf samples were used as control, since the *aba3-1* line should not produce ABA in any of the plant tissues. ABA quantification in the leaves of *aba3-1* showed indeed this mutant plant is not able to produce more ABA under saline growth conditions (Fig. 4.9F). However, p*MYB60::ABA3* plants do elevate ABA levels in guard cells and leaves on 1x salt growth conditions (Fig. 4.9E and F). thus confirming guard cell autonomous ABA biosynthesis under saline growth conditions. Altogether, internal ABA quantification in intact guard cells, and external ABA quantification in intact and destroyed guard cells, and washing solutions confirm that both plant species do elevate ABA content in guard cells under saline growth conditions.

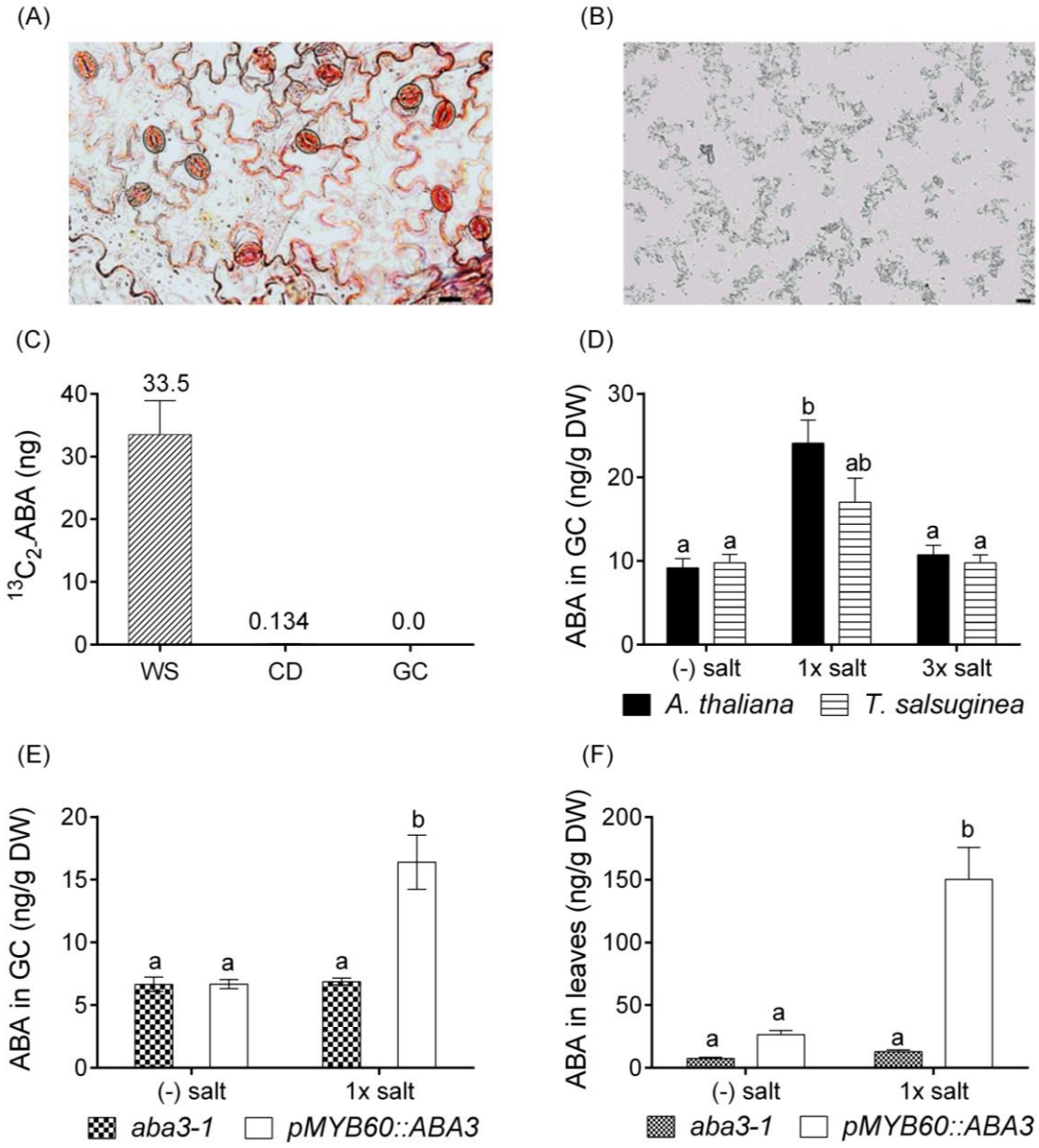

Fig. 4.9 ABA in guard cells

(A) and (B) neutral red stained bright field images of intact and destroyed guard cells respectively. (C) External ABA with carbon isotope $^{13}C_2$-ABA in the washing solution (WS), Cell debris (CD) and guard cells (GC). (D) Internal ABA content in guard cells of both plant species (E) guard cells and, (F) leaves ABA of *A. thaliana*-Col-0 ABA biosynthesis deficient mutant and its complement line with rescued ABA biosynthesis in guard cells during absence and presence of saline growth conditions. Data represent the means ± SE of 13-19 samples collected from 3 individual experiments. Two-way ANOVA followed by Bonferroni post-hoc test for mutiple test corrections was performed for statistical anaylsis. Bars with different letters show significant difference at P-value ≤ 0.05 from each other.

4.4.3. Gas Exchange Parameters are Differently Affected in Both Plant Species

As gas exchange and water loss are directly controlled by guard cells therefore both plant species grown on saline and non-saline soils were subjected to analyse transpiration and CO_2 assimilation rates.

4.4.3.1. Transpiration of Arabidopsis is Strongly Reduced under Both Salt Concentrations

It was hypothesized that plants undergone saline treatments should reduce transpiration rates because of the increased ABA levels in leaves and guard cells (Fig. 4.7 and Fig. 4.9). Transpiration rates recorded on whole rosette leaves (see section 3.5.3) demonstrated indeed *A. thaliana* water loss is strongly decreased under both saline conditions during light and dark-adapted phases when compared to non-salt grown plants (Fig. 4.10A). However, they showed similar transpiration rates in first dark and light phase of the experiment under both salt conditions. Yet, significant differences were observed between the two salt treatments (i.e 1x and 3x) when light adapted *A. thaliana* plants were subjected to dark phase (Fig. 4.10A).

T. salsuginea cultivated on both salt and non-salt growth conditions in general showed higher transpiration rates (both in light and dark) compared to *A. thaliana* (Fig. 4.10A and B). *T. salsuginea* plants grown under both saline conditions exhibited lower levels of transpiration in first dark phase compared to the control plants (Fig. 4.10B). Interestingly, in the light adapted phase, only 3x salt grown plants showed significant decrease in transpiration. Moreover, these plants not only showed significant reduction in transpiration than control but also than 1x salt grown plants in the light phase. In line with first dark phase, *T. salsuginea* plants also demonstrated significant decrease in transpiration rates in the second dark phase (after light adapted phase) compared to control plants. *T. salsuginea* plants cultivated on both saline environments displayed similar transpiration rates during the initial phase of the second dark period. However, significant reduction in 3x salt grown plants were observed during the steady state of second dark phase (Fig. 4.10B).

Transpiration rates are directly related to the stomatal apertures and densities. Therefore, validation of the transpiration rates of both plant species was performed by measuring these two parameters. As the stomatal apertures were measured by taking microscopic pictures therefore, avoiding of light was not possible. This is why data could only be correlated with the light adapted transpiration rates. Stomatal apertures of *A. thaliana* plants grown under both saline conditions were significantly smaller in comparison to non-salt grown plants (Fig. 4.10C). *T. salsuginea* plants grown on 1x salt conditions did not significantly change their stomatal apertures compared to the non-salt treated plants (Fig. 4.10C). However, *T. salsuginea* plants cultivated on 3x salt conditions significantly reduce their stomatal apertures compared to the non-and 1x salt growth conditions (Fig. 4.10C). These data are in line with the light adapted transpiration rates from the gas exchange measurements. Comparison of stomatal apertures of the plants and transpiration rates in the light phase demonstrate that indeed saline growth conditions caused reduction in stomatal apertures which in turn affects transpiration rates. Stomatal apertures of both plant species were very much similar under non-saline conditions, but under saline conditions *T. salsuginea* plants showed larger apertures than *A. thaliana* (Fig. 4.10C). Counting stomata per unit area showed 1.4 and 1.6 times more stomata on the abaxial and adaxial surface of Thellungiella leaves respectively than Arabidopsis (Fig. 4.10D).Taken together stomatal apertures and densities from both plant species demonstrate that higher transpiration rates of *T. salsuginea* than *A. thaliana* are due to higher abundance of stomata in the former plant species.

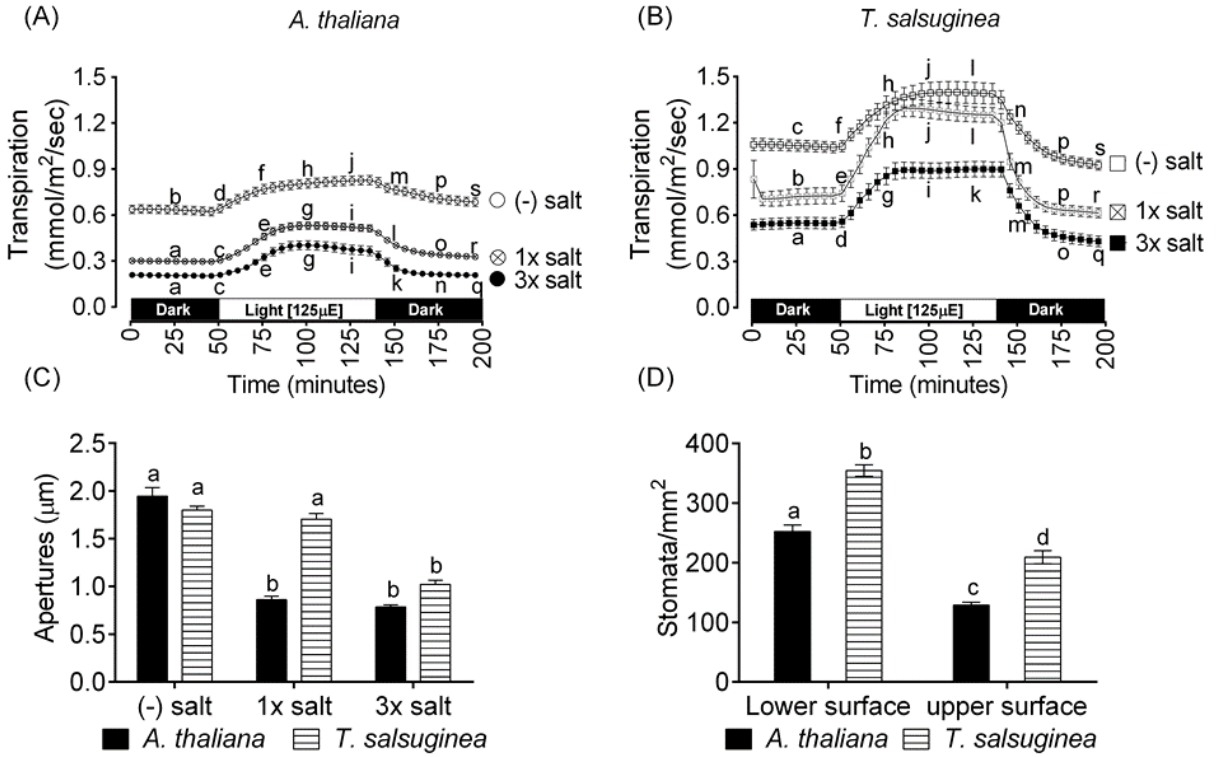

Fig. 4.10 Transpiration of both plant species on saline growth conditions

Transpiration rates of (A) *A. thaliana* and (B) *T. salsuginea* plants during dark and light phases, (C) stomatal apertures of both plant species under light adapted conditions, and (D) Stomatal density from lower and upper leaf surfaces of both plant species. Data represents mean ±SEM of 8-19 plants from 3-4 independent experiments (for A and B) and average of 4-5 plants ±SEM of three independent experiments (for C and D). Statistical analysis was performed using one-way ANOVA (for A and B) after every 25 minutes time point and two-way ANOVA (for C and D) followed by Bonferroni post-hoc test at P-value ≤0.05 for multiple test corrections. Bars with different letters denote significant difference at P-value ≤ 0.05.

4.4.3.2. Thellungiella CO_2 Assimilation Rates were Higher than Arabidopsis under Both Salt Concentrations

Plants take up environmental CO_2 via stomata that is then used in the photosynthesis process, hence CO_2 assimilation of both plants under saline conditions was measured to see if salinity also affects this mechanism. The data showed decrease in CO_2 assimilation rates only in 3x salt grown *A. thaliana* where it reached to half of the basal rates (Fig. 4.11). However, assimilation rates of *A. thaliana* remain similar under both salt conditions. Salt tolerant, *T. salsuginea* plants did not show any significant changes in the assimilation rates under both saline conditions when compared to non-salt cultivated plants (Fig. 4.11). Moreover, *T.*

salsuginea displayed higher CO_2 assimilation rates than *A. thaliana* under both saline conditions (Fig. 4.11).

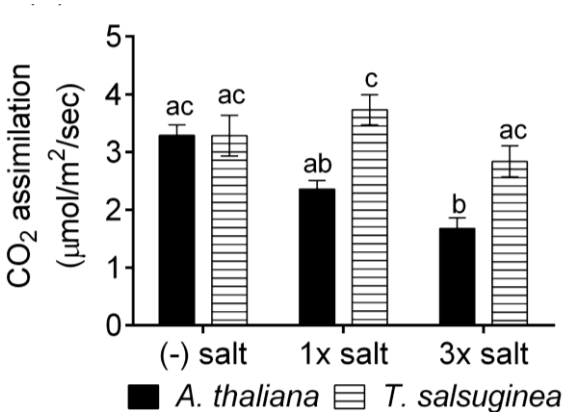

Fig. 4.11 CO_2 assimilation of both plant species on saline growth conditions

Assimilation of CO_2 in light adapted plants of *A. thaliana* and *T. salsuginea* under salt and non-salt growth conditions. Data represents mean ±SEM of 8-19 plants from 3-4 independent experiments. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test at P-value ≤0.05. Bars with different letters denote significant difference at P-value ≤ 0.05.

4.4.3.3. Intrinsic Water Use Efficiency (WUE_i) of both Plant Species Remain Unchanged under Both Saline Conditions

Intrinsic water use efficiency (WUE_i) considered as an important indicator for plant adaptation to unfavourable conditions (Mckay et al. 2003). Hence, WUE_i was calculated as ratios between CO_2 assimilation and transpiration rates which provides the efficiency of consumed water molecules per molecule of carbon assimilated under the provided conditions. WUE_i of both plant species did not change significantly under saline conditions. Interestingly, *A. thaliana* plants showed a higher WUE_i under non-saline and short-time saline conditions than *T. salsuginea* (Fig. 4.12). But, on 3x salt growth conditions both plant species demonstrated similar levels of WUE_i. A tendency in increased WUE_i was observed in both plant species under saline conditions compared to their respective controls. Irrespective of reduced CO_2 assimilation rates in *A. thaliana* under saline growth conditions, the WUEi remained unchanged due to the reduced transpiration rates. Thus, the results of the transpiration rates, CO_2 assimilation and WUEi suggest that both plant species do not compromise their WUEi under the saline conditions. Moreover, Arabidopsis ability to use water more effectively than Thellungiella for CO_2 assimilation is helpful in more biomass production in this plant type which is evident from plant phenotypes (Fig. 4.2A).

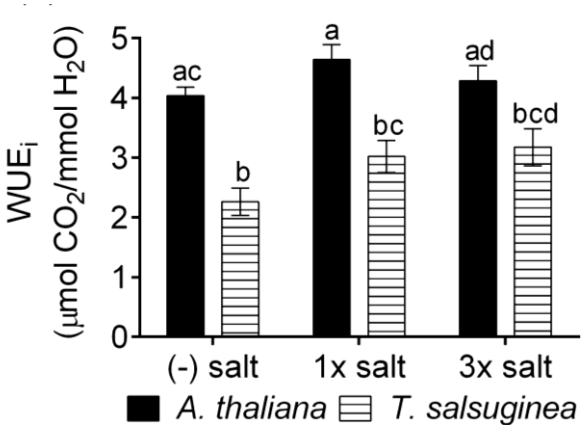

Fig. 4.12 Intrinsic Water Use Efficiency of both plant species on saline conditions

WUE_i in light adapted plants of *A. thaliana* and *T. salsuginea* under salt and non-salt growth conditions. Data represents mean ±SEM of 8-19 plants from 3-4 independent experiments. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc test at P-value ≤0.05. Bars with different letters denote significant difference at P-value ≤ 0.05.

4.4.4. Gas Exchange Parameters of Both Plant Species during Increasing Light Intensities

Both plant species undergone different salt growth conditions were further investigated to observe guard cell responses to gradually increasing light exposures. This experiment was designed to inspect if the salt induced negative effects could be neglected by the progressively light exposures. Therefore, dark adapted plants were exposed to gradually increasing light intensities (see 3.5.3). Transpiration rates, CO_2 assimilation and WUEi were recorded at each light intensity.

4.4.4.1. Short-time Salt Induced Decrease in Arabidopsis Transpiration was Neglected during Gradual Increased Light Exposures

Both saline conditions caused decrease in transpiration in *A. thaliana* under single light intensity (125 µE) (Fig. 4.10A). Contrastingly, Arabidopsis plants from 1x salt treatment exposed to gradual increased light intensities did not show any significant differences in the transpiration levels at all light intensities compared to their non-salt treated plants (Fig. 4.13A). In line with single light recordings, *T. salsuginea* from 1x salt treatment showed unchanged transpiration at higher light intensities. Both plant species from 3x salt treatment clearly showed contrasting patterns. *A. thaliana* plants belonging to this group showed significantly lower levels of transpiration than non-salt treated plants up to 850 µE light intensity while *T. salsuginea* showed non-significant decrease in transpiration till 850 µE light. However, when *A. thaliana* plants were exposed to 1050 and 1250 µE light intensity, transpiration levels did not show any differences under all cultivated conditions. However, 3x salt grown *T. salsuginea* plants on high light intensities (i.e 1050 and 1250 µE) exhibited

significant decrease in transpiration compared to non, and 1x salt treated *T. salsuginea* plants (Fig. 4.13A). It was obvious from the light saturation curves that both plant species increase their transpiration levels with increasing light intensities irrespective of salt treatments (Fig. 4.13A). However, induction was stronger in *T. salsuginea* than *A. thaliana.* Moreover, under all conditions, *A. thaliana* plants attain saturation levels earlier than *T. salsuginea*, thus showing maximal guard cells opening. Under non-saline conditions the fold change increase in the levels of transpiration from dark (0 µE) to the highest light intensity (1250 µE) was 2.7 and 4.1 for *A. thaliana* and *T. salsuginea* plants respectively. Change in transpiration from dark (0 µE) to highest light intensity(1250 µE) was stronger in salt treated plants of both species compared to its respective non salt grown plants. It was observed that exposure of highest light intensity (1250 µE) resulted in 2.6, 4.0 and 3.4 times increase in transpiration rates of (-), 1x and 3x salt grown *A. thaliana* plants (Fig. 4.13A). Similarly, 4.1, 6.3 and 7.2-fold increase in transpiration was observed in (-), 1x and 3x salt grown *T. salsuginea* plants respectively (Fig. 4.13A).

4.4.4.2. Both Plant Species Exposed to Long-time Salt Treatment Decrease CO_2 Assimilation at High Light Intensities

During single light exposure (125 µE), 3x salt treatment caused reduction in CO_2 assimilation only in Arabidopsis (Fig. 4.11). Interestingly, both plant species from 3x salt group exposed to gradually increasing light intensities demonstrated decline in CO_2 assimilation especially at higher light intensities. In line with single light exposure, CO_2 assimilation levels of *A. thaliana* plants from 3x salt treatment remained significantly lower than non-salt treated plants at all light exposures. However, under extremely high light intensities (i.e 850, 1050 and 1250 µE) they also showed significantly lower levels of CO_2 assimilation when compared to 1x salt treated plants (Fig. 4.13B). Similarly, *T. salsuginea* plants from 3x salt conditions demonstrated lower CO_2 assimilation levels than 1x salt treated plants only at higher light intensities (i.e 500-1250 µE). Both plant species from 1x salt treatment did not show significant differences in assimilation rates compared to their respective non-salt treated plants under all light intensities (Fig. 4.13B). Interestingly, *T. salsuginea* grown on 1x salt concentrations displayed a tendency in increased assimilation levels compared to non-salt treated plants. Like transpiration, *A. thaliana* plants attain saturation levels for CO_2 assimilation earlier than *T. salsuginea* especially under saline growth conditions. Fold changes calculated from lowest

light exposure (75 µE) to the highest light exposures (1250 µE) revealed that *T. salsuginea* plants are more prone to changes than *A. thaliana* under all conditions. *T. salsuginea* showed 7.3, 6.7 and 10-fold increase in CO_2 assimilation under non, 1x and 3x saline conditions respectively. Whereas *A. thaliana* showed 4.5, 3.6 and 2.6-fold increase in assimilation levels under non, 1x and 3x saline conditions respectively (fig. 4.4.4B).

4.4.4.3. Intrinsic Water Use Efficiency (WUE_i) was Decreased in long-term salt Treated Arabidopsis Plants at High Light Intensities

Both salt and non-salt treated *T. salsuginea* plants exhibited non-significant differences in WUE_i levels under all light conditions (Fig. 4.13C). Similarly, *A. thaliana* plants grown under non and short-time saline conditions showed similar levels of WUE_i at all light exposures (Fig. 4.13C). However, *A. thaliana* plants belonging to 3x salt group demonstrated significant decline in WUEi compared to non-salt treated plants. Both plant species on salt and non- salt treatments achieved maximum WUEi levels at 300 µE light. Light exposures beyond this light intensity resulted in decrease of WUEi levels only in 3x salt treated *A. thaliana* plants. Other groups of both plant species maintain their maximum WUEi levels beyond 300 µE light. Fold changes calculated from lowest light exposure (75 µE) to the highest light exposures (1250 µE) revealed that *T. salsuginea* plants are more prone to changes in WUE_i than *A. thaliana* under all conditions. *T. salsuginea* showed 3.26, 2.49 and 3.32-fold increase in WUEi on non, 1x and 3x salt mediums respectively. Whereas *A. thaliana* showed only 2.4, 1.5 and 0.9-fold increase under non, 1x and 3x salt conditions respectively (Fig. 4.13C).

Taken together, the adverse effects of salt on transpiration during 1x and 3x salt growth conditions of *A. thaliana* and *T. salsuginea* (Fig. 4.10A and B) respectively are nullified when the plants are exposed to gradually increasing light intensities (Fig. 4.13A). However, exposure to extremely high light intensities poses equally damaging effects on transpiration and CO_2 assimilation to both plant species treated with 3x salt. Unchanged levels in WUEi of *T. salsuginea* even at high light intensities under all saline conditions demonstrates its better mechanisms to cope light and heat exposures than *A. thaliana*.

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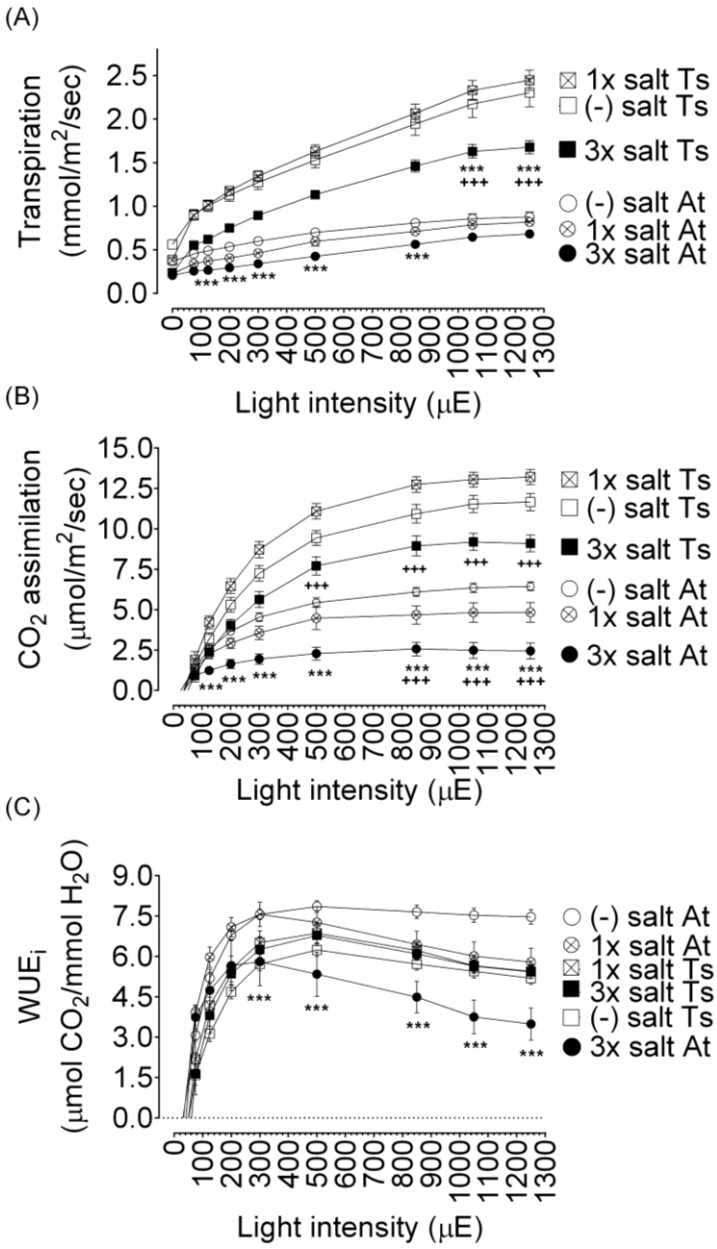

Fig. 4.13 Gas exchange parameters under increasing light intensities

Changes in (A) transpiration rates, (B) CO_2 assimilation rates and (C) WUE_i during increasing light intensities. Data represents means of 6-12 plants ±SEM of three independent experiments. Statistical analysis was performed separately for each light intensity using one-way ANOVA followed by Bonferroni post-hoc test at P-value ≤ 0.05. Whereas *** indicate significant differences between (-) and 3x salt and +++ denotes significant differences between 1x and 3x salt within same plant species. Traces belonging to each treatment is mentioned in parallel. Boxes represent *T. salsuginea* and circles denote *A. thaliana*. Where *** and +++ shows p-value ≤ 0.0001.

4.4.5. Salinity Causes Changes in Light Energy Consumption in *A. thaliana* Guard Cells

The effect of saline conditions on light energy consumption of PSII was analysed in guard cells from both plant species by using the microscopic PAM. On non-saline conditions, dark-adapted guard cells of both plant types resulted in a similar optimal quantum yield of PSII (Fv/Fm) upon exposure to a saturating light pulse (Fig. 4.14A). Under both saline conditions, the optimal quantum yield of *A. thaliana* guard cells decreased by 9.6% under 1x- and 17% under 3x salt treatment whereas, guard cells of salt treated *T. salsuginea* plants did not represent any change in the optimal quantum yield compared to non-saline conditions.

Moreover, guard cells of *T. salsuginea* demonstrated higher levels of optimal quantum yield under saline conditions compared to *A. thaliana* (Fig. 4.14A).

Effective quantum yield (ΔF/Fm') is another parameter that measures the efficiency of PSII in illuminated guard cells. Only the salt sensitive Arabidopsis guard cells displayed decrease in effective quantum yield on both saline conditions compared to non-salt growth environments (Fig. 4.14B). Guard cells of both plant species exhibited similar effective quantum yield on non- and 3x salt mediums. Major differences were observed in short time salt growth conditions. Where, effective quantum yield of Thellungiella guard cells (of 1x salt) was significantly higher than the guard cells of salt grown Arabidopsis plants (both 1x and 3x salt) (Fig. 4.14B).

Non-Photochemical Quenching (NPQ) of guard cells was also compared to determine a possible difference in the ability of heat dissipation for protection of PSII against high light energy irradiation. Arabidopsis guard cells showed linear increase in NPQ and did not attain saturation levels during the whole experiment under all growth conditions (Fig. 4.14C). However, guard cells of non-salt grown Arabidopsis plants demonstrated consistent increase in NPQ levels compared to other salt treated groups (i.e 1x and 3x). As the variation among the samples was high therefore, no significant differences were observed among all treatments in this plant species. In contrast to Arabidopsis, Thellungiella guard cells from non and 1x salt grown plants reach saturation levels during the initial three minutes of the experiment. However, Thellungiella guard cells from 3x salt group tend to increase NPQ levels without reaching saturation level. Comparing both plant species, no significant differences in NPQ levels under non and long-time saline conditions were observed. But, on 1x salt conditions, Arabidopsis guard cells illustrated significantly lower NPQ levels than Thellungiella guard cells. Thellungiella guard cells from 1x salt group displayed significantly higher NPQ levels than non- and 3x salt treated Arabidopsis during the initial time of the experiment. The levels did not change significantly after two minutes and forty seconds (Fig. 4.14C, encircled). Similarly, during initial phase of the experiment, guard cells of (-) salt grown Thellungiella plants exhibited significantly higher NPQ levels than 1x salt treated Arabidopsis guard cells. In summary, guard cells of salt tolerant Thellungiella can better cope the adverse effects caused by soil salinity on energy consumptions which is evident from their unchanged levels of both photochemical and non-photochemical quenching measurements. Moreover, higher NPQ

levels under saline conditions in this plant type (than Arabidopsis guard cells) demonstrates better protection mechanisms for PSII at high energy irradiations than Arabidopsis guard cells.

Taken together, increase in leaf and guard cells salt ions under saline conditions caused reduced osmotic potential which led to ABA-mediated reduction in transpiration especially in the salt sensitive plant species. Smaller stomatal apertures also give rise to yield penalties in plants, as they are the gateway of environmental CO_2 uptake. Reduced photosynthetic pigment contents (Chl. and car.) and CO_2 uptake resulted in *A. thaliana* plant phenotype (smaller surface area) under 3x salt growth conditions. Irrespective of reduced transpiration in the salt tolerant *T. salsuginea* on 3x salt medium, CO_2 assimilation was not negatively affected. Therefore, its biomass production remained similar under saline and non-saline growth conditions. Similarly, guard cells of salt tolerant plant species also better deal with heat dissipation damage and have higher quantum yield than *A. thaliana* guard cells under saline growth conditions. Higher WUE_i, in *A. thaliana* might be the reason of greater biomass production as compared to *T. salsuginea*.

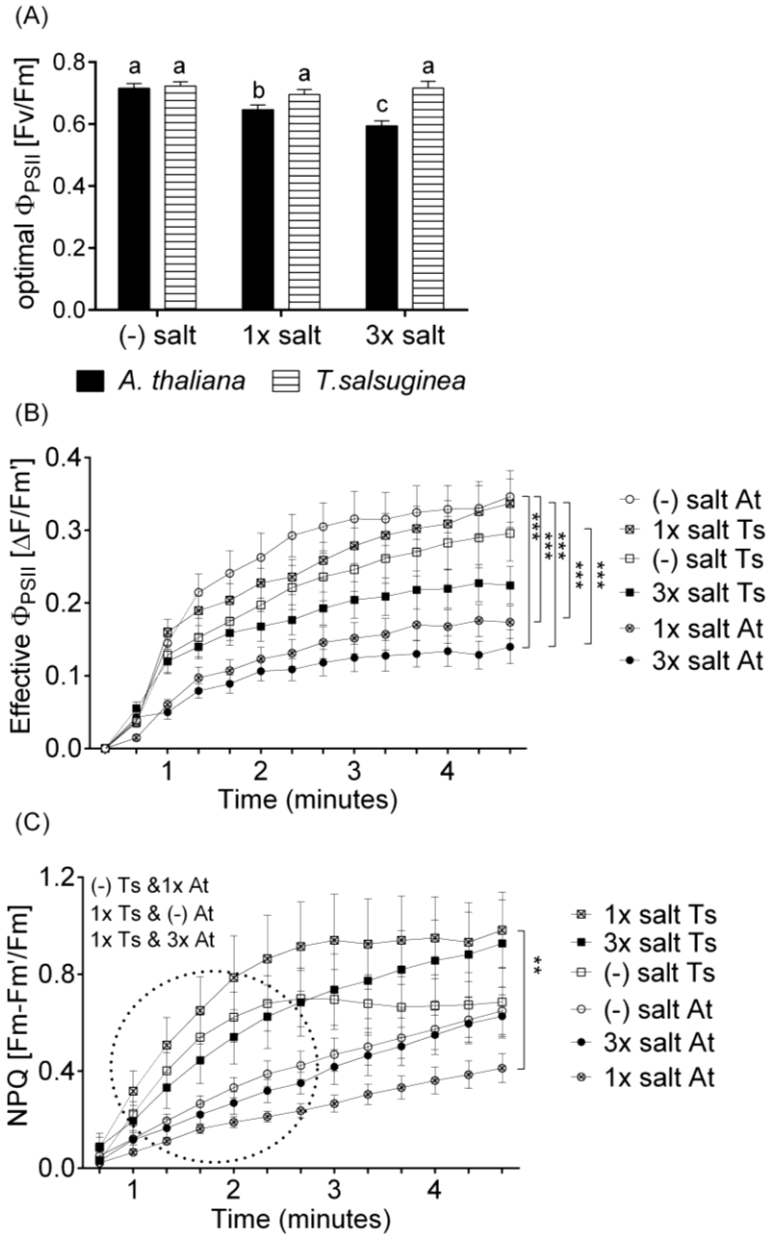

Fig. 4.14 PAM measurements of guard cells

(A) Optimal quantum yield (Fv/Fm), (B) Effective quantum yield(ΔF/Fm') and (C) Non-photochemical quenching (NPQ) of guard cells from both plant species extracted from salt and non-salt treated plants. Encircled area in (C) represents the time points where significant differences were observed between the groups of both plant species (Groups that showed significant differences are cited on top of the encircled area). Each data point represents the mean ±SEM of ten samples. Statistical analysis was performed using Two-way ANOVA followed by Bonferroni post-hoc test at P-value ≤ 0.05. Curve belonging to each treatment is mentioned in parallel and in the similar order as it is ending. Boxes represent *T. salsuginea* and circles represent *A. thaliana*. Where ** and *** represent P- value < 0.01 and ≤ 0.0001 respectively

4.5. Salt induced Gene Expression Changes in Guard Cells

Guard cells of both plant species were further investigated at genome-wide gene expression levels. Physiological and transcriptomic data of both plant species were correlated to get insights into the physiological processes during saline growth conditions.

A set of differentially expressed genes were chosen (based on their p-values or known effects) for qPCR to validate the guard cells microarrays data of both plant species. Correlation performed on the log2 fold changes of gene expression obtained by qPCR and microarrays showed significant linear relationship for both plant species (Fig. 4.15).

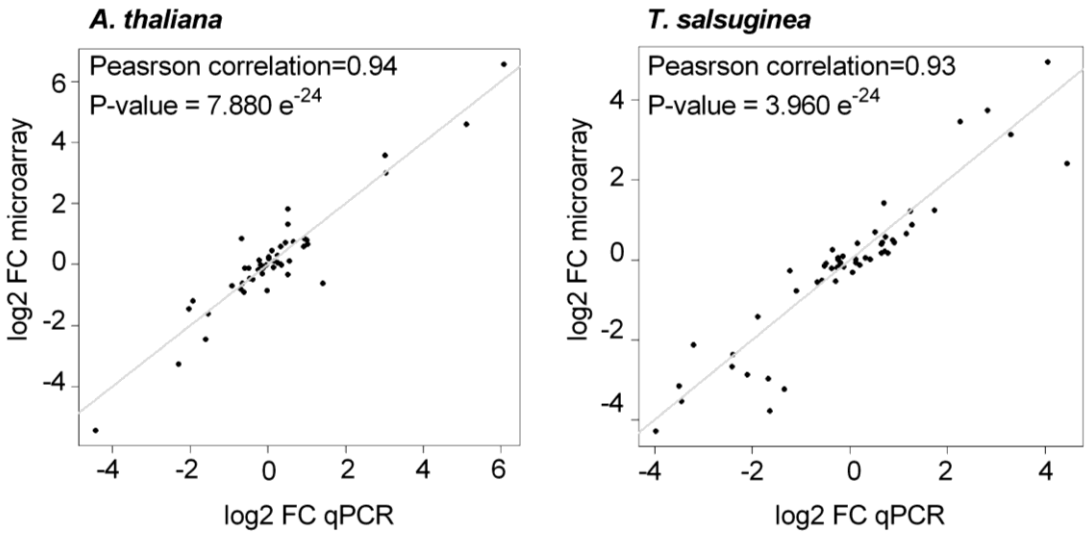

Fig. 4.15 Verification of microarray data

Guard cell microarray data of both plant species were validated by qPCR. Pearson correlation and P-values for each plant species are indicated in its respective box. Genes used for the validation assay are listed in supplement table 8.1.

4.5.1. Salinity Caused more Changes in Gene Expressions of *A. thaliana* Guard Cells than of *T. salsuginea*

Transcriptomic data were obtained from the samples collected from light and dark-adapted plants grown under various degrees of saline conditions. Hence, there were more than one factor that could influence the gene expression patterns i.e. salt and light/dark. Therefore, the transcriptomic data were first analysed to find out the major factors that caused changes in gene expression levels in both plant species. For that, Principal Component Analysis (PCA) was performed. The main component that induced changes in the gene expression of *A. thaliana* guard cells was salt which accounted for 34.3 % of the total variance. Whereas in *T. salsuginea*, it was light/dark which contributed 51.3% of the total variation in gene expression (Fig. 4.16A). This observation implicates that *A. thaliana* is more sensitive to saline growth conditions than *T. salsuginea* as the former showed more differential expression of genes on provided saline environments.

Transcriptomic data of guard cells were further processed to get deeper insight into the differentially expressed genes (DEGs). For that comparisons between each saline growth condition were made (i.e 1x vs (-) salt and 3x vs (-) salt) within the same plant species, separately for light- and dark-adapted growth environments. A gene was considered as

differentially expressed when P-value was < 0.05 and had log2 fold change ≥ 1. *A. thaliana* guard cells displayed more changes in gene expression than *T. salsuginea* under all conditions except of dark phase from 1x salt environments (Fig. 4.16B and C). Long-time salt growth conditions resulted in more DEGs in *A. thaliana* guard cells than of *T. salsuginea* compared to their respective short-time saline environments (Fig. 4.16B and C). Compared to their respective short-time salt grown plants, altogether (i.e up and down regulated genes) *A. thaliana* showed 1.67 and 4.68, and *T. salsuginea* represented 1.37 and 1.39 times more DEGs in light and dark-adapted phases respectively (Fig. 4.16B and C). Among upregulated gene categories, *A. thaliana* exhibited 1.48 and 5.16-fold more DEGs in light and dark-adapted plants respectively on 3x salt growth. Similarly, 1.85 and 4.24 times more down regulated DEGs in light and dark adapted 3x salt grown plants were observed respectively in this plant species. Long-time salt treated *T. salsuginea* showed 1.49 and 1.43 times additional up regulated DEGs in light and dark-adapted growths respectively. Similarly, 3x salt grown plants of *T. salsuginea* exhibited 1.31 and 1.37-fold extra down regulated DEGs in light and dark-adapted phases respectively.

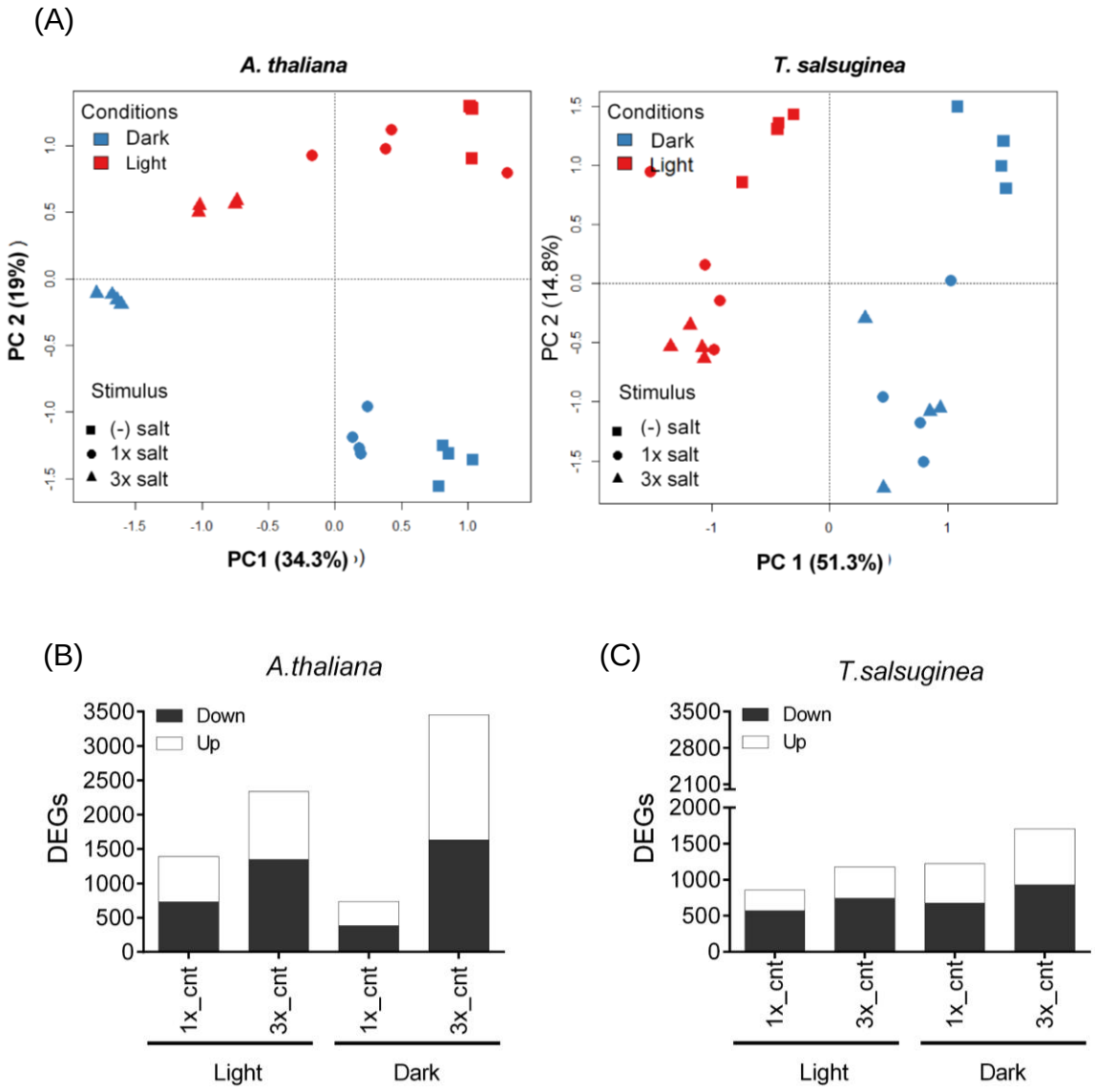

Fig. 4.16 Genome wide gene expression in guard cells of both plant species

(A) Principal Component Analysis of transcriptomic profiles of 24 guard cells samples (12 each from light and dark-adapted plants) from individual plant species grown on control [(-) salt)] and different saline conditions [1x and 3x]. Salt-induced DEGs in guard cells of (B) *A. thaliana* and (C) *T. salsuginea* plants under light and dark-adapted phase. Where 1x_cnt, 3x_cn represent number of DEGs during short and long-time saline growth conditions compared to non-saline growth conditions respectively.

4.5.2. Genes Involved in Membrane-Related Transport Processes are Strongly Influenced in Arabidopsis Guard Cells

Guard cells elements quantifications demonstrated accumulation of sodium in both plant types whereas of chloride only in Arabidopsis under both saline conditions. Similarly,

decrease in guard cells potassium levels was also evident in both plant species under the provided saline environments (Fig. 4.8). Guard cells turgor is modulated by movement of ions, water fluxes and sugars which in turn regulate stomatal apertures. Therefore, genes related to these transport processes were investigated in both guard cell types.

Cation transporters/channels

Overall, expression patterns of the cation transport proteins were more changed in the guard cells of *A. thaliana* than *T. salsuginea* under the given saline conditions (Fig. 4.17). More pronounced changes were observed on 3x salt environments where overall a greater number of cation transporters were upregulated. Arabidopsis gene encoding plasma membrane localized potassium inward channel (KAT2- member of Shaker type potassium channels) was down-regulated and the potassium outward channel (GORK- shaker type potassium channel) was upregulated only in light-adapted guard cells upon 3x salt treatment (Fig. 4.17). Similarly, another member from the same family KAT3, which acts as regulatory subunit for the inward rectifying potassium channel KAT1 (PM located), showed induction in light adapted Arabidopsis guard cells only in 3x salt environments. KAT1 (PM located K^+ inward channel) was the only differentially expressed shaker type potassium channel in Thellungiella guard cells (Fig. 4.17). Expression of this gene was reduced in dark-adapted Thellungiella guard cells after 3x salt treatment. Gene of the tonoplast (TP) localized Na^+/H^+ exchanger NHX4 was the only member from NHX gene family which was differentially expressed (downregulated) in Arabidopsis guard cells upon 1x salt exposure in dark-adapted conditions (Fig. 4.17). Moreover, none of the NHX members were differentially expressed in Thellungiella guard cells under the provided saline environments. The majority of DEGs that encode cation/H^+ or cation/Ca^{2+} exchangers (CAX/CCXs), cyclic nucleotide gated channels (CNGCs) all of which are important for cation and pH homeostasis in guard cells, were up-regulated particularly after 3x salt application in Arabidopsis guard cells (Fig. 4.17). However, only one member (CNGC4) of these gene families was differentially expressed in Thellungiella guard cells (Fig. 4.17). The ATPases genes, VHA-E3 (TP located) and HA2 (PM located) were down-regulated in both guard cell types where former showed reduced expression in Arabidopsis (upon 3x salt) and later in Thellungiella (only in dark upon both salt treatments) guard cells (Fig. 4.17).

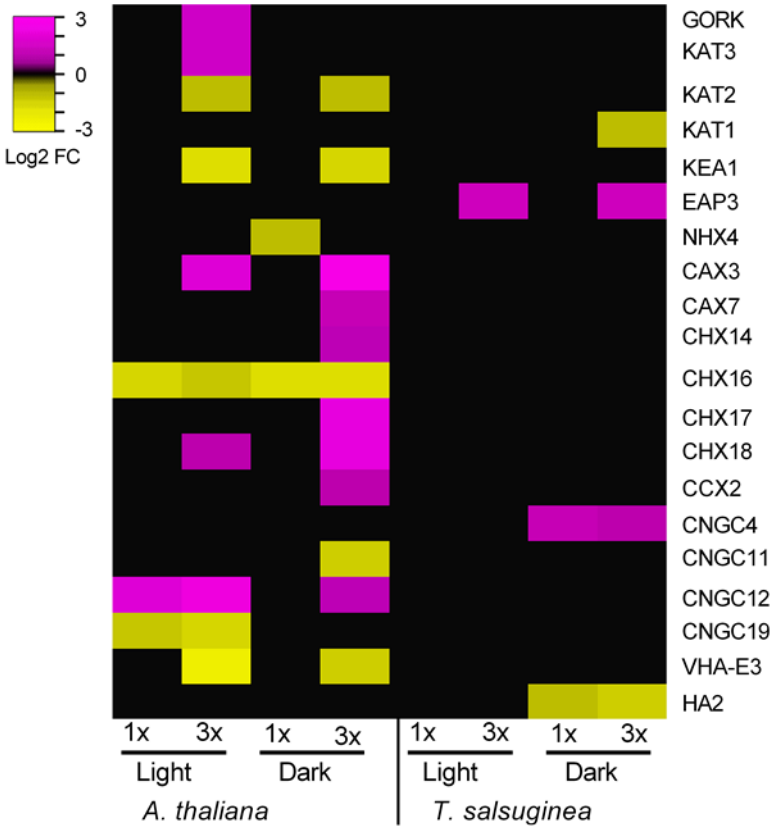

Fig. 4.17 Salt induced changes in expression of cation transport genes

Transcriptomic analysis of cation transport in the light and dark-adapted guard cells of both plant species under short (1x) and long-time (3x) salt conditions. Common names of the genes are represented here in the heat maps. Gene identifiers for each gene from both plant species are listed in the supplementary table 8.2. Genes that are differentially expressed (i.e. p-value ≤ 0.05) and having log2 fold change ≥ 1 are shown in this figure.

Anion transporters/channels

Several genes of the four anion channel classes, Slow Anion Channels (SLACs/SLAHs), Nitrate Transporters (NRTs/NPFs), Chloride Channels (CLCs) and Aluminum activated Malate Transporters (ALMTs) were differentially expressed in guard cells of both plant species under the provided saline growth conditions (Fig. 4.18). SLACs/SLAHs and several NRTs/NPFs were up-regulated in Arabidopsis guard cells under all conditions, except of 1x salt in dark (Fig. 4.18). In contrast, a smaller number of genes from these families were differentially expressed in Thellungiella guard cells under the provided saline conditions. Among these, PM located SLAC1, the tonoplast localized NRT2.7, NRT1.3, and NPF6.4 were the candidates induced in Thellungiella guard cells and all other genes from SLACs/SLAHs and NRTs/NPFs were down-regulated (Fig. 4.18). The CLC genes and ALMTs showed reduced expression in Arabidopsis and Thellungiella guard cells, except CLCa and ALMT14 which were induced in Arabidopsis guard cells (Fig. 4.18).

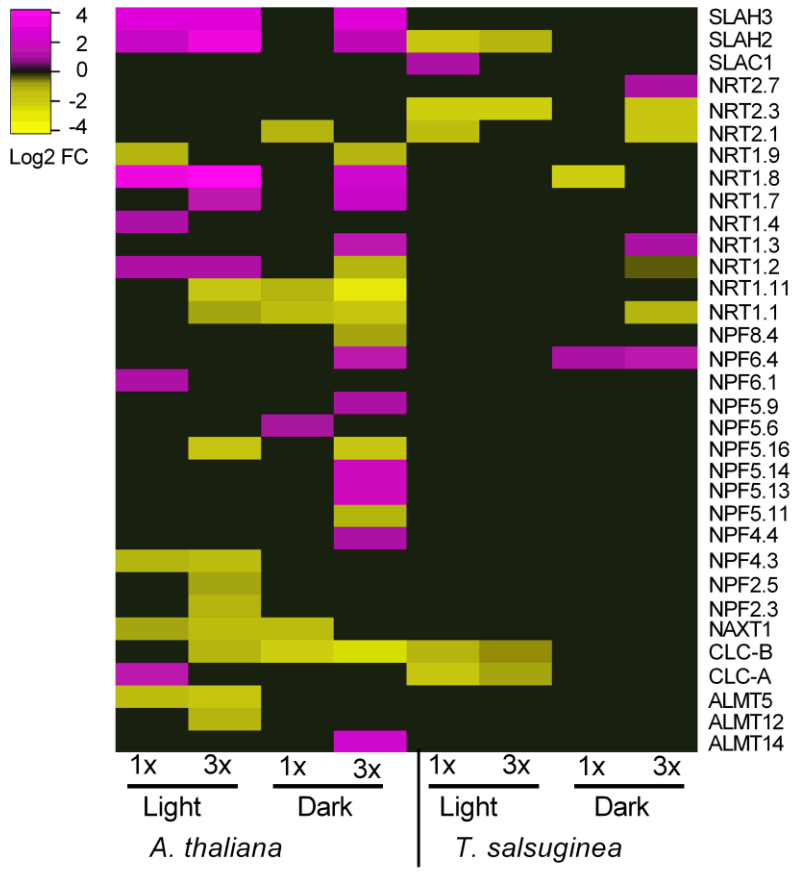

Fig. 4.18 Salt induced changes in expression of anion transport genes

Transcriptomic analysis of anion transport in the light and dark-adapted guard cells of both plant species under short (1x) and long-time (3x) salt conditions. Common names of the genes are represented here in the heat maps. Gene identifiers for each gene from both plant species are listed in the supplementary table 8.3. Genes that are differentially expressed (i.e. p-value ≤ 0.05) and having log2 fold change ≥ 1 are shown in this figure.

Water channels

Transport of ions leads to changes in the osmolarity which influences water flow. Aquaporins are known to be involved in water transport processes in the cells. A total number of 16 and 15 genes encoding aquaporins were found to be differentially expressed in guard cells of *A. thaliana* and *T. salsuginea* respectively under the provided growth conditions (Fig. 4.19). The genes of the tonoplast-localized aquaporins TIPs (tonoplast intrinsic protein) were altogether up-regulated in both plant species except TIP2.1 in light-adapted Arabidopsis guard cells after 1x salt exposure (Fig. 4.19). Genes of the PM located aquaporin family PIP (Plasma membrane intrinsic proteins) showed a contrasting expression pattern in Arabidopsis and Thellungiella guard cells. All differentially expressed PIPs were upregulated in Arabidopsis guard cells after 1x salt application in light adapted plants (Fig. 4.19). Strikingly, no aquaporin gene was differentially expressed after 1x salt in dark-adapted guard cells of Arabidopsis. In Thellungiella guard cells, none of the PIPs were upregulated in 1x salt treated plants. Contrastingly, 3x salt exposure led to induction of more PIPs in guard cells of Thellungiella where three genes were up-regulated and only one was down-regulated (Fig. 4.19).

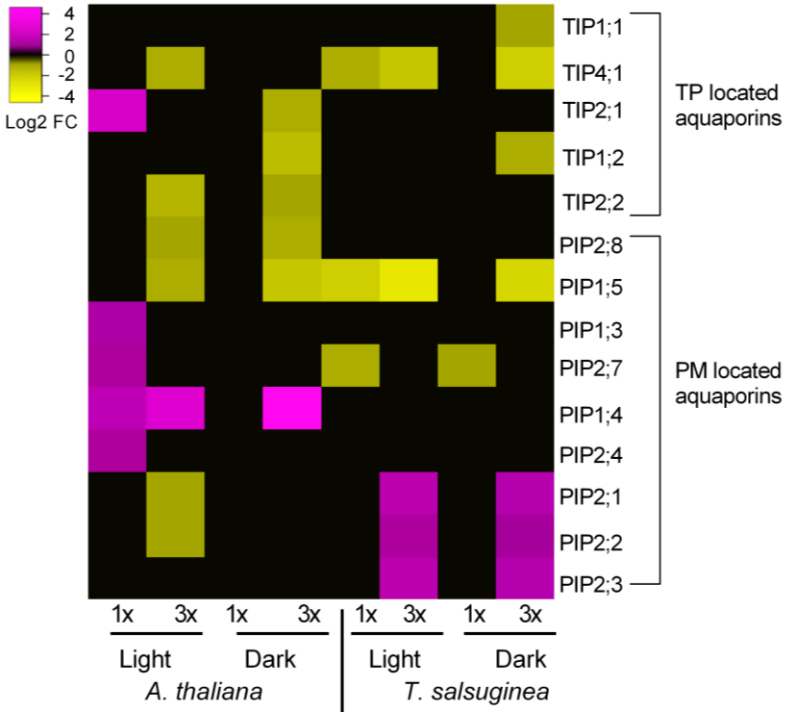

Fig. 4.19 Salt induced changes in expression of water transport genes

Transcriptomic analysis of water transport in the light and dark-adapted guard cells of both plant species under short (1x) and long-time (3x) salt conditions. Common names of the genes are represented in the figure. Gene identifiers for each candidate from both plant species are listed in the supplementary table 8.4. Genes that are differentially expressed (i.e. p-value ≤ 0.05) and having log2 fold change ≥ 1 are shown in this figure.

Sugar transporters

Sugars play important role in the guard cell functioning. The number of differentially expressed genes encoding sugar transporters was also higher in Arabidopsis than in Thellungiella guard cells under the provided saline growth conditions. Altogether, total of 21 and 17 number of genes encoding sugar transporters were found to be differentially expressed in guard cell of *A. thaliana* and *T. salsuginea* respectively (Fig. 4.20). In Arabidopsis guard cells, most of the sugar transporters were induced upon salt exposure in each condition (Fig. 4.20). In contrast, a greater number of sugar transport related genes showed reduced expression in Thellungiella guard cells in both salt concentrations (Fig. 4.20). Only two genes, INT2 and an ERD6-like monosaccharide transporter, were induced in Thellungiella guard cells and all others down-regulated (Fig. 4.20). Moreover, PM located, INT2 was the only salt induced sugar transporter which was induced in Arabidopsis guard cells under all conditions.

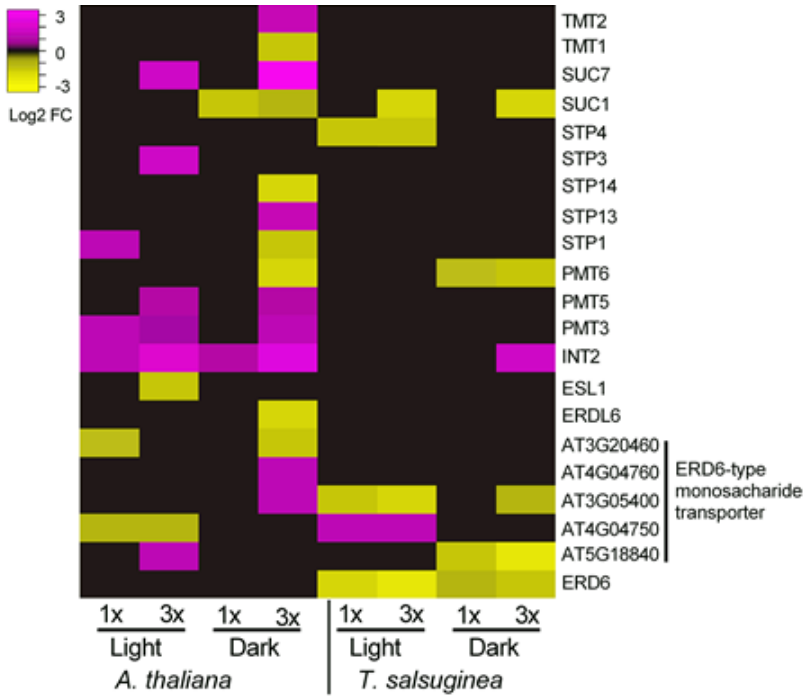

Fig. 4.20 Salt induced changes in expression of sugar transport genes

Transcriptomic analysis of sugar transport in the light and dark-adapted guard cells of both plant species under short (1x) and long-time (3x) salt conditions. Common names of the genes are represented in the figure. Gene identifiers for each candidate from both plant species are listed in the supplementary table 8.5. Genes that are differentially expressed (i.e. p-value ≤ 0.05) and having log2 fold change ≥ 1 are shown in this figure.

4.5.3. Pigments, Energy and Compatible Osmolyte related Metabolisms are Strongly Affected in *A. thaliana* Guard Cells

Whole plant response showed decrease in pigment levels (Chl. a and anthocyanin) (Fig. 4.4) and CO_2 assimilation rates (Fig. 4.11). Plant pigments are essential for light harvesting. Reduced efficiency of Arabidopsis guard cells for light energy consumption suggests decreased chlorophyll pigments in this plant type. Light harvesting is also vital for photosynthesis and hence for energy. To get further insights into both plant species, transcriptomic data were further investigated for pigments, light reaction and energy related pathways. Moreover, increased salt ions in guard cells especially of Arabidopsis suggests changes in osmotic potential. Cells produce compatible osmolytes under osmotic stress conditions. Therefore, metabolism of well-known osmo-protectants were also taken into consideration to observe their role in both guard cells types.

Pigment metabolism

Biosynthesis and biodegradation of chlorophylls (i.e. chlorophyll a and b) were more affected in *A. thaliana* than *T. salsuginea* guard cells at gene expression level (Fig. 4.21). Most of the genes related to biosynthesis of these pigments were strongly reduced in dark phase

under 3x salt environment. However, only one gene was significantly induced in the light phase under both saline conditions. In contrast biosynthesis related genes for chlorophylls remained unchanged in guard cells of *T. salsuginea* (Fig. 4.21). Interestingly, chlorophyll biodegradation was induced in dark adapted Thellungiella guard cells upon 1x salt exposure. Carotenoids, along their other biological functions, play important role in chlorophyll's stability. In *A. thaliana*, genes involved in biosynthesis of carotenoids were upregulated only under 3x salt environment in dark phase (Fig. 4.21). Their expression remained unchanged in all other conditions. Genes responsible for the biodegradation of these pigments were strongly upregulated in light (on both 1x and 3x salt) and in dark (on 3x salt). In contrast, salt tolerant *T. salsuginea*, did not show any changes in gene expression belonging to biosynthesis of carotenoids under all conditions. However, only one gene responsible for carotenoids biodegradation was upregulated at 1x salt growth conditions in dark adapted plants (Fig. 4.21). Anthocyanins are the pigments which are considered as stress response by the plants. Data revealed increased levels of biosynthesis related genes in both plant species under both saline concentrations. However, in *A. thaliana* they were upregulated in dark phase and in *T. salsuginea* in light phase (Fig. 4.21). Biodegradation pathway for the anthocyanins are not known, therefore further insights into the metabolism of anthocyanin was not possible.

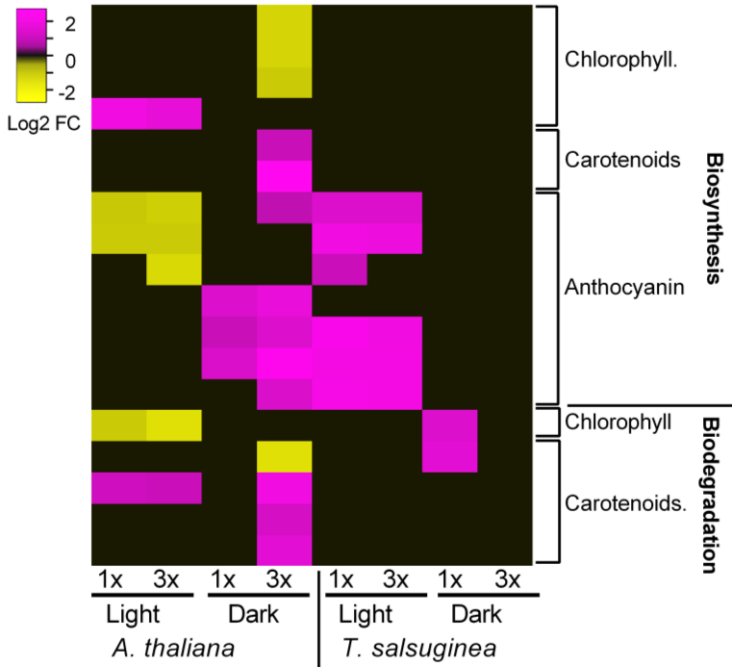

Fig. 4.21 Salt induced changes in plant pigments metabolism

Gene expression patterns involved in biosynthesis and biodegradation of chlorophylls, carotenoids and anthocyanin in the light and dark-adapted guard cells of both plant species under short (1x) and long (3x) term saline conditions. Genes shown in these heat maps are listed in supplementary table 8.6

Light reaction

Chlorophyll fluorescence determined by Pulse Amplitude Modulation (PAM) method revealed that saline conditions induced more changes (decreased optimum and effective quantum yield) in *A. thaliana* guard cells than *T. salsuginea* (Fig. 4.14). This physiological response was further investigated at the gene expression levels. For that genes involved in light harvesting complexes, electron transport and polypeptide subunits were focused. Genes related to light harvesting complexes of both photosystem I and II were strongly reduced under 3x salt conditions in light and dark-adapted *A. thaliana* guard cells (Fig. 4.22). Similarly, expression pattern of genes related to polypeptide subunits that form core complexes in the photosystem I and II were also strongly decreased upon 3x salt application in *A. thaliana* guard cells. In addition, gene coding Cytochrome b6d (cytb6f), an enzyme involved in the transfer of electrons from plastoquinole to plastocyanin, was upregulated only at 1x salt growth in dark. However, genes encoding proteins involved in the electron transfer were strongly downregulated at the transcriptomic level at 3x salt exposure in light and dark-adapted *A. thaliana* guard cells. In contrast, salt growth conditions did not cause any changes in light reaction related genes of Thellungiella guard cells(Fig. 4.22), which is also consistent with their physiological function (Fig. 4.14). Hence these findings are consistent with the PAM results which showed a reduction in effective Φ_{PSII} of chlorophyll fluorescence in Arabidopsis guard cells under both saline conditions but not in guard cells of Thellungiella.

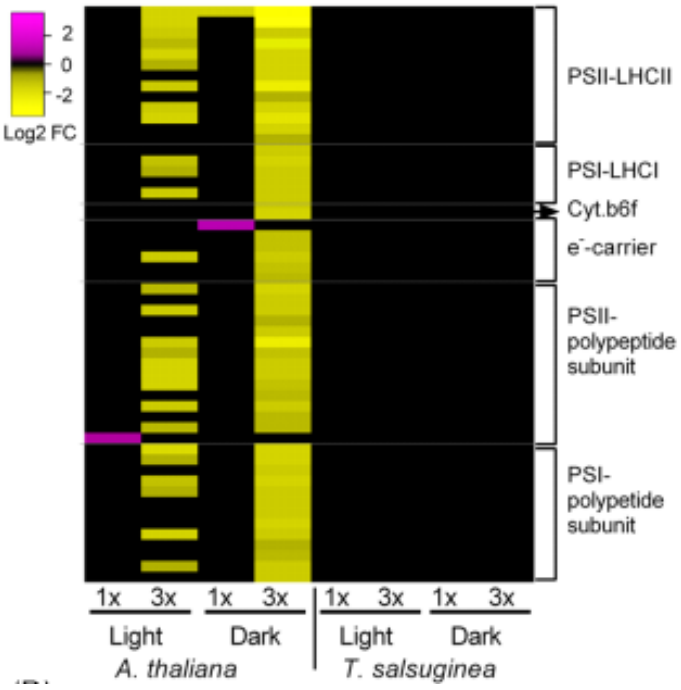

Fig. 4.22 Salt induced changes in light reaction

Gene expression patterns involved in light harvesting and electron transport in PSI and PSII in the light and dark-adapted guard cells of both plant species under short (1x) and long (3x) term saline conditions. Genes shown in these heat maps are listed in supplementary table 8.7. Where PS II-LHCII = Light harvesting complex II of Photosystem II, PS I LHC I = Light harvesting complex I of photosystem I, Cyt. b6f =Cytochrome b6f complex, e^- carrier = electron carrier.

Calvin cycle

Genes involved in the Calvin cycle (carbon fixation for energy production) exhibited reduced expression in *A. thaliana* guard cells under both saline conditions (Fig. 4.23). suggesting that photosynthetic efficiency of the plants is largely affected under the provided saline conditions. In contrast, only one gene belonging to Calvin cycle was induced in light adapted *T. salsuginea* guard cells under both saline environments (Fig. 4.23). These findings were consistent with the decreased CO_2 assimilation rates where it was only affected in Arabidopsis (Fig. 4.11).

Photorespiration

Photosynthesis and photorespiration work in opposite directions (Taiz and Zeiger 2010), hence induction in one would lead to reduction of the other process. Interestingly, *A. thaliana* guard cells showed similar direction (reduction) in the changes of their gene expression for both metabolic processes under provided saline conditions (Fig. 4.23). Contrastingly, *T. salsuginea* did not represent any changes in gene expression belonging to photorespiration.

Tricarboxylic Acid Cycle (TCA)

Gene expression patterns in the Tricarboxylic Acid Cycle (TCA), an energy conserving pathway, revealed reduction in light and dark-adapted *A. thaliana* guard cells under 3x salt conditions. However, the expression patterns did not alter under 1x salt treatment in *A. thaliana* guard cells (Fig. 4.23). In contrast, *T. salsuginea* guard cells showed overall induction in gene transcripts under all conditions except of light adapted 1x salt treated plants where they showed reduced expression (Fig. 4.23).

Carbohydrate metabolism (CHO metabolism)

Gene expression patterns belonging to CHO metabolism were overall reduced in *A. thaliana* guard cells on both saline conditions in light phase (Fig. 4.23). Where five out of nine and eleven out of sixteen were down regulated under 1x and 3x salt conditions respectively (Fig. 4.23). Similarly, dark adapted *A. thaliana* plants showed down regulation of four out of seven and fourteen out of twenty-nine DEGs in guard cells under 1x and 3x salt growth

conditions respectively (Fig. 4.23). In contrast to *A. thaliana*, overall genes belonging to CHO metabolism were induced in *T. salsuginea* guard cells under both saline conditions in light and dark-adapted plants (Fig. 4.23). In short, guard cells CHO metabolism was reduced and induced in Arabidopsis and Thellungiella guard cells respectively upon salt exposures.

Glycolysis and Fermentation

Genes belonging to this pathway were only significantly expressed (up-regulated) in dark-adapted *A. thaliana* guard cells under 3x saline conditions (Fig. 4.23). Whereas, *T. salsuginea* guard cells showed down regulation (1x salt in light) and upregulation of genes under both saline environments (Fig. 4.23). Genes related to fermentation were induced in *A. thaliana* guard cells under all conditions except of dark at 1x salt. Contrastingly, *T. salsuginea* guard cells showed reduction in gene expression for fermentation process. Moreover, only guard cells of dark-adapted *T. salsuginea* plants exhibited significant changes in expression of genes related to this process under both salt concentration (Fig. 4.23).

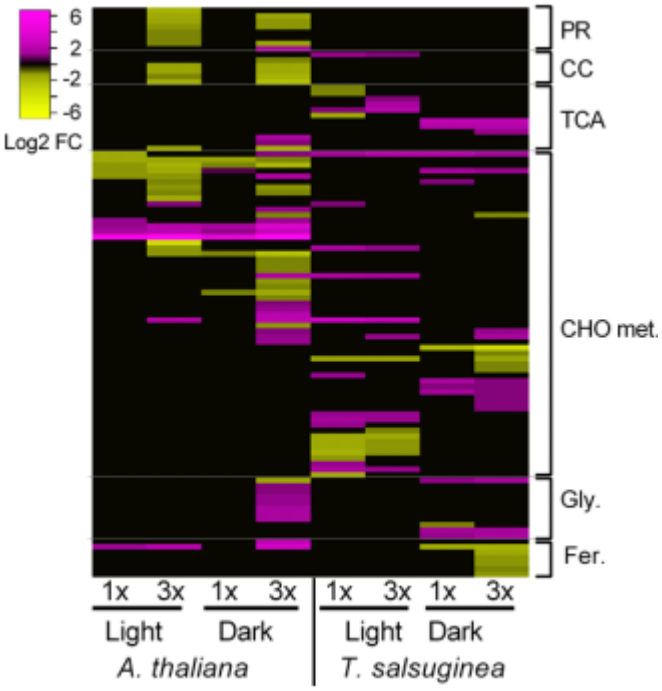

Fig. 4.23 Salt induced changes in energy related pathways

Gene expression patterns for energy production and consumption related pathways in the light and dark-adapted guard cells of both plant species under short (1x) and long (3x) time saline growth conditions. Genes shown in the figure are listed in table 8.8. Where PR = photorespiration, CC = Calvin cycle, TCA = tricarboxylic acid cycle, CHO met. = carbohydrate metabolism, Gly. = glycolysis, and Fer. = fermentation

Metabolism of Compatible Osmolytes

Plant cells produce compatible osmolytes that function as osmo-protectants and maintain cell volume especially under drought conditions (Basu et al. 2016; Munns and Tester 2008). Increased ionic content of both guard cell types especially of Arabidopsis suggests changes in osmotic potential like in leaves. Plants produce proline, glycine betaine, Gamma

Aminobutyric Acid (GABA), Branched chain amino acids (BCCAs) such as leucine, isoleucine and valine as an osmo-protectants. Metabolism of these metabolites were investigated in guard cells of both plant species.

Proline biosynthesis was induced at transcriptomic level in guard cells of both plant species under all the provided conditions except of light adapted Thellungiella guard cells after 1x salt treatment (Fig. 4.24). More number of biosynthesis related genes were induced in Arabidopsis guard cells under 3x salt. None of the proline biodegradation related genes were induced in Thellungiella guard cells however, mix pattern of gene expression was observed in Arabidopsis guard cells. BCAAs biosynthetic related genes were reduced in guard cells of both plant species (Fig. 4.24). BCAAs biodegradation was overall induced at transcriptomic level in Arabidopsis guard cells except 1x saline conditions in light. However, Thellungiella guard cells showed opposite pattern. GABA another osmo-protectant was induced at transcriptomic level in Arabidopsis guard cells under all conditions except in 1x salt treatment under light phase (Fig. 4.24). In contrast to Arabidopsis, Thellungiella guard cells either showed unchanged transcriptomes or reduction in transcriptomes related to GABA biosynthesis (Fig. 4.24). None of the genes involved in GABA biodegradation were significantly changed in both plant species under the provided growth conditions.

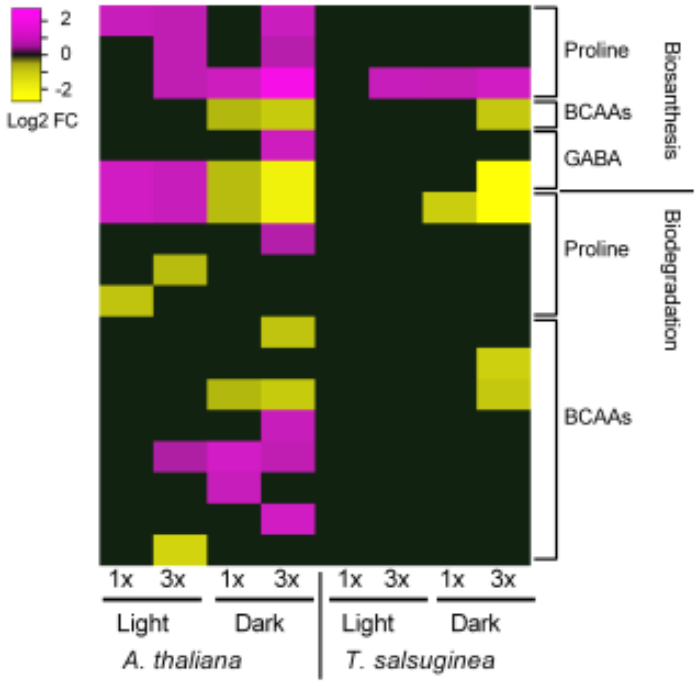

Fig. 4.24 Salt induced changes in metabolism of compatible osmolytes

Gene expression patterns for biosynthesis and biodegradation of compatible osmolytes in the light and dark-adapted guard cells of both plant species under short (1x) and long (3x) time saline growth conditions. Genes shown in the figure are listed in table 8.9. Where BCAAs = Branched Chain Amino Acids, GABA = Gamma Aminobutyric Acid, FC = Fold Change.

In summary, biosynthesis of osmo-protectants suggests osmotic stress in guard cells of both plant species. However, metabolism processes related to energy were more negatively affected in *A. thaliana* than *T. salsuginea* under both saline conditions which ultimately would have impact on growth and development.

4.5.4. Salt Caused more Changes in ROS Scavenging Related Gene expression in Arabidopsis C*ompared to* Thellungiella Guard Cells

An increase in reactive oxygen species (ROS) has been reported under abiotic stress conditions including salt stress. Stomatal closure under saline growth conditions resulted in reduced transpiration, CO_2 assimilation and hence less fixation by Calvin cycle especially in Arabidopsis plants which was evident in the guard cells transcriptomic data. Under these conditions, excessive light excitation affects the rate of electron transport (Bose et al. 2013) which was also evident in Arabidopsis guard cells gene expression data (Fig. 4.22). Under these circumstances, ROS production via O_2 reduction is increased (Bose et al. 2013). To get an overview on ROS homeostasis, genes involved in ROS biosynthesis and scavenging were investigated in the guard cells of both plant species. Arabidopsis guard cells displayed more changes than Thellungiella in ROS homeostasis related genes (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Moreover, Thellungiella guard cells represented overall downregulation in ROS metabolism under the provided conditions. A greater number of genes were upregulated in light-adapted Arabidopsis guard cells under both saline environments. However, dark adapted Arabidopsis guard cells exhibited down regulation of genes in 1x salt and a mix pattern in gene regulation was observed under 3x salt conditions **(**Fehler! Verweisquelle konnte nicht gefunden werden.**)**. Differentially expressed genes in both plant species were involved both in conversion of O_2 to superoxide (O_2^-) (e.g by oxidases, ascorbate) and its further conversion to H_2O_2 (e.g by ascorbate, glutathione, thioredoxin, heme, superoxide dismutase). A greater number of H_2O_2 scavengers (catalases and peroxidases) were differentially expressed in Arabidopsis guard cells under the provided saline growth conditions.

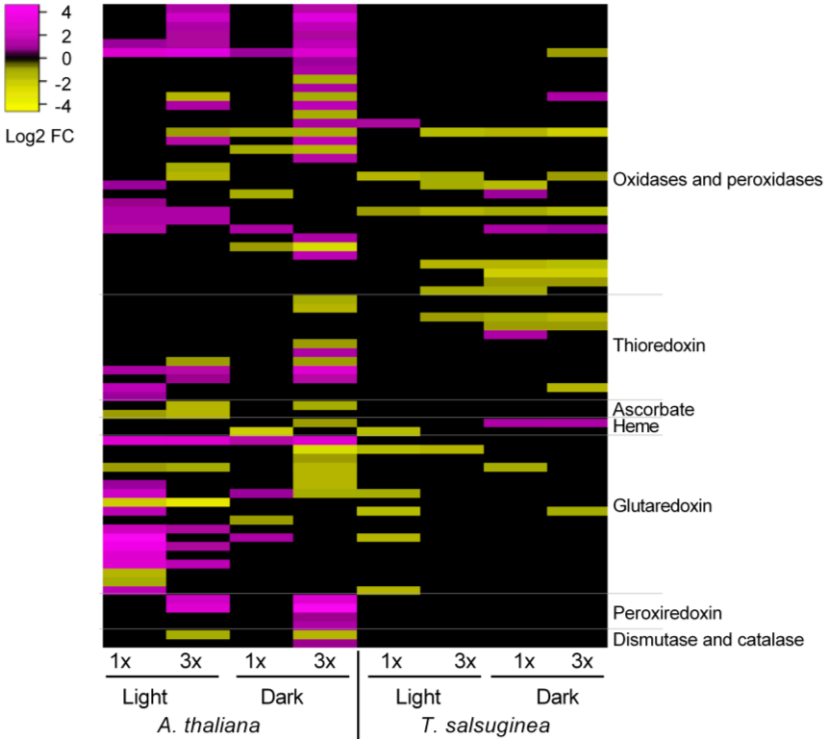

Fig. 4.25 Salt induced changes in ROS

Gene expression analysis for both enzymatic (dismutase, catalase, ascorbate peroxidase) and non-enzymatic (Ascorbate and glutathione) ROS scavenging mechanisms in light and dark-adapted guard cells of *A. thaliana* and *T. salsuginea* under 1x and 3x salt growth conditions. Gene from both plant species shown in the figure are listed in the supplementary table 8.10.

4.5.5. Salt Induced Phytohormones Metabolism and Signaling was more Profound in *A. thaliana* Guard Cells

Phytohormones play pivotal role in plant development, growth and stress induced responses. Plants phenotypic data showed reduction in rosette surface area (Fig. 4.5) and leaf colour (Fig. 4.2A) especially of *A. thaliana* on 3x salt growth conditions. Similarly, the reduction in transpiration rates (Fig. 4.10) and increase in ABA levels in guard cells (Fig. 4.9) of both plant species were also observed. Altogether, phenotypic and physiological observation gives hint in the changes of metabolism and signalling of phytohormones under saline growth conditions. To get further insights into changes of metabolism and signalling of phytohormones in the guard cells of both plant species, transcriptomic data was analysed for the genes involved in these processes.

Along with their role in growth and development phytohormones are also involved in stomatal regulation (Misra et al. 2015; Acharya and Assmann 2009). Both biosynthesis and biodegradation related genes for phytohormones were differentially expressed especially in Arabidopsis guard cells under the provided salt growth conditions. Contrasting responses were observed in gibberellic acid biosynthesis related genes which showed downregulation in Arabidopsis and upregulation in Thellungiella guard cells under all conditions and at 1x salt of

dark phase respectively (Fig. 4.26A). Moreover, none of the phytohormones degradation related genes were differentially expressed in Thellungiella guard cells under all conditions (Fig. 4.26A). In line with phytohormone biosynthesis, biodegradation related genes were also differentially expressed in Arabidopsis guard cells. A mix pattern of up and down regulated genes for phytohormones biodegradation was observed in the provided conditions except for cytokinin, jasmonate and gibberellic acid where all the differentially expressed genes were downregulated. As all the phytohormones were not quantified therefore the data could not be verified at physiological level.

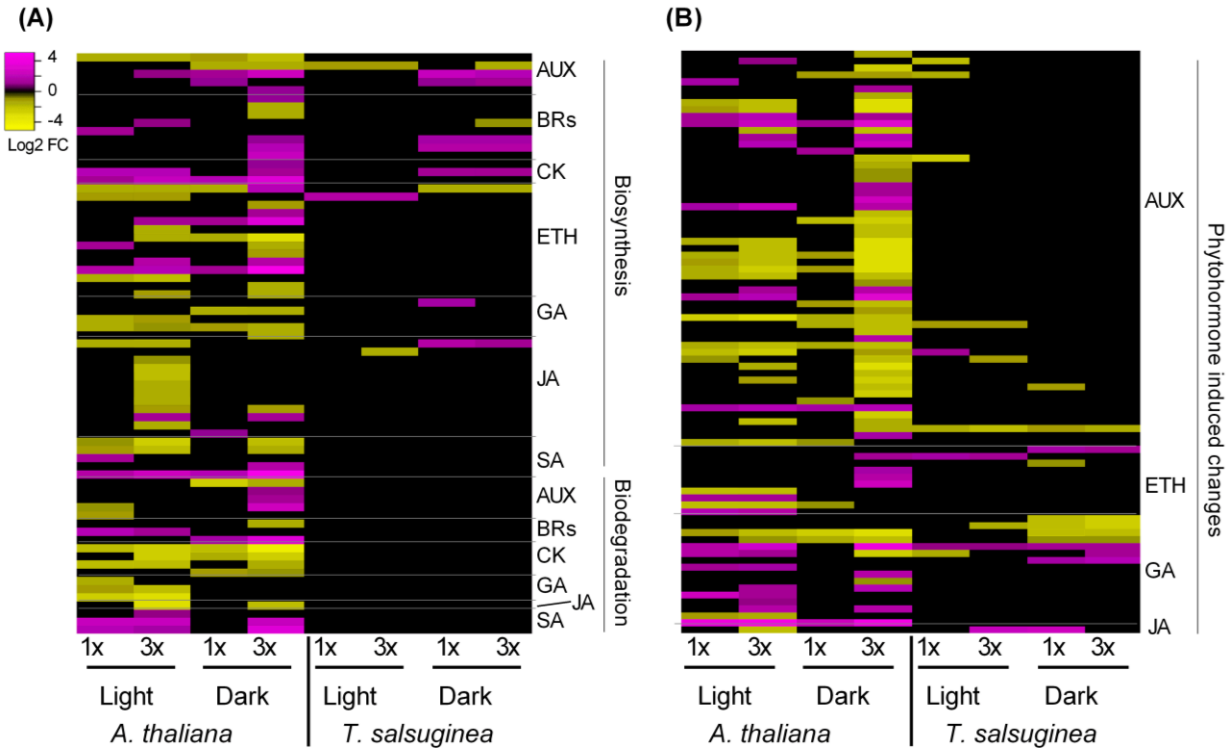

Fig. 4.26 Salt induced changes in metabolism and signalling of phytohormones in the guard cells

Transcriptomic analysis of the genes involved in phytohormones (A) Biosynthesis and biodegradation, and (B) induced changes n guard cells of both plant species during saline growth conditions. Gene identifiers for each gene involved in different pathways from both plant species shown in the figure are listed in the supplementary table 8.11. Where AUX= Auxin, BRs = Brassinosteroids, ETH= ethylene CK = Cytokinins, GA= Gibberellic Acid, JA= Jasmonic Acid, SA = Salicylic Acid.

Among all phytohormones, only the genes under the control of auxin, ethylene gibberellic acid (GA) and jasmonic acid (JA) were significantly altered in expression in guard cells of both plant species under salinity. However, Arabidopsis guard cells showed more profound changes in the gene expression under the provided conditions (Fig. 4.26B).

Arabidopsis plants undergone 3x salt conditions represented greater number of differential gene expression compared to 1x salt growth environment (Fig. 4.26B). This salt specific pattern was not consistent in Thellungiella guard cells where equal number of genes were differentially expressed in both saline environments. Role of these phytohormones has been shown in stomatal regulation (Misra et al. 2015). Auxin has been shown to be involved in stomatal opening (Levitt et al. 1987; Song et al. 2006). Similarly, its role in inhibiting ABA induced stomatal closure via promoting ethylene biosynthesis has also been reported in *A. thaliana* (Tanaka et al. 2006). Ethylene has dual role in stomatal regulation where it can open and close the stomata depending on the conditions (Misra et al. 2015). Similarly, GA and JA induced stomatal regulation has also been reported. Among all phytohormones, genes responsive to auxin were found to be more differentially expressed in both plant species under the provided saline growth conditions.

Taken together, the pattern of gene expression indicates that phytohormone metabolism and signalling was significantly changed under the provided saline conditions. However, *A. thaliana* guard cells represented more changes in metabolic and signalling related pathways. Although gene expression patterns controlled by all phytohormones were observed in both plant species, yet, auxin induced changes in gene expressions were more obvious compared to other phytohormones. Genes under the control of auxin, GA, JA and ethylene showed differential expression in both guard cells which demonstrates their role in guard cell physiology. This finding is persistent with the previous reports where they showed their role in stomatal movement (Misra et al. 2015). Moreover, vital role of these phytohormones has been well established in growth and development, therefore salt induced changes in their metabolism and signalling suggest abruption in optimum plant growth.

4.5.5.1. The Number of Genes Involved in ABA Metabolism was Higher in Arabidopsis Guard Cells than Thellungiella

ABA is known as stress phytohormone and plays important role in stomatal closure by decreasing the turgor in the guard cells. Both guard cells types were investigated in detail for ABA metabolism and under the provided growth conditions. Physiological data represented ABA accumulation in guard cells which led to stomatal closure in both plant species. Guard cells transcriptomic data demonstrated differential expression of ABA biosynthesis related

genes in both plant species under salinity. Interestingly, no DEGs were detected in dark-adapted Arabidopsis guard cells under 1x salt while 3x salt caused differentially gene expression of almost all genes of the ABA biosynthesis pathway under both light and dark-adapted phases (Fig. 4.27). Furthermore, a smaller number of ABA biosynthesis genes were induced in light adapted Arabidopsis guard cells under both salt environments. In contrast to Arabidopsis, Thellungiella guard cells displayed induction of only the NCED biosynthesis genes in dark-adapted conditions under both saline conditions (Fig. 4.27).

Genes involved in ABA transport processes across the membranes were also investigated to get to know whether the ABA increase in guard cells is due to biosynthesis or its import. None of the transporters were differentially expressed in the guard cells of *T. salsuginea* under the provided conditions (Fig. 4.27). *A. thaliana* guard cells exhibited altogether four ABA transporters on provided salt conditions. These included the members of the ATP-binding cassette (ABC) and DTX/MATE type transporters. Among these ABCC2, ABCG30and ABCG40 are known to be ABA importers whereas DTX50 is known as ABA exporter. All of them were induced in the guard cells of dark-adapted plants on 3x salt medium (Fig. 4.27). However, none of the transporters were differentially expressed in the dark-adapted plants of 1x salt group (Fig. 4.27). In the light adapted phase, only ABCG30 was induced and ABCG40 was reduced in expression in *A. thaliana* guard cells on 3x salt conditions (Fig. 4.27). Similarly, plants from 1x salt growth conditions showed differential expression of only ABCG40 (reduction) during light adapted environments (Fig. 4.27). Genes involved in ABA catabolism were also included in the investigation to get to know whether ABA is being biodegraded under the provided conditions. Members of CYP707As were differentially expressed in both types of guard cells. However, CYP707A4 was the only candidate induced in Thellungiella (under all conditions) and Arabidopsis guard cells (under 3x salt dark) (Fig. 4.27).

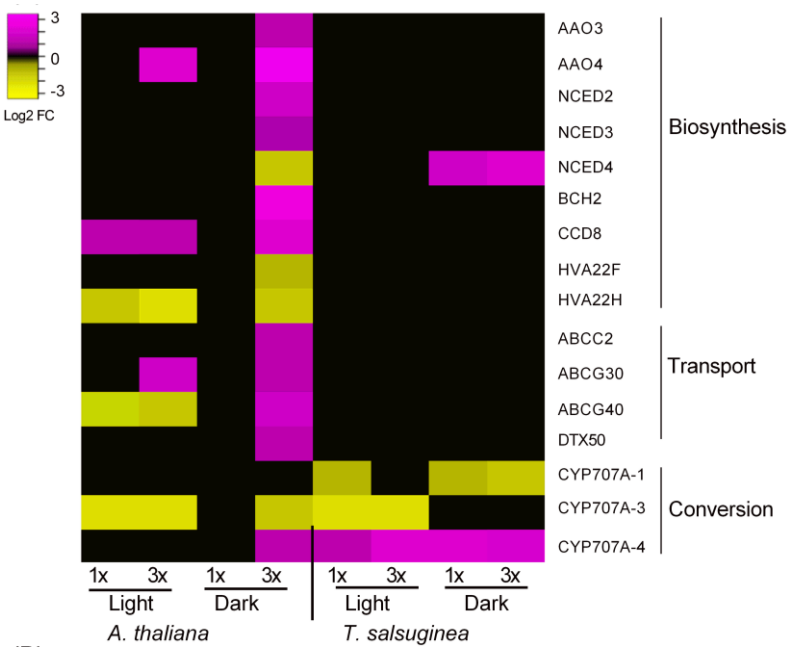

Fig. 4.27 Salt induced changes in ABA metabolism

Gene expression patterns related to ABA biosynthesis, transport and its conversion to inactive forms in the light and dark-adapted guard cells of both plant species under short (1x) and lomg (3x) term saline growth conditions. Identifiers of the genes from both plant species shown in the figure are listed in the supplementary table 8.12.

4.5.5.2. ABA Signalling was more Affected in Arabidopsis Guard Cells on Salt Growth Medium

Genes involved in the ABA signalling pathway such as ABA receptors, phosphatases, and kinases displayed contrasting expression patterns in guard cells of both plant species (Fig. 4.28). In light adapted conditions, Arabidopsis guard cells of 1x salt group represented induction of only two ABA receptor genes (PYL5 and 6) whereas none of the other were differentially expressed under these conditions. In contrast, 3x salt grown Arabidopsis plants exhibited reduced expression of two ABA receptor genes (PYL2 and 9) and induction of only one gene (PYL5) in light adapted environments. In contrast to light conditions, all differentially expressed ABA receptors in Arabidopsis guard cells presented decreased expression on both salt conditions. Thellungiella guard cells exhibited reduction in gene expression of all ABA receptors under all conditions (Fig. 4.28).

Phosphatases, another major component in the ABA signalling showed induction in *A. thaliana* guard cells under all conditions. Among nine members of the group A PP2C type phosphatases, altogether seven were differentially expressed in *A. thaliana* guard cells under the provided growth environments (Fig. 4.28). In line with ABA biosynthesis and receptor genes expression, a greater number of genes encoding phosphatases were differentially expressed in dark adapted Arabidopsis guard cells of the 3x salt group (Fig. 4.28). Interestingly none of the phosphatases encoding genes were reduced in expression in both guard cell types.

HAI1 was the only phosphatase that was equally induced in Arabidopsis guard cell under all conditions. Furthermore, this was also the only candidate gene that was induced in Thellungiella guard cells, hence was shared in both guard cell types (Fig. 4.28). Similarly, genes encoding PIA1 was the only phosphatase that was shared during both salt exposures in dark adapted guard cells of Arabidopsis.

Protein kinases are important players in the ABA signalling. These include calcium dependent, CBL interacting serine-threonine and SNF1 related protein kinases. A greater number of CBL interacting serine-threonine protein kinases (altogether 12) was found to be differentially expressed in the guard cells of *A. thaliana* under the provided growth conditions (Fig. 4.28). *A. thaliana* guard cells showed increase in gene expression encoding CIPK15 and CIPK19 in light phase and decrease gene expression of CIPK17 after 1x salt exposure (Fig. 4.28). Genes encoding CIPK4, 15,16,19 and 22 were induced whereas CIPK17 and 20 were reduced upon 3x salt exposure in guard cells of light adapted *A. thaliana* plants (Fig. 4.28). CIPK 15 and 19 were the only kinases that showed induction and CIPK17 showed reduction in gene expression in *A. thaliana* guard cells under both saline conditions in light phase. Similarly, in the dark-adapted Arabidopsis guard cells demonstrated increase in only CIPK5 gene transcripts on both saline environments (Fig. 4.28). None of the other CIPKs were differentially expressed in dark phase of 1x salt. However, a greater number of CIPKs were differentially expressed in *A. thaliana* guard cells under 3x salt conditions in dark adapted phase. These included the induction in gene expression of CIPK10, 16, 18, 19, 22 and 25 whereas reduction of CIPK17, 20 and 21 respectively (Fig. 4.28). Transcripts of CIPK21 were reduced in the guard cells of light adapted *T. salsuginea* after 1x salt growth conditions (Fig. 4.28). Rest of the CIPKs were not differentially expressed in *T. salsuginea* guard cells. Similarly, none of the CIPKs were differentially expressed in the light adapted *T. salsuginea* guard cells on 3x salt growth conditions. CIPK1 was the only candidate that showed differential expression in dark-adapted *T. salsuginea* guard cells under both saline conditions (Fig. 4.28). Whereas rest of the CIPKs were not differentially expressed under these conditions.

Among SNF1 related protein kinase (SnRKs), SnRK 2.8 was the only candidate that displayed differential expression (expression was reduced) in *A. thaliana* guard cells in light adapted phase under both salt concentration (Fig. 4.28). Other members from the same class remain unchanged in 1x salt conditions. In the light adapted *A. thaliana* guard cells, expression

of SnRK2.5 was reduced and SnRK2.8 was induced after 3x salt conditions (Fig. 4.28). None of the members from the same group were differentially expressed on 1x salt growth in dark adapted phase. Similarly, only one gene i.e SnRK2.9 showed induction in the dark-adapted *A. thaliana* guard cells on 3x salt growth (Fig. 4.28). None of the members of SnRK2s were differentially expressed in *T. salsuginea* guard cells under 1x and 3x saline conditions in light and dark-adapted phases respectively. SnRK2.9 was the only candidate that showed differential expression (expression was induced) in the guard cells of *T. salsuginea* under 1x and 3x salt growth in dark and light adapted phases respectively (Fig. 4.28). Other members remain unchanged under these conditions.

Among the calcium dependent kinases (CPKs), CPK28 and CPK32 were reduced in expression on both salt concentrations in guard cells of light adapted *A. thaliana* plants (Fig. 4.28). None of the CPKs were differentially regulated in dark adapted plants after 1x salt conditions (Fig. 4.28). However, 3x salt growth led to decrease in CPK31 transcripts in dark adapted *A. thaliana* guard cells. Guard cells of *T. salsuginea* exhibited reduction in gene expression of CPK1 and CPK32 in light adapted plants under 1x and 3x saline conditions respectively.

In summary ABA receptors exhibited contrasting patterns in guard cells of both plant species. Moreover, less members of the phosphatase and kinases (both CIPKs and CPKs) were differentially expressed in *T. salsuginea* guard cells thus suggesting regulation of genes is more changed in *A. thaliana* guard cells under saline conditions.

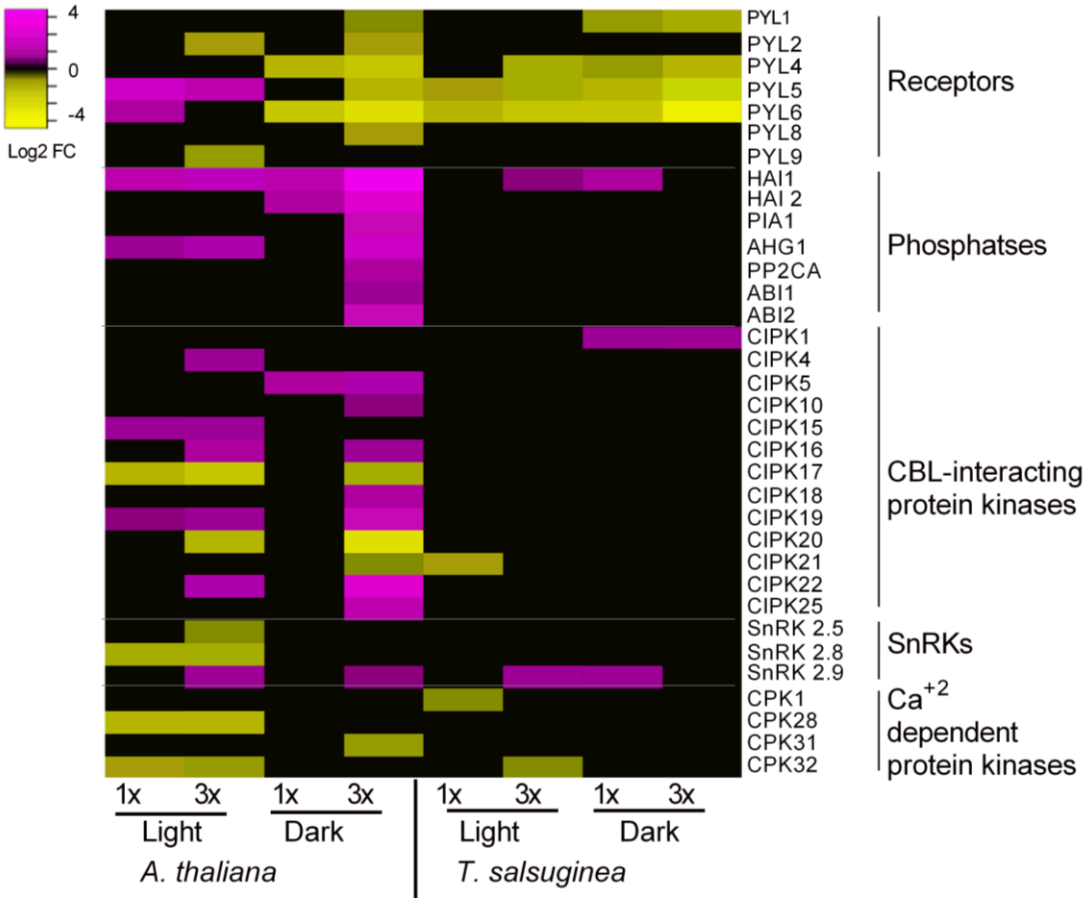

Fig. 4.28 Salt induced changes in ABA signalling

Gene expression patterns for ABA receptors, phosphatase and kinases in the light and dark-adapted guard cells of both plant species under short (1x) and long (3x) term saline growth conditions. Identifiers of the genes from both plant species shown in the figure are listed in the supplementary table 8.13.

5. Discussion

Guard cells are key regulators for gaseous exchange and hence are vital for crop productivity as well as for ecosystem. In the past they have been well characterized under different climatic conditions such as illumination, CO_2, humidity and temperature. However, their function under saline growth conditions is scant. Climate change also gives rise to increasing soil salinity along with other adverse effects, therefore understanding guard cells functioning on these conditions would be useful for crop yield improvement. For that comparative studies of salt sensitive and salt tolerant model plants is highly advantageous. Present study demonstrates whole plant responses of salt sensitive *A. thaliana* and salt tolerant *T. salsuginea* under single and multiple times of salt exposures. Moreover, guard cells physiological and transcriptomic responses have been documented which explains the possible differences in both plant species under diverse conditions of saline growth environments.

5.1. Salt Induced Changes in Leaves

Whole plant responses of both plant species were recorded, and comparisons were made to get differences in both plant species under saline growth mediums. For that, phenotypic, chemical and biochemical parameters of rosette leaves were documented, and correlated with the guard cells performance in terms of their additive effect on plant functioning.

5.1.1. Selective Uptake of Salt Ions and Higher Leaf Potassium Levels is the Key for Salt Tolerance in *T. salsuginea*

T. salsuginea plants accumulated less salt ions in leave than *A. thaliana* under the provided saline conditions. This suggests that this plant has developed mechanisms to exclude ions from the photosynthetic tissues. This could be achieved by keeping low translocation of these ions from roots to shoots and/or sequestering these ions into the vacuoles. Different studies have shown that *T. salsuginea* have higher expression of SOS1 (Na^+/H^+ antiporter) in roots and shoots as compared to *A. thaliana* (Oh et al. 2010; Taji et al. 2004; Kant et al. 2006) which make possible for this plant species to keep lower levels of sodium in the aerial parts. One of the major negative effects of NaCl stress is the decrease in potassium concentration

and the resulting drastic shift in the K^+/Na^+ ratio in the cytosol of plant cells which negatively effects celluar functioning and plant growth. The ability to keep the potassium concentration constant under saline environments is therefore considered a key feature of tolerant plant species (Munns and Tester 2008). Again, no negative effect of salt application on *T. salsuginea* leaf potassium content was observed in the present study during the 1x salt application, but only in the 3x salt conditions. Present study demonstrated higher levels of potassium in *T. salsuginea* leaves than *A. thaliana* leaves during non-saline conditions which was consistent with the earlier studies conducted by (Inan et al. 2004; Prerostova et al. 2017; Stepien and Johnson 2009). Higher potassium content in the plant tissue is maintained by increasing potassium uptake from the soil. It has been reported earlier that *T. salsuginea* has an effective mechanism to discriminate between sodium and potassium which makes it possible to keep high K^+/Na^+ ratios in the plant tissues (Volkov et al. 2004). Earlier reports also suggest that *T. salsuginea* has higher root/shoot ratios of potassium than *A. thaliana* under both non-saline and saline growth environments (Aleman et al. 2009). Moreover, potassium uptake and transportation in *T. salsuginea* was also higher than *A. thaliana* under higher saline conditions. Similarly in another study it was shown that the K^+ inward channel HAK5, is more expressed in roots of salt tolerant *T. salsuginea* than salt sensitive *A. thaliana* which makes possible to keep higher levels of potassium in the plant tissues (Alemán et al. 2009).

Being a micronutrient, chloride levels are not required that high, and most of the plants contain it in range of 1-20 mg/g DW in the aerial parts (Geilfus 2018a) which is also reflected in our studies. Chloride being a micronutrient is required for different functions such as photosynthesis, osmoregulation, growth, turgor regulation in higher plants (Geilfus 2018b). However, elevated levels of this anion lead to dysfunction of these processes. Less accumulation of leaf chloride (Cl^-) in leaves of *T. salsuginea* than *A. thaliana* under short and long- time saline growth conditions suggest tight control over its uptake in salt tolerant plant species. *T. salsuginea* has been suggested as chloride excluder by reducing chloride loading to the xylem stream (Geilfus 2018a). Low accumulation of Cl^- in *T. salsuginea* leaves suggests this plant is excluder of chloride which could be achieved by restricting chloride loading into the xylem. When grown on saline soils, the accumulation of chloride ions (Cl^-) in shoot tissues is more commonly associated with a reduction in plant growth and fruit yield than the accumulation of sodium ions (Na^+) in the shoot (Walker et al. 1997; Munns and Tester 2008;

Teakle and Tyerman 2010; Storey and Walker 1998). Therefore, small rosette leaves and dead phenotype of *A. thaliana* plants after 3x 200 mM saline conditions could also be due to increased levels of chloride.

5.1.2. Long-time Exposure of Salt Results in Reduced Plant Growth of *A. thaliana*

Elevated levels of salt ions in leaves of both plant species led to reduction in osmotic potential especially in *A. thaliana* on 3x salt growth. Plants do accumulate compatible osmolytes (e.g proline, GABA) during osmotic stress conditions. (Sanders and Arndt 2012). *T. salsuginea* has previously been reported to have more proline content in leaves even under non-stressed conditions and the levels even increase under saline conditions (Lee et al. 2016). Higher levels of proline in roots and shoots under non-salt conditions suggest its pre-adaptation to extreme abiotic stress conditions (Kant et al. 2006; Lee et al. 2016; Bartels and Dinakar 2013). Similarly, sugars (e.g. sucrose, glucose, fructose) were also reported to be increased under saline conditions. Salt induced increase in phytohormone ABA in leaves had negative effect in CO_2 assimilation in A. thaliana plants especially on 3x salt medium. However, under control conditions, the assimilation rates were similar (4.4.2 E). Higher CO_2 assimilation rate in *T. salsuginea* has also been reported earlier by (Stepien and Johnson 2009) in saline conditions. Despite of lower transpiration rate, *T. salsuginea* manged to keep CO_2 assimilation levels under 3x salt conditions. Stomatal density of *T. salsuginea* is almost the twice of *A. thaliana*, this feature may help in even distribution of CO_2 to the photosynthetic mesophyll cells which might lead to higher photosynthetic activity of salt tolerant plant (Stepien and Johnson 2009). Unchanged CO_2 assimilation of salt tolerant, T. salsuginea, on 3x salt conditions led to increase intrinsic water use efficiency in this plant species. Though the differences were not obvious when compared to control plants, however, when compared to *A. thaliana*, *T. salsuginea* eliminated the differences in WUEi on 3x salt growth (Fig. 4.12). *A. thaliana* plants exhibited more biomass than *T. salsuginea* under all conditions which could be due to higher WUE_i. Uptake of CO_2 is used in carbohydrate synthesis in the Calvin cycle. Reduced CO_2 assimilation in A. thaliana plants on 3x salt medium might led to limited food supply which resulted in reduced growth in this plant species. Moreover, Rubisco activity in *T.*

salsuginea has been reported higher than of A. thaliana (Wiciarz et al. 2015). This feature is useful to combat with limited CO_2 supply in this plant species.

Long-time salt treatment caused reduction in plant pigments content (i.e chlorophyll a and carotenoids in *A. thaliana* plants. The decrease in the amount of these pigments suggests changes in their metabolism related processes. Plant pigments are vital for light harvesting and ultimately photosynthesis. Though the whole leaf photosynthesis was not quantified in the present study, but it is known from earlier experiments that salt stress causes decrease in photosynthesis and hence carbohydrate supply (Stepien and Johnson 2009; Chaves et al. 2009) which in turn affects the growth of plant. In the experiment conducted by P. Stepien & Johnson in 2009 on salt tolerant *T. salsuginea* and salt sensitive *A. thaliana* showed chlorophyll degradation and inhibition in electron transport of photosystem II only in *A. thaliana* on high salt exposure.

Apart from plant pigments degradation, increased leaf salt ions in *A. thaliana* caused changes in the metabolic functioning of the cells. Potassium is an important nutrient for plant growth. Increased sodium to potassium ratios in *A. thaliana* leaves causes osmotic imbalance, reduction in cell division and expansion along with inhibition in activity of essential enzymes (Tuteja 2007; Munns and Tester 2008). As *T. salsuginea* had more leaf potassium therefore less harmful effects were observed in this plant species. Altogether increase in leaf salt ions, degradation in chlorophyll content, reduced CO_2 assimilation and carbohydrate supply ratios in *A. thaliana* leaves led to the inhibition in plant growth.

5.2. Salt Induced Changes in Guard cell Functioning

Salinity effects on guard cell physiology were examined for both plant species. For that comparisons were made at molecular and physiological levels. Molecular mechanisms for ABA biosynthesis and induced signalling, ion and water transport, major and minor metabolisms were examined and correlated with the guard cell functioning.

5.2.1. Salt induces ABA Production and Signaling in Both Guard Cell Types

ABA, also known as stress phytohormone has been shown to be produced in abiotic stress conditions especially during drought and saline conditions (Christmann et al. 2007; Fernando and Schroeder 2016; Wang et al. 2011; McAdam and Brodribb 2018; Pilarska et al.

2016; Jia et al. 2002; McAdam and Brodribb 2016). However, experiments conducted on guard cell ABA quantification are old and very few. In one of the experiments conducted three decades ago demonstrated 8- and 18-times higher ABA levels in the guard cells of water stressed leaves of *V. faba* and *P. sativum* respectively than their respective controls (Cornish and Zeevaart 1986). Cornish and Zeevart concluded that rapid stomatal closure is because of the ABA movement from mesophyll to the guard cell apoplast. Furthermore, guard cell ABA production is not required for stomatal closure. Similarly another study conducted on *V. faba* guard cells showed nine fold increase in ABA after fifteen minutes of drought conditions (Harris and Outlaw 1991). However, they could not demonstrate if the increase in ABA levels is because of accumulation from the mesophyll cells or autonomous biosynthesis by the guard cells. Research conducted in recent times showed that guard cells are equipped with autonomous ABA production and during reduced air relative humidity ABA biosynthetic machinery is activated which leads to ABA induced responses (Bauer et al. 2013). Bauer et al. did not report ABA quantification in the guard cells. Here, we demonstrated that guard cells of *A. thaliana* and *T. salsuginea* showed 2.6- and 1.7-times higher ABA content after two days of saline conditions respectively.

ABA quantification from guard cells of i) *A. thaliana* wild type, ii) ABA mutant (*aba3-1*) and iii) the rescued line (able to synthesize ABA in guard cells only) pMYB60-ABA3 nicely explains that guard cells do synthesize ABA during saline growth conditions. Moreover, presence of exogeneous ABA only in the washing solution of guard cells confirms removal/washing of ABA from other cells during blending method of guard cells extraction and hence eliminates the risk of contamination. Activation of ABA biosynthetic machinery under saline conditions in both plant species further validates that increase in guard cell ABA levels are due to autonomous ABA production by the guard cells which is consistent with the findings of Bauer et. al (2013). Along with guard cell ABA quantification from the ABA deficient mutant (aba3-1) and its compliment line (pMYB60-ABA3), leaf ABA levels were also quantified from the plant leaves. Interestingly, ABA levels in the pMYB60-ABA3 leaves were like that of wild type. This leads to the conclusion that ABA production in the guard cells rescue the ABA content in the foliar parts of plants, which is consistent with the observations made by (Merilo et al. 2018). They found that ABA production restricted to guard cells can rescue the plant growth phenotype, stomatal conductance and leaf ABA levels. Similar results were also

obtained when ABA biosynthesis was rescued in immature epidermis and stomatal lineage cells of *nced3* and *nced5* mutants of *A. thaliana* (Chater et al. 2015). Altogether these reports show that ABA is transported from guard cells to the vasculature and vice versa thus, indicating the plasticity to mobilize ABA as a signalling molecule. Increased guard cell ABA content (Fig. 4.9) along with no differential expression of ABA importer genes in *T. salsuginea* guard cells (Fig. 4.27) further strengthens guard cell autonomous ABA production. Similarly, drastic increase in *A. thaliana* guard cells ABA on 1x salt medium and reduced gene expression of ABA importer (ABCG40) under similar conditions reinforce our conclusion of guard cells ABA production on saline conditions. Although a greater number of ABA biosynthesis related genes were induced on 3x salt growth (Fig. 4.27) however, lower guard cell ABA levels on 3x than 1x salt conditions were determined. This suggests that guard cells may either transport ABA to other cells or store it in inactive form for future use under 3x salt conditions. This was confirmed by the induction of DTX50 (ABA exporter) and CYP707A4 (coverts ABA to inactive form) where former was found in *A. thaliana* and latter was observed in both plant species.

The genes of key components (receptors, phosphatases and kinases) involved in core ABA signalling (Fernando and Schroeder 2016; Li et al. 2017b) were expressed in guard cells of both plant species under saline conditions. However, contrasting expression patterns of ABA receptors in both guard cell types suggest that receptors in both plant species may differently regulate downstream signalling. Among all significant differentially expressed receptors, PYL2, PYL8 and PYL9 were specific to *A. thaliana* guard cells. Moreover, PYL2 was found to be differentially expressed only after 3x saline conditions in *A. thaliana* guard cells thus suggesting its role in long term salt stress in this plant species. *A. thaliana* transcriptomic data exhibited ABA receptors that were formally shown not to be guard cell specific, such as PYL1, PYL5 and PYL6 (Gonzalez-Guzman et al. 2012). Our findings are consistent with the reports of Bauer et al., 2013. They also reported differential expression of these ABA receptor genes under different abiotic stress conditions. Discrepancy between our studies and reported by Gonzalez-Guzman et al. 2012 could be because of obtaining data under different conditions. Reports presented here and of Bauer et al., 2013 are from the intact guard cells whereas, Gonzalez-Guzman et al. 2012 used guard cell protoplast. Moreover, the data presented here are normalized to the control samples which would ignore the basal expression of these genes under control conditions and therefore may lead to different result

to the earlier report. It has been reported that dimeric receptors (PYL1, PYL2, PYR1) have lower affinity to ABA whereas monomeric receptors (PYL5, PLY6, PYL8) have high affinity, however, in presence of clade A of type 2 C protein phosphatases (PP2Cs), both kinds of receptors show high affinity with ABA (Ma et al. 2009; Santiago et al. 2009a; Santiago et al. 2009b). Differential expression of phosphatases (ABI1, ABI2, PP2cA and PIA) only after 3x salt conditions in *A. thaliana* guard cells suggests their role of regulating gene expression in long time saline environments.

In the provided experimental conditions transpiration rates of *T. salsuginea* plants were higher than *A. thaliana* irrespective of saline treatments. *T. salsuginea* exhibited higher transpiration rates than *A. thaliana* under all conditions. Higher transpiration is not because of wider stomatal apertures but higher stomatal density. Demonstration of higher stomatal density is consistent with the earlier reports (Inan et al. 2004; Orsini et al. 2010). However, it has been reported that *T. salsuginea* has lower transpiration rates than *A. thaliana* and therefore has tight control over stomatal regulation (Stepien and Johnson 2009; Inan et al. 2004). Discrepancy between our findings and the earlier reports earlier is most probably due to the differences in plant age, growth and experimental conditions. Furthermore, reports about *T. salsuginea* stomatal responses under saline conditions is not consistent (Amtmann 2009). Change in transpiration from dark to light was more pronounced in *T. salsuginea* than of *A. thaliana* under all conditions. There is also discrepancy in the light induced stomatal opening among the literature. For example, reports from (Orsini et al. 2010) showed that under control conditions, transpiration shift from dark to light is stronger in *A. thaliana* than *T. salsuginea*, whereas (Inan et al. 2004) demonstrated opposite response.

Salt induced ABA induction in guard cells led to stomatal closure and hence caused decline in transpiration rates. *A. thaliana* plants showed decrease in transpiration rates under both saline conditions whereas *T. salsuginea* demonstrated decline in transpiration only under 3x salt. Moreover, only dark adapted Thellungiella plants from 1x salt medium displayed decrease in transpiration. Transpiration in both plant species was strongly decreased during long-time saline conditions despite that, the guard cell ABA content did not increase significantly. This could be indication of increased ABA sensitivity. It has been reported earlier that multiple stress lead to adaptation (Bruce et al. 2007; Savvides et al. 2016) where initial stress activates the defence responses thus serves as “priming” which helps plants to better

combat further stress. This pattern was displayed in 3x salt conditions where a greater number of genes involved in ABA signalling were differentially expressed in guard cells of both plant species. Further validation of salt caused increased ABA sensitivity can be made by recording transpiration rates of detached leaves from 3x salt grown plants in the presence of exogeneous ABA feeding with different concentrations. Differential expression of more ABA receptors and phosphatases related genes in dark adapted *A. thaliana* guard cells from 3x salt conditions was prominent which could be because of darkness, as it is well known that rapid closure of stomata under dark conditions require ABA induced signalling (Merilo et al. 2013). Moreover, greater representation of ABA signalling components in dark also suggests that plants become more sensitive to dark under saline growth conditions which is also evident from the transpiration rates.

5.2.2. *A. thaliana* Guard cells Face Problems at High Light Intensities

T. salsuginea exposed to both single light intensity (125 $\mu molm^{-2}sec^{-1}$) and increasing light intensities showed higher transpiration and CO_2 assimilation than *A. thaliana* under all conditions. This consistent behaviour further validates that gaseous exchange and transpiration in *T. salsuginea* guard cells is stronger than *A. thaliana* under our cultivation conditions. Under control conditions, transpiration rate of *A. thaliana* tended to reach saturation level at light intensities above 1000 $\mu molm^{-2}sec^{-1}$, which is consistent with the findings of (Müller et al. 2017). Whereas, *T. salsuginea* transpiration continued to increase and no saturation was observed even at the highest applied light intensity, and hence no complete opening of the stomata reached. This behavior could be an adaptation to the even higher light intensities and along with the saline natural habitat of halophytes (Müller et al. 2017). Similar transpiration rate of non and 1x salt treated *A. thaliana* during increasing light intensities suggests that gradually increasing light exposure is helpful to combat salt induced lower transpiration. This could also mean, that in nature where light intensity has a diurnal rhythm, salt shock (1x saline conditions) may not impose drastic effects on stomatal movement. Long-time salt treatment did not lead to maximal opening of *A. thaliana* stomata at the light intensities (above 1000 $\mu molm^{-2}sec^{-1}$) where it is used to achieve saturation point under non saline conditions. This could be due to Reactive Oxygen Species (ROS) abundance. *A. thaliana* plants tended to reach saturation levels for CO_2 assimilation earlier than *T. salsuginea*, moreover, change in levels was also stronger in *T. salsuginea* suggesting that its

photosynthetic components are better prepared than *A. thaliana*. In sensitive species, high light intensities can lead to so-called photooxidative stress, especially under hot, saline and water-limiting conditions where the stomata are only minimally or not opened (Rennenberg et al. 2006; Munné-Bosch et al. 2013). In this case, the light energy absorbed by the photosystems exceeds the need for reduction equivalents of the Calvin cycle, and the formation of ROS occurs. ROS is known to play role in stress induced responses and programmed cell death in plants therefore optimal levels of ROS should be maintained. ROS metabolism and biochemical quantification was not examined under higher light intensities in the present study therefore it is hard to correlate physiological data with that of molecular/biochemical data. However, very efficient antioxidant protection mechanism and fine tuning of hormones to suppress programmed cell death has been reported in T. salsuginea (Pilarska et al. 2016) which could be vital for stronger responses of this plant species to high light intensities.

5.2.3. Salt Caused Negative Effects on *A. thaliana* Guard Cells Photochemistry and Induces ROS Scavenging Mechanisms

Guard cell transcriptomic data suggests that salt led to decline in chlorophylls biosynthesis related genes increase in carotenoid biodegradation related genes in *A. thaliana*. As the plant pigments are vital for light harvesting and ultimately photosynthesis, therefore, reduced quantum yield of *A. thaliana* guard cells was observed in saline growth conditions. Higher quantum yield and NPQ levels of Thelungiella guard cells suggests its efficient use of light energy and btter protection from heat dissipation than of *A. thaliana* guard cells. These features make them fit to survive under extreme conditions of light and drought. Similarly, reduced CO_2 assimilation in only *A. thaliana* plants under both saline conditions and also influenced expression of genes related to calvin cycle especially after 3x saline conditions suggests energy starvation in this plant species under salinty. Light energy absorbed by the photosystems exceeds the need for reduction equivalents of the Calvin cycle, and thus the formation of ROS occurs. ROS metabolism related genes were more differentially expressed in *A. thaliana* guard cells suggesting ROS production and scavenging under saline growth conditions which is in correlation with the genes involved in light harvesting complexes and guard cell quantum yield recordings. Most of the ROS metabolism related genes were

downregulated in *T. salsuginea* guard cells which is due to better utilization of light energy and antioxidant mechanism in this plant spcies that is also in corelation with the guard cell quantum yields. Other metabolic processes involved in energy production such as CHO metabolism, TCA and glycolysis were also strongly reduced at gene expression level in *A. thaliana* guard cells under 3x salt growth conditions. These findings suggest that *A. thaliana* face severe interruption in energy production.

5.2.4. *T. salsuginea* Guard Cells have Selective Uptake Mechanism for Chloride Ions

Along with other factors, uptake of ions is vital for guard cells osmoregulation and hence adjustments of stomatal apertures. Both guard cell types accumulated similar levels of sodium under saline mediums. The increase in sodium was on the expense of potassium in both guard cell types. However, only *A. thaliana* guard cells accumulated chloride under saline conditions whereas *T. salsuginea* maintained similar chloride levels under all conditions. This suggests better uptake mechanism of salt ions in *T. salsuginea* guard cells. Moreover, this also proposes different role (e.g selectivity of nitrate over chloride) of Thellungiella anion transporters than of Arabidopsis. Interestingly, Thellungiella accumulated less sodium in leaves under 1x salt medium, however they displayed similar sodium levels as that of Arabidopsis in guard cells. Unchanged levels of Thellungiella transpiration under 1x salt compared to control ones, and increased guard cells sodium levels on 1x salt suggests this plant species might use sodium along with potassium for guard cell osmoregulation under these conditions. However, under 3x salt medium transpiration rates of salt tolerant plants were significantly reduced which suggests sodium to potassium ratios are vital for utilization of sodium as osmoregulatory in these motor cells. To get better insight whether halophytic guard cells could completely replace potassium with sodium for osmotic adjustments EDXA must be performed on completely opened and closed guard cells of salt treated plants.

Elevated levels of salt ions in guard cells of both plant species would led to reduction in osmotic potential especially in *A. thaliana* under3x saline conditions. Plants do accumulate compatible osmolytes during osmotic stress conditions. (Sanders and Arndt 2012). Early studies reported that the movement and differential distribution of these compounds within and between cells, is regulated by membrane-associated transporters; most of these

transporters are secondary active transporters. Induction of proline (in *T. salsuginea* guard cells) and GABA biosynthesis related genes in *A. thaliana* guard cells suggests that under saline conditions both plant species adjust osmolarity by osmo-protectants. Thellungiella guard cells metabolome is not available however, *T. salsuginea* has previously been reported to have more proline content in leaves even under non-stressed conditions and the levels even increase under saline conditions (Lee et al. 2016). Higher levels of proline in roots and shoots under non-saline conditions suggest its pre-adaptation to extreme abiotic stress conditions (Kant et al. 2006; Lee et al. 2016; Bartels and Dinakar 2013). Similarly, sugars (e.g. sucrose, glucose, fructose) were also reported to be increased under saline conditions. We found induction of PM localized sugar: H^+ symporters in both plant species under both saline conditions. Induction of plasma membrane localized import proteins under 1x and 3x salt conditions strongly suggests increase in sugar molecules in the cytoplasm of *A. thaliana* guard cells. This increase will help cells to combat the osmotic stress induced by salt. Contrasting patterns of sugar transporters in *T. salsuginea* guard cells proposes its pre-metabolic adaptation to saline conditions.

5.2.5. Salt Caused more Changes in Ions and Water Related Transcriptomes in *A. thaliana* Guard cells than of *T. salsuginea*

PCA demonstrated salt as a main component giving rise to changes in gene expression patterns of *A. thaliana* guard cells. Membrane related transport genes were also more influenced by salt applications in *A. thaliana* guard cells. Stomatal apertures are regulated by turgor pressure in the pair of guard cells forming the stomata. ABA induced stomatal closure under the provided saline conditions results in reduced transpiration. As the transpiration rates of *A. thaliana* plants were more affected in 3x saline conditions, therefore more changes in membrane transport related processes are obvious in this plant species. *A. thaliana* guard cells showed induction of potassium exporter, GORK (Gated outward rectifying potassium channel) on one hand and reduction of KAT2 (potassium inward channel) transcripts on the other hand under 3x salt conditions thus suggesting lower level of potassium in guard cells. Efflux of cations always lead to efflux of anions as charge balance of ions in the cell. Therefore, PM localized anion exporter SLAH3, SLAH2 and NPF6.1 were also induced under 3x saline conditions. Altogether, efflux of these ions from the guard cell leads to efflux of water which

was evident in the upregulation of PM localized aquaporins related genes thus resulting in closed stomata. PM located aquaporin PIP1;4 was the only water channel that showed upregulation under both saline conditions in light and dark which suggests its role in water efflux in *A. thaliana* guard cells under short and long-time saline environments. Similarly, upregulation in PM located aquaporins (PIP2;1, PIP2;2 and PIP2;3) in light and dark-adapted *T. salsuginea* guard cells on 3x salt medium suggests their role in long-time saline exposures in this plant species. Calcium is important for cell signalling especially under saline conditions and its levels in plant cells are increased during biotic and abiotic stress environments (Kader and Lindberg 2010; Manishankar et al. 2018). Cellular pH is very crucial for proper protein functioning in the living cells. Changes in cytosolic pH, either alone, or in coordination with changes in cytosolic Ca^{2+} concentration, induce a wide range of cellular functions in plants including signal transduction in stress conditions (Kader and Lindberg 2010). Similarly, regulation of pH gradient across the plasma membrane is crucial for salt tolerance responses, as this gradient represents the driving force for the SOS1-mediated Na^{+} extrusion from the cytosol (Manishankar et al. 2018). Likewise, sodium sequestration into the vacuole is also dependent on vacuolar pH hence, its pH regulation of vacuole is also important under saline conditions. Upregulation of PM, TP and ER located calcium transporters in *A. thaliana* guard cells shows salt induced calcium signalling in this plant type. However, none of the calcium transporters were differentially expressed in *T. salsuginea* guard cells.

6. Summary

The greatest problems faced during the 21st century is climate change which is a big threat to food security due to increasing number of people. The increase in extreme weather events, such as drought and heat, makes it difficult to cultivate conventional crops that are not stress tolerant. As a result, increasing irrigation of arable land leads to additional salinization of soils with plant-toxic sodium and chloride ions. Knowledge about the adaptation strategies of salt-tolerant plants to salt stress as well as detailed knowledge about the control of transpiration water loss of these plants are therefore important to guarantee productive agriculture in the future. In the present study, I have characterized salt sensitive and salt tolerant plant species at physiological, phenotypic and transcriptomic level under short (1x salt) and long-time (3x) saline growth conditions. Two approaches used for long-time saline growth conditions (i.e increasing saline conditions (3x salt) and constant high saline conditions (3x 200 mM salt) were successfully developed in the natural plant growth medium i.e soil. Salt sensitive plants, *A. thaliana*, were able to survive and successfully set seeds at the toxic concentrations on the increasing saline growth mediums, with minor changes in the phenotype. However, under constant high saline conditions they could not survive. This was due to keeping low potassium, and high salt ions (sodium and chloride) in the photosynthetic tissue i.e leaf. Similarly, high potassium and low salt ions in salt tolerant *T. salsuginea* on both saline environments were the key for survival of this plant species. Being salt tolerant, *T. salsuginea* always kept high potassium levels and low sodium (during 1x) and chloride levels (during both 1x and 3x) in the leaf tissue.

A strict control over transpirational water loss via stomata (formed by pair of guard cells) is important to maintain plant water balance. Aperture size of the stomata is regulated by the turgidity of the guard cells. More turgid the guard cells, bigger the apertures are and hence more transpiration. Under osmotic stress, the water loss is reduced which was evident in the salt sensitive *A. thaliana* plants under both short and long-time saline growth conditions. As the osmotic stress was only increased during long time saline growth conditions in *T. salsuginea* therefore, water loss was also decreased only under these saline conditions. Environmental CO_2 assimilation also takes place via stomata in plants which then is used for photosynthesis. Stomatal apertures also influence CO_2 assimilation. As the light absorbing photosynthetic pigments were more affected in *A.*

thaliana, therefore photosynthetic activity of the whole plant was also reduced. Similarly, both short and long-time saline growth conditions also reduced the effective quantum yield of *A. thaliana* guard cells. Growth of the plant is dependent on energy which comes from photosynthesis. Reduced environmental CO_2 assimilation would affect photosynthesis and hence growth, which was clearly observed in *A. thaliana* guard cells under long-time saline growth conditions.

Major differences in both guard cells types were observed in their chloride and potassium levels. Energy Dispersive X-Ray Analysis (EDXA) suggested strict control of chloride accumulation in *T. salsuginea* guard cells as the levels remain unchanged under all conditions. Similarly, use of sodium in place of potassium for osmotic adjustments seems to be dependent on Na^+/K^+ rations in both guard cell types. Increased salt ions and reduced potassium levels in *A. thaliana* guard cells posed negative effect on photochemistry which in turn increased ROS metabolism and reduced energy related pathways at transcriptomic level in this plant species. Moreover, photosynthesis was strongly affected in *A. thaliana* guard cells both at transcriptomic and physiological levels. Similarly, global phytohormones induced changes were more evident in *A. thaliana* guard cells especially on 3x salt medium. Among all phytohormones, genes under the control of auxin were more differentially expressed in *A. thaliana* guard cells which suggests wide changes in growth and development in this plant species under salinity.

Phytohormone, ABA is vital for closing the stomata under abiotic stress conditions. Increased levels of ABA during saline conditions led to efflux of potassium and counter anions (chloride, malate, nitrate) from the guard cells which caused the outward flow of water and hence reduction in turgor pressure. Reduced turgor pressure led to reduced water loss and CO_2 assimilation especially in *A. thaliana*. Guard cells of both plant species synthesized ABA during saline conditions which was reflected from transcriptomic data and ABA quantification in the guard cells. ABA induced signaling in both plant species varied at the ABA receptor (PYL/PYR) levels where totally contrasting responses were observed. PYL2, PYL8 and PYL9 were specific to *A. thaliana,* furthermore, PYL2 was found to be differentially expressed only under 3x salt growth conditions thus suggesting its role during long term salt stress in this plant species. Protein phosphatases, which negatively regulate ABA signaling on one hand and act as ABA sensor on the other hand were found to be more differentially expressed in *A. thaliana* than *T. salsuginea* guard cells, which suggests their diverse role in both plant species

under saline conditions. Differential expression of more ABA signaling players in long time saline conditions was prominent which could be because of darkness, as it is well known that rapid closure of stomata under dark conditions require ABA signaling. Moreover, representation of these components in dark also suggests that plants become more sensitive to dark under saline conditions which is also evident from the transpiration rates.

Altogether, increased salt ions in *A. thaliana* guard cells and leaves led to pigment degradation and ABA induced reduction in transpiration which in turn influenced its growth. In contrast, *T. salsuginea* is the salt excluder and therefore keeps low levels of salt ions especially the chloride both in leaves and guard cells which mildly affects its growth. Guard cells of *A. thaliana* encounter severe energy problems at physiological and transcriptomic level. Main differences in the ABA signalling between both plant species were observed at the ABA receptor level.

7. Zusammenfassung

Das größte Problem des 21. Jahrhunderts ist der Klimawandel, der aufgrund der wachsenden Zahl von Menschen eine große Bedrohung für die Ernährungssicherheit darstellt. Die Zunahme extremer Wetterereignisse wie Dürre und Hitze erschwert den Anbau konventioneller, nicht stressresistenter Pflanzen. Eine zunehmende Bewässerung von Ackerland führt daher zu einer zusätzlichen Versalzung der Böden mit pflanzentoxischen Natrium- und Chloridionen. Kenntnisse über die Anpassungsstrategien salztoleranter Pflanzen an Salzstress sowie detaillierte Kenntnisse über die Kontrolle des Wasserverlusts durch Transpiration dieser Pflanzen sind daher wichtig, um eine produktive Landwirtschaft auch in Zukunft zu gewährleisten. In der vorliegenden Studie habe ich salzempfindliche und salztolerante Pflanzenarten auf physiologischer, phänotypischer und transkriptioneller Ebene unter kurzen (1x Salz) und langen (3x) Salzwachstumsbedingungen charakterisiert. In dem natürlichen Pflanzenwachstumsmedium, dh. dem Boden, wurden zwei Ansätze erfolgreich entwickelt, die für lang anhaltende Salzwachstumsbedingungen (dh zunehmende Salzbedingungen (3x Salz) und konstant hohe Salzbedingungen (3x 200 mM Salz) verwendet wurden. Die Pflanzen waren in der Lage, Samen bei den toxischen Konzentrationen auf den ansteigenden Salzwachstumsmedien zu überleben und erfolgreich zu setzen, wobei geringfügige Änderungen des Phänotyps auftraten. Unter konstant hohen Salzbedingungen konnten sie jedoch nicht überleben. Dies lag daran, dass wenig Kalium und hohe Salzionen vorhanden waren (Natrium und Chlorid) im photosynthetischen Gewebe, dh im Blatt. Ebenso stellten hohe Kalium- und niedrige Salzionen in salztoleranten T. salsuginea in beiden salzhaltigen Umgebungen den Schlüssel zum Überleben dieser Pflanzenart dar. Da T. salsuginea salztolerant war, blieb der Kaliumspiegel stets hoch und der Natrium- (während 1x) und Chloridspiegel (während 1x und 3x) im Blattgewebe niedrig.

Eine strikte Kontrolle des transpirationelen Wasserverlusts über Stomata (gebildet von zwei Schließzellen) ist wichtig, um den Wasserhaushalt der Pflanzen aufrechtzuerhalten. Die Öffnungsgröße der Stomata wird durch den Turgor der Schutzzellen reguliert. Je praller die Schließzellen, desto größer die Öffnungen und damit die Transpiration. Unter osmotischem Stress wird der Wasserverlust verringert, was bei den salzempfindlichen A. thaliana-Pflanzen sowohl unter kurz- als auch langfristigen Salzwachstumsbedingungen

offensichtlich war. Da der osmotische Stress in T. salsuginea nur über einen langen Zeitraum unter Salzwachstumsbedingungen anstieg, verringerte sich auch der Wasserverlust nur unter diesen Salzbedingungen. Die Aufnahme von CO2 in die Umwelt erfolgt auch über die Stomata und wird dann für die Photosynthese verwendet. Stomata beeinflussen daher auch die CO2-Assimilation. Da die lichtabsorbierenden photosynthetischen Pigmente in A. thaliana stärker betroffen waren, war auch die photosynthetische Aktivität der gesamten Pflanze verringert. In ähnlicher Weise verringerten sowohl kurz- als auch langzeitige Salzwachstumsbedingungen auch die effektive Quantenausbeute von A. thaliana-Schließzellen. Das Wachstum der Pflanze hängt von der Energie ab, die aus der Photosynthese stammt. Eine verringerte CO2-Assimilation aus der Umwelt würde die Photosynthese und damit das Wachstum beeinträchtigen, was bei A. thaliana-Schließzellenn unter lang andauerenden Salzwachstumsbedingungen deutlich zu beobachten war.

Wesentliche Unterschiede bei beiden Schließzelltypen wurden in ihren Chlorid- und Kaliumspiegeln beobachtet. Die energiedispersive Röntgenanalyse (EDXA) ergab eine strikte Kontrolle der Chloridakkumulation in T. salsuginea Schließzellen, da die Chloridkonzentrationen unter allen Bedingungen unverändert bleiben. In ähnlicher Weise scheint die Verwendung von Natrium anstelle von Kalium für osmotische Anpassungen von Na + / K + -Verhältnissen in beiden Schließzelltypen abhängig zu sein. Erhöhte Salzionen und verringerte Kaliumspiegel in A. thaliana-Schließzellen wirkten sich negativ auf die Photochemie aus, was wiederum den ROS-Metabolismus erhöhte und die energiebezogenen Wege auf transkriptomischem Niveau bei dieser Pflanzenart verringerte. Darüber hinaus war die Photosynthese in A. thaliana-Schließzellen sowohl auf transkriptioneller als auch auf physiologischer Ebene stark beeinträchtigt. In ähnlicher Weise waren globale Phytohormon-induzierte Veränderungen in A. thaliana-Schließzellen, insbesondere auf 3 × Salzmedium, deutlicher. Unter allen Phytohormonen wurden Gene unter der Kontrolle von Auxin in A. thaliana-Schließzellen differenzierter exprimiert, was auf weitreichende Veränderungen im Wachstum und in der Entwicklung dieser Pflanzenart unter Salzgehalt hindeutet.

Das Phytohormon ABA ist für das Schließen der Stomata unter abiotischen Stressbedingungen von entscheidender Bedeutung. Erhöhte ABA-Spiegel unter Salzbedingungen führten zum Austritt von Kalium und Gegenanionen (Chlorid, Malat, Nitrat) aus den Schließzellen, was den Wasserfluss nach außen und damit eine Verringerung des Turgordrucks bewirkte. Reduzierter Turgordruck führte insbesondere bei A. thaliana zu einem

geringeren Wasserverlust und einer geringeren CO2-Aufnahme. Die Schließzellen beider Pflanzenarten synthetisierten ABA unter Salzbedingungen, was sich aus den Transkriptomdaten und der ABA-Quantifizierung in den Schließzellen widerspiegelte. Die ABA-induzierte Signalübertragung in beiden Pflanzenarten variierte bei den ABA-Rezeptor- (PYL / PYR-) Spiegeln, bei denen völlig unterschiedliche Reaktionen beobachtet wurden. PYL2, PYL8 und PYL9 waren spezifisch für A. thaliana. Darüber hinaus wurde festgestellt, dass PYL2 nur unter dreifachen Salzwachstumsbedingungen unterschiedlich exprimiert wird, was auf seine Rolle bei langfristigem Salzstress bei dieser Pflanzenart hindeutet. Es wurde gefunden, dass Proteinphosphatasen, die einerseits die ABA-Signalübertragung negativ regulieren und andererseits als ABA-Sensor wirken, in A. thaliana differenzierter exprimiert werden als in T. salsuginea-Schließzellen, was auf ihre vielfältige Rolle in beiden Pflanzenarten unter Salzbedingungen hindeutet. Eine differenzierte Expression von mehr ABA-Signalgebern unter Bedingungen mit langer Salzwasserbewässerung war auffällig, was auf Dunkelheit zurückzuführen sein könnte, da bekanntlich ein schnelles Schließen der Stomata unter dunklen Bedingungen eine ABA-Signalgebung erfordert. Darüber hinaus deutet die Darstellung dieser Komponenten im Dunkeln auch darauf hin, dass Pflanzen unter salzhaltigen Bedingungen empfindlicher gegenüber Dunkelheit werden, was auch aus den Transpirationsraten hervorgeht.

Insgesamt führten erhöhte Salzionen in A. thaliana-Schließzzellen und Blättern zu einem Pigmentabbau und einer durch ABA verursachten Reduktion der Transpiration, was deren Wachstum beeinflusste. Im Gegensatz dazu ist T. salsuginea in der Lage Salz auszuschließen und hält daher geringe Mengen an Salzionen, insbesondere das Chlorid sowohl in Blättern als auch in Schließzellen, dass sein Wachstum geringfügig beeinflusst. Schließzellen von A. thaliana stoßen auf physiologischer und transkriptomischer Ebene auf schwerwiegende Energieprobleme. Hauptunterschiede in der ABA-Signalgebung zwischen beiden Pflanzenarten wurden auf der ABA-Rezeptorebene beobachtet.

8. Supplement

Table 8.1: Primers list

A. thaliana

AT3G02480	ABR-fwd	GGTGGAATGATGGACAAG
	ABR-rev	ATAAAGATCCAAATGGACG
AT1G49240/AT3G18780	AtACT2/8-fwd	GGTGATGGTGTGTCT
	AtACT2/8rev	ACTGAGCACAATGTTAC
AT2g40610	Exp8_-fwd	CCT CTC CAA CGA TAA TGG
	Exp8_-rev	CGC TGG TCG TTA CCT G
AT5G37500	GORK-fwd	CCTCCTTTAATTTAGAAG
	GORK-rev	GCTCCATCCGATAG
	AtHVA22E-fwd	GCG TCG GTG ATA G
	AtHVA22E-rev	GAT GCC GTA CTT CT
AT1g08810	MYB60(3)-fwd	ATGCTGTGACAAGATAGG
	MYB60(3)-rev	AAAGTTTCCACGTTTAAT
AT5g27150	NHX1-fwd	CAAAATCCATACATATCCC
	NHX1-rev	AAGCCTTACTAAGATCAG
AT4g33950	OST1-fwd	ACGATAACACGATGAC
	OST1-rev	TCCTGTGAGGTAATGG
AT1g12480	SLAC1-fwd	CCGGGCTCTAGCACTCA
	SLAC1-rev	TCAGTGATGCGACTCTT
AT2g01980	SOS1-fwd	TCACGGTAGTACATTGG
	SOS1-rev	GCGGGAGATGATATAAG

T. salsuginea

Thlg0012198	TsABAR-fwd	GCAAACGCACAGGTGAGG
	TsABAR-rev	GACCTGACGTTGCACTCCA
Thlg0346143	TsMYB60-fwd	GCACGGCTACCTTCTTGGA
	TsMYB60-rev	TGCTATGACCCTCTCCCCA
Thlg0105522	TsNHX1-fwd	AGCACCGTGGTGTTTGGTAT
	TsNHX1-rev	GTCAGGCCGAGGCACATT
Thlg0210869	TsSOS1-fwd	CGGAAGGCTCAATGGCAAC
	TsSOS1-rev	CGCTTGCTCACACATCGTTC
Thlg0111393	TsEXP8-fwd	CGCACCGTCGTTAGCAATG
	TsEXP8-rev	ATGGCGGGTGTTTACGAGC
Thlg0010987	TsActin2-fwd	ACATCGTGCTCAGTGGTGG
	TsActin2-rev	ACCTGCTGGAATGTGCTGA
Thlg0371084	TsLTP4 -FWD	CTTGGCTCCATGTGCAGG

	TsLTP4 -rev	TGATGGCATGAACTACTCCC
Thlg0039173/Thlg016477	TsPYL6 -fwd	TCCGTGAGAGAGGTCCGAG
	TsPYL6 -rev	TTAGGCGGGTTTCTCTGCG
Thlg0032797	TsXTH15-fwd	GTTTCCGGGTCGGGTTTCA
	TsXTH15-rev	TGTTGCCTTTCCCTTGAGCA

Table 8.2: Cation transport

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
GORK	AT5G37500		1.5							Thlg0192603
KAT3	AT4G32650		1.5							Thlg0110292
KAT2	AT4G18290		-1.0		-1.0					nd
KAT1	AT5G46240								-1.0	Thlg0395676
KEA1	AT1G01790		-1.8		-1.5					Thlg0194922
EAP3	AT3G09030						1.3		1.4	Thlg0382484
NHX4	AT3G06370			-1.1						nd
CAX3	AT3G51860		1.9		3.0					Thlg0019934
CAX7/CCX1	AT5g17860				1.2					Thlg0141301
CHX14	AT1G06970				1.1					nd
CHX16	AT1G64170	-1.6	-1.2	-1.8	-1.9					nd
CHX17	AT4G23700				2.1					Thlg0100764
CHX18	AT5G41610		1.0		2.1					Thlg0163637
CCX2	AT5g17850				1.0					nd
CNGC4	AT5G54250							1.4	1.2	Thlg0064806
CNGC11	AT2G46440				-1.3					Thlg0093272
CNGC12	AT2G46450	1.9	2.2		1.1					Thlg0162485
CNGC19	AT3G17690	-1.2	-1.6							Thlg0163585
VHA-E3	AT1G64200		-2.2		-1.4					Thlg0362589
HA2	AT4G30190.2							-1.0	-1.3	Thlg0117119
VHA-E3	AT1G64200		-2.2		-1.4					Thlg0362589
AHA2	AT4G30190							-1	-1.3	Thlg0117119

Table 8.3: Anion transport

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
SLAH3	AT5G24030	3.0	2.8		2.8					Thlg0090363
SLAH2	AT4G27970	1.9	3.5		1.7	-1.6	-1.1			Thlg0382734
SLAC1	AT1G12480					1.2				Thlg0071394
NRT2.7	AT5G14570								1.2	Thlg0381029
NRT2.3	AT5G60780					-2.3	-2.2		-1.6	Thlg0314109
NRT2.1	AT1G08090			-1.2		-1.7			-1.5	Thlg0193651
NPF2.9/NRT1.9	AT1G18880	-1.1			-1.1					nd
NPF7.2/NRT1.8	AT4G21680	3.7	4.2		2.5			-2.2		Thlg0363525
NPF2.13/NRT1.7	AT1G69870		1.5		2.0					Thlg0051991
NPF6.2/NRT1.4	AT2G26690	1.2								Thlg0029590
NPF-6/NRT1.3	AT3G21670				1.5				1.3	Thlg0388016
NPF1.1/NRT1.12	AT3G16180	1.1	1.1		-1.1					Thlg0184376
NPF1.2/NRT1.11	AT1G52190		-1.8	-1.1	-2.8					Thlg0054338
NPF6.3/NRT1.1	AT1G12110		-1.0	-1.7	-1.9				-1.1	Thlg0040895
NPF8.4	AT2G02020				-1.0					Thlg0186883
NPF6.4	AT3G21670				1.5			1.0	1.4	Thlg0031699
NPF6.1	AT5G13400	1.0								Thlg0397052
NPF5.9	AT3G01350				1.0					Thlg0393597
NPF5.16	AT1G22550		-1.5		-1.9					Thlg0184264
NPF5.14	AT1G72120				2.1					nd
NPF5.13	AT1G72125				2.1					nd
NPF5.11	AT1G72130				-1.1					Thlg0245366
NPF4.4	AT1G33440				1.0					nd
NPF4.3	AT1G59740	-1.1	-1.3							Thlg0207624
NPF2.5	AT3G45710		-1.0							nd
NPF2.3	AT3G45680		-1.1							nd
NAXT1	AT3G45650	-1.0	-1.3	-1.3						nd
CLC-B	AT3G27170		-1.2	-2.3	-2.6	-1.2				Thlg0151044
CLC-A	AT5G40890	1.4				-1.5				Thlg0204786
ALMT5	AT1G68600	-1.3	-1.5							nd
ALMT12	AT4G17970		-1.2							nd
ALMT14	AT5G46610				2.3					nd

Table 8.4: Aquaporins

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
TIP1;1	AT2G36830								-1.0	Thlg0117303
TIP4;1	AT2G25810		-1.3			-1.1	-1.8		-2.0	Thlg0010975
AQP1	AT3G16240	2.4			-1.1			-3.1		Thlg0031865
TIP2	AT3G26520				-1.7				-1.2	Thlg0138139
DELTA-TIP2;TIP2;2	AT4G17340		-1.4		-1.1					Thlg0073638
PIP2;8;PIP3B	AT2G16850		-1.0		-1.2					nd
PIP1;5;PIP1D	AT4G23400		-1.1		-1.8	-2.1	-3.1		-2.4	Thlg0031865
PIP1;3;PIP1C;TMP-B	AT1G01620	1.3								Thlg0072315
PIP2;7;PIP3;PIP3A;SIMIP	AT4G35100	1.2				-1.2		-1.0		Thlg0205394
PIP1;4;PIP1E;TMP-C	AT4G00430	1.7	2.8		4.6					Thlg0005952
PIP2;4;PIP2F	AT5G60660	1.1								Thlg0127493
AtPIP2;1;PIP2;PIP2;1;PIP2A	AT3G53420		-1.1				1.5		1.4	Thlg0108520
PIP2;2;PIP2B	AT2G37170		-1.0				1.1		1.1	Thlg0412665
PIP2;3	AT2G37180						1.5		1.4	Thlg0001925
NIP6;NIP6;1;NLM7	AT1G80760	-1.4	-1.8		-1.2					Thlg0043823
SIP1;2;SIP1B	AT5G18290		-1.7	-1.0	-1.2					Thlg0401518

Table 8 5 Sugars transporters

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
TMT2	AT4G35300				1.6					Thlg0039424
TMT1	AT1G20840				-1.5					Thlg0086077
SUC7	AT1G66570		1.7		3.4					nd
SUC1	AT1G71880			-1.0	-1.2		-1.0		-1.1	Thlg0032433
STP4	AT3G19930					-1.4	-1.6			Thlg0054911
STP3	AT5G61520		1.1							Thlg0186765
STP14	AT1G77210				-1.7					nd
STP13	AT5G26340				1.6					Thlg0042698
STP1	AT1G11260	1.4			-1.4					Thlg0075365
PMT6	AT4G36670				-1.8	-1.3	-1.4			Thlg0168798
PMT5	AT3G18830		1.0		1.0					Thlg0073214
PMT3	AT2G18480	1.3			1.4					nd
INT2	AT1G30220	1.0	2.3	1.0	2.6				1.1	Thlg0104374
ESL1	AT1G08920		-1.4							Thlg0211170
ERDL6	AT1G75220				-1.1					Thlg0108399
ERD6-type monosaccharide transporter	AT3G20460	-1.3			-1.5					nd
ERD6-type monosaccharide transporter	AT4G04760				1.3					nd
ERD6-type monosaccharide transporter	AT3G05400				1.3	-1.5	-1.7		-1.1	Thlg0104724
ERD6-type monosaccharide transporter	AT4G04750	-1.0	-1.2			1.4	1.4			Thlg0175944
ERD6-type monosaccharide transporter	AT5G18840		1.3					-1.6	-2.2	Thlg0381418
ERD6	AT1G08930					-1.5	-1.7		-1.1	Thlg0104724

Table 8.6: Gene expression patterns for plant pigment metabolism

			A. thaliana				*T. salsuginea*				
			Light		Dark		Light		Dark		
	At-ID	Name	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
CHlorophyl biosynthesis	AT3G59400	GUN4				-1.0					Thlg0004112
	AT4G27440	PORB				-1.3					Thlg0019574
	AT4G25080	CHLM				-1.1					Thlg0393828
	AT5G54190	PORA	2.2	1.9							nd
Carotenoid biosynthesis	At4g25700	BCH1				1.1					Thlg0276193
	At5g52570	BCH2				2.7					Thlg0065994
Anthcyanin biosynthesis	AT4G22880	ANS	-1.1	-1.3			1.6	1.6			Thlg0048267
	AT5G17050	UFGT	-1.0	-1.2			2.2	2.1			Thlg0380034
	AT5G08640	FLS		-1.5			1.2				Thlg0116893
	AT5G13930	CHS			1.6	2.0	1.2				Thlg0006508
	AT3G51240	F3H			1.1	1.6	2.4	2.2			Thlg0068114
	AT5G42800	DFR			1.5	2.6	2.3	2.3			Thlg0044038
	AT5G54060	UF3GT				1.5	2.4	2.3			Thlg0198197
Chlorophyl degradation	AT1G19670	CLH1	-1.2	-1.7					1.6		Thlg0010254
Carotenoid degradation	AT4G19170	NCED4				-1.7			1.8	2.0	Thlg0019016
	AT4G32810	CCD8	1.3	1.2		2.2					nd
	AT3G14440	NCED3				1.0					nd
	AT3G63520	NCED1									Thlg0033743
	AT4G18350	NCED2	1.3	1.2		2.2					nd

Table 8.7: Gene expression patterns related to light reaction

Light Reaction									
	A. thaliana				T. salsuginea				
	Light		Dark		Light		Dark		
At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
AT3G01440		-1.4		-1.4					Thlg0012891
AT2G40100		-1.4		-1.4					Thlg0243209
AT4G39710		-1.6		-2.0					Thlg0004431
AT2G05100		-1.7		-1.9					Thlg0412349
AT5G58260		-1.4							Thlg0005589
AT4G37925		-1.1							Thlg0082592
AT5G02120		-1.4		-1.4					Thlg0006649
AT3G27690		-1.9	-1.1	-3.0					Thlg0132307
AT5G43750		-1.8		-1.9					Thlg0004939
AT2G05070		-1.8		-2.1					Thlg0408288
AT2G34430		-1.7		-3.6					Thlg0008082
AT1G14150		-1.7		-1.3					Thlg0039081
AT1G20020		-1.6		-1.4					Thlg0015565
AT4G05180		-1.5		-2.1					Thlg0151578
ATCG01010		-1.4		-2.0					Thlg0040888
AT1G15820		-1.4		-2.1					Thlg0002345
AT1G19150		-1.4		-1.5					Thlg0058645
AT5G21430		-1.4		-1.3					Thlg0009288
AT5G64040		-1.3		-1.8					Thlg0316265
AT3G54890		-1.3		-1.9					Thlg0015629
AT1G67740		-1.2		-1.2					Thlg0359637
AT4G21280		-1.2		-1.7					Thlg0028915
AT1G75690		-1.2		-1.6					Thlg0002217
AT4G12800		-1.1		-1.4					Thlg0112607
ATCG00480		-1.1		-1.4					Thlg0414111
AT1G08380		-1.1		-1.8					Thlg0410033
AT1G70760		-1.1		-1.2					Thlg0002418
AT2G34420		-1.1		-2.4					Thlg0363464
AT2G39470		-1.1		-1.4					Thlg0005835
AT1G52230		-1.1		-1.8					Thlg0000039
AT1G55670		-1.1		-1.6					Thlg0115470
AT1G06680		-1.1		-1.4					Thlg0106606
AT5G54270		-1.0		-1.9					Thlg0104261
ATCG01040		-1.0		-1.6					Thlg0042825
AT4G04640				-1.0					Thlg0006882
AT3G08940				-1.9					Thlg0413154

Continue….

AT3G61470				-1.4					Thlg0171922
AT1G54780				-1.0					Thlg0000390
AT4G02770				-1.6					Thlg0297869
AT1G15980				-1.2					Thlg0070841
AT3G16140				-1.4					Thlg0036642
ATCG00350				-1.5					Thlg0333770
AT2G30570				-1.3					Thlg0363002
AT1G61520				-1.4					Thlg0094298
AT4G21445				-1.6					Thlg0037049
AT5G66190				-1.2					Thlg0009093
AT3G18890				-1.0					Thlg0074478
ATCG01110				-1.2					Thlg0202166
AT5G01530				-1.1					Thlg0226633
AT3G50820				-1.2					Thlg0000209
AT5G66570				-1.2					Thlg0414530
AT4G09650				-1.4					Thlg0000909
AT1G60950				-1.3					Thlg0410421
AT3G47470				-1.4					Thlg0366605
AT4G28750				-1.2					Thlg0255931
AT1G20340				-1.2					Thlg0381554
ATCG01100				-1.1					Thlg0075346
AT2G20260				-1.1					Thlg0087171
AT3G16250				-1.3					Thlg0013312
AT1G51400				-1.4					Thlg0414888
ATCG00150				-1.1					Thlg0301360
AT5G52100				-1.1					Thlg0112552
ATCG00580				-1.1					Thlg0356676
AT1G34000				-1.3					Thlg0358269
AT1G03130				-1.5					Thlg0001040
AT1G30380				-1.1					Thlg0000120
AT4G10340				-1.1					Thlg0361269
AT3G01480				-1.3					Thlg0030186
ATCG00120				-1.2					Thlg0011777
AT3G21055				-1.1					Thlg0000225
AT2G26500				-1.1					Thlg0001022
AT1G29920				-1.8					Thlg0382199
AT2G27510				1.2					Thlg0001786
AT3G22840		2.1	2.7	5.8					Thlg0414927
AT5G45040			1.2						Thlg0007629
AT4G14690	2.6	2.3	1.2	3.8					Thlg0412821

Table 8.8 Energy related pathways

		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Carbohydrate metabolism	AT1G56600	-1.5			1.0		2.1	2.1	1.4	Thlg0094816
	AT2G22190	-1.4	-1.3	-1.3	-1.0					nd
	AT5G11920	-1.2	-1.8	-1.1	-2.0					Thlg0227632
	AT2G32290	-1.2	-1.5	0.6				1.3	1.1	Thlg0401487
	AT1G35910	-1.1	-1.2		1.7					nd
	AT1G32900		-1.1					1.1		Thlg0029669
	AT4G17090		-1.1		-1.2					Thlg0021854
	AT2G35840		-1.1		-1.1					Thlg0047365
	AT1G70820		-1.6							Thlg0057094
	AT2G21590		1.0			1.1				Thlg0264694
	AT1G51420				1.1					Thlg0364748
	AT1G50460				-1.0				-1.0	Thlg0379481
	AT5G65140	1.1			1.1					Thlg0113374
	AT4G02280	1.2	2.0	1.5	3.6					Thlg0019782
	AT5G11110	1.5	2.0	1.1	2.6					nd
	AT3G13784	5.0	6.6	4.0	6.4					nd
	AT4G15210		-3.7							Thlg0217916
	AT2G47180		-1.3			1.6	1.2			Thlg0039298
	AT4G39770		-1.2	-1.0	-2.3					Thlg0257275
	AT5G19220				-1.1					Thlg0005827
	AT5G37180				-1.0					nd
	AT5G46110				-1.1					Thlg0011593
	AT5G40390				1.4	1.4	1.3			Thlg0091053
	AT5G04360				-1.2					Thlg0174008
	AT1G05610				-1.0					nd
	AT1G47840			-1.0	-1.4					Thlg0211500
	AT5G18670				-1.1					Thlg0054848
	AT5G20830				1.0					Thlg0025655
	AT5G20280				1.2					Thlg0135973
	AT3G23920				2.1					Thlg0043433
	AT1G60470		1.6		2.2	2.7	2.1			Thlg0034425
	AT5G51460				-1.2					Thlg0388946
	AT4G25000				1.0				1.2	Thlg0292687
	AT4G39210				1.1		1.2		1.5	Thlg0069472
	AT5G10100				1.3					nd
	AT2G19800							-2.1	-3.6	Thlg0060980
	AT1G59950								-1.0	Thlg0115572
	AT5G20250					-1.3	-1.3		-1.4	Thlg0098034
	AT4G03550.1								-1.0	Thlg0073593
	AT1G78090.1								-1.0	Thlg0141701
	AT2G22240.1					1.3				Thlg0164517
	AT5G08380.1							1.5	1.1	Thlg0173791
	AT3G52180.1							1.1	1.0	Thlg0027945
	AT1G32900.1							1.3	1.0	Thlg0020491
	AT3G46970.1								1.1	Thlg0028950
	AT2G37770								1.1	Thlg0024778
	AT4G39210.1								1.1	Thlg0115416
	AT4G39800.1					1.2	1.1			Thlg0258637
	AT2G22240.1					1.3	1.2			Thlg0027922
	AT2G22240.1					1.2				Thlg0049780
	AT4G25900						-1.1			Thlg0156014
	AT1G23870					-1.5	-1.4			Thlg0109713
	AT1G23870.1					-1.4	-1.2			Thlg0322330
	AT1G70290.1					-1.4	-1.2			Thlg0246054
	AT2G18700.1					-1.7	-1.2			Thlg0283748
	AT1G60140.1					-1.2				Thlg0392383
	AT4G22592.1					1.2				Thlg0121322
	AT2G22240.1					1.4	1.1			Thlg0399804
	AT2G18700.1					-1.8				Thlg0061187

		A. Thaliana				T. Salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Photorespiration	AT1G36370		-1.8							nd
	AT4G37930		-1.5		-1.4					Thlg0009121
	AT1G11860		-1.3		-1.2					Thlg0018837
	AT1G68010		-1.2		-1.2					Thlg0014886
	AT2G13360		-1.1							Thlg0009445
	AT5G36790		-1.0							Thlg0023068
	AT1G32470		-1.0		-1.4					Thlg0102976
	AT4G18360				1.5					Thlg0178802

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Calvin Cycle	AT2G45290					1.2	1.0			Thlg0290370
	AT1G67090				-1.0					Thlg0412085
	AT3G55800		-1.4		-1.4					Thlg0008019
	AT1G32060		-1.3		-1.4					Thlg0011389
	AT5G38410		-1.0		-1.7					Thlg0410128
	AT5G38420		-1.8		-2.4					Thlg0351426

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
TCA	AT5G14740.1					-1.1				Thlg0168528
	AT5G14740.1					-1.1				Thlg0091728
	AT2G19900						1.1			Thlg0103842
	AT3G49360						1.4			Thlg0005539
	AT5G24420					1.1	1.9			Thlg0024003
	AT1G24180					-1.4			-1.0	Thlg0314989
	AT3G52720.1							2.2	2.3	Thlg0120335
	AT3G52720							1.9	1.9	Thlg0101836
	AT5G44520.1								1.2	Thlg0064636
	AT5G66760				1.4					Thlg0049366
	AT3G15020				1.1					nd
	AT5G50950		-1.5		-1.9					Thlg0186573

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Glycolysis	AT4G26530				-1.5			1.2	1.5	Thlg0268337
	AT3G04050				1.0					Thlg0191810
	AT4G26270				1.1					Thlg0390235
	AT5G56350				1.2					Thlg0138672
	AT3G04120				1.2					Thlg0002488
	AT1G74030				1.4					Thlg0131032
	AT2G36460				1.4					Thlg0030072
	AT1G79530				1.5					Thlg0041030
	AT4G32840.1							-1.0		Thlg0112204
	AT1G70820.1							1.3	1.3	Thlg0057094
	AT4G26520.1							1.2	1.5	Thlg0274958

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Fermentation	AT4G33070				2.3					Thlg0405208
	AT1G77120	1.3	2.2		3.2			-1.8	-1.9	Thlg0026882
	AT4G17260								-1.4	Thlg0025984
	AT4G17260.1								-1.3	Thlg0129349
	AT4G17260.1								-1.2	Thlg0033876
	AT4G17260.1								-1.0	Thlg0396771
	AT4G17260.1								-1.2	Thlg0277962

Table 8.9: Gene expression patterns for compatible osmolytes metabolism

			A. thaliana				*T. salsuginea*				
			Light		Dark		Light		Dark		
	At-ID	Name	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Proline Biosynthesis	AT3G55610	P5CS2	1.5	1.3		1.6					Thlg0262174
	AT5G14800	P5CSR		1.3		1.2					Thlg0022753
	AT2G39800	P5CS1		1.0	1.7	3.3		1.5	1.4	1.8	Thlg0014619
BCAAs biosynthesis	AT5G23020	MAM3	-1.0								Thlg0265395
	AT3G58990	IPMI1		-1.6							nd
	AT3G49580	LSU1	-3.1								nd
	AT1G10060	BCAT			-1.1	-1.5				-1.0	Thlg0203949
GABA biosynthe	AT3G30775	PDH1	1.8	1.5	-1.2	-2.1				-3.0	Thlg0055385
	AT1G17745	GDH				1.7					Thlg0232226
Proline catabolism	AT3G30775	PDH1	1.8	1.5	-1.2	-2.1				-3.0	Thlg0055385
	AT5G46180	OAT				1.1					Thlg0070963
	AT5G38710	PDH2		-1.2							nd
	AT5G62530	P5CDH	-1.3								Thlg0288635
BCAAs catabolism	AT4G13360	crotonase family protein				-1.3					
	AT1G55510	BCDH BETA1								-1.1	Thlg0148423
	AT4G16760	ACX1				1.5					Thlg0030811
	AT3G05190	PLP-dependent enzymes superfamily protein			1.0	1.3					Thlg0326719
	AT3G45300	IVDH			1.4						Thlg0047638
	AT2G33150	PKT3				1.1					Thlg0255352
	AT5G48880	PKT1		-1.9							Thlg0058503
	AT3G60510	crotonase family protein				-1.0					nd

Table 8.10 ROS metabolism and scavenging

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Oxidases and peroxidases	AT5G20960		1.5		1.6					Thlg0067796
	AT1G04580		2.3		3.4					nd
	AT4G11230		1.5		2.1					nd
	AT2G22420		1.2		1.2					Thlg0200216
	AT5G15180	1.2	1.2		1.7					Thlg0089424
	AT4G33420	3.1	3.5	1.1	2.9				-1.1	Thlg0080625
	AT1G44970				1.1					nd
	AT5G06730				1.5					nd
	AT2G34060				-1.3					nd
	AT3G49120				1.3					Thlg0202440
	AT4G21960		-1.4		-1.2				1.3	Thlg0411659
	AT5G19890		1.3		1.9					Thlg0204986
	AT2G18980				-1.3					nd
	AT1G68850				1.4	1.2				Thlg0102252
	AT5G64120		-1.1	-1.3	-1.4		-2.0	-1.6	-2.2	Thlg0413110
	AT2G38380		1.6		2.0					Thlg0409190
	AT5G39580			-1.2	-1.4					nd
	AT5G05340				1.6					Thlg0035762
	AT5G06720		-1.2							Thlg0381778
	AT2G41480		-1.5			-1.6	-1.3		-1.1	Thlg0375237
	AT3G32980	1.1					-1.3	-1.8		Thlg0011241
	AT2G37130			-1.3				1.1		Thlg0006296
	AT3G28200	1.0								nd
	AT4G37520	1.5	1.4			-1.1	-1.6	-1.3	-1.9	Thlg0063886
	AT4G37530	1.5	1.3							nd
	AT5G51890	1.6		1.3				1.3	1.1	Thlg0154759
	AT4G18360				1.5					Thlg0178802
	AT1G72610			-1.0	-2.7					nd
	AT5G13700				1.8					nd
	AT5G64110						-1.5	-1.5	-2.0	Thlg0038172
	AT4G08770							-2.3	-2.5	Thlg0150580
	AT5G64100							-1.0	-1.1	Thlg0188125
	AT3G49110.1						-1.2	-1.3		Thlg0107370

		A. thaliana				T. salsuginea				
		Light		Dark		Light		Dark		
		1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Thioredoxin	AT5G16400				-1.3					Thlg0052013
	AT5G06690				-1.5					Thlg0021164
	AT2G42580.1						-1.0	-1.2	-1.5	Thlg0120505
	AT1G04980.1							-1.1	-1.1	Thlg0143578
	AT4G12170							1.3		Thlg0062697
	AT1G03680				-1.0					Thlg0411992
	AT2G40790				1.3					nd
	AT3G02730		-1.0		-1.1					Thlg0088656
	AT1G69880	1.5	1.6		2.9					Thlg0022105
	AT1G59730		1.0		1.7					Thlg0022131
	AT5G61440	1.7							-1.5	Thlg0332353
	AT4G08930	1.1								Thlg0156927
Ascorbate and glutathione	AT1G06640		-1.6		-1.2					nd
	AT1G26100	-1.1	-1.5							Thlg0033509
Redox Heme	AT3G10520				-1.1			1.4	1.5	Thlg0123257
	AT2G16060			-2.2		-1.8				Thlg0181696
Glutaredoxin	AT2G47870	3.0	2.6	1.6	3.1					nd
	AT1G03850				-2.7	-1.8	-1.4			Thlg0102987
	AT5G06470				-1.0					Thlg0182530
	AT1G06830	-1.1	-1.2		-1.6			-1.3		Thlg0018669
	AT5G18600				-1.5					Thlg0004527
	AT5G14070	1.1			-1.5					nd
	AT3G62950	2.2		1.1	-1.3	-1.2				Thlg0101514
	AT1G28480	-2.1	-3.2							Thlg0173267
	AT3G62930	1.9				-1.9			-1.3	Thlg0076222
	AT1G03020			-1.0						nd
	AT4G15690	2.5	1.2							Thlg0341620
	AT3G21460	4.6		1.3		-1.5				Thlg0199954
	AT4G15660	4.0	1.3			-1.7				Thlg0098668
	AT4G15670	2.7				-1.5				Thlg0194200
	AT4G15700	2.9	1.7							nd
	AT5G11930	-1.4								Thlg0224684
	AT4G33040	-1.2								Thlg0219658
	AT4G15680	1.7				-1.5				Thlg0414855
Peroxiredoxin	AT1G60740		2.2		3.3					Thlg0371195
	AT1G65970		3.1		4.6					Thlg0119094
	AT1G65980				1.0					Thlg0125553
	AT1G65990				1.4					nd
Dismutase and catalses	AT4G25100		-1.2		-1.4					Thlg0351314
	AT1G08830				1.0					Thlg0206306

Table 8.11 Phytohormones

		A. thaliana				T. salsuginea				
		Light		*Dark*		*Light*		*Dark*		
	AGI	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Ethylene induced reponse	AT3G62550							1.4	1.4	Thlg0001511
	AT3G24500				1.1	1.4	1.3			Thlg0040161
	AT1G05710							-1.0		Thlg0084917
	AT1G09740				1.4					Thlg0004786
	AT4G37140				1.7					nd
	AT4G37150				2.4					nd
	AT5G44350	-2.1	-1.8							Thlg0239048
	AT3G11930	1.1	1.3							Thlg0186364
	AT1G04370	-2.2	-2.1	-1.0						nd
	AT2G31730	1.8	1.6							Thlg0170592
Gib. Induced responses	AT2G18420							-2.0	-2.5	Thlg0382505
	AT1G75750						-1.6	-1.9	-2.5	Thlg0000164
	AT1G22690	-1.1	-1.8	-2.3	-3.6			-2.2	-1.9	Thlg0004023
	AT5G15230		-1.0	-1.3	-2.2			-1.0	-1.0	Thlg0363522
	AT5G59845	1.7	2.9		3.8	1.0	1.0	1.1	1.2	Thlg0353896
	AT1G74670	1.7	1.3		-2.7	-1.5			1.3	Thlg0002281
	AT2G05380							1.4	1.9	Thlg0366176
	AT5G14920	1.2	1.4							Thlg0282505
	AT5G11740				1.6					nd
	AT1G14920				-1.0					Thlg0131180
	AT1G68570		1.3		1.8					Thlg0393760
	AT2G44810	2.8	1.1							nd
	AT3G55560		1.0							Thlg0405959
	AT4G09610		1.8		1.8					nd
	AT3G10185	-1.2	-1.4							Thlg0179161
	AT4G09600	4.0	5.0	2.5	5.2					nd
JA induced responses	AT3G16470		-2.1				2.1	2.3		Thlg0371482

		A. thaliana				T. salsuginea				
		Light		*Dark*		*Light*		*Dark*		
	AGI	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Auxin induced responses	AT2G34680				-1.3					Thlg0103816
	AT2G33830		1.0			-1.8				Thlg0016319
	AT4G38850				-2.4					
	AT2G44500			-1.1	-1.2	-1.5				Thlg0129209
	AT4G03400	1.4								Thlg0131762
	AT1G28130				1.1					Thlg0143202
	AT2G47750				-1.3					nd
	AT5G18030	-1.3	-2.1		-3.5					Thlg0016398
	AT1G11803	-1.2	-1.9		-3.3					nd
	AT1G60680	1.2	1.9		1.3					nd
	AT5G13370	1.4	2.2	1.3	3.2					nd
	AT4G12980		-1.7		-1.9					Thlg0132772
	AT4G17280		1.1		2.0					Thlg0305173
	AT1G60730		2.1		2.4					nd
	AT2G04850			1.1						nd
	AT3G15450				-2.1	-2.6				Thlg0110762
	AT4G27450				-1.3					Thlg0011130
	AT5G43830				-1.0					Thlg0409892
	AT5G27771				-1.0					nd
	AT5G13360				1.1					nd
	AT5G65470				1.4					Thlg0068425
	AT3G25290				2.3					nd
	AT2G45210	1.4	2.1		1.5					nd
	AT4G34770				-1.8					nd
	AT4G38825			-1.7	-2.5					nd
	AT4G38840				-2.1					Thlg0151060
	AT4G38860				-1.8					Thlg0026512
	AT5G18010	-1.4	-2.0		-3.5					Thlg0099386
	AT5G18020		-1.9		-3.2					nd
	AT5G18050	-1.4	-2.1	-1.1	-3.7					Thlg0066565
	AT5G18060	-1.3	-2.1		-3.5					Thlg0190903
	AT5G18080	-1.6	-2.6	-1.1	-3.6					Thlg0034568
	AT3G03850	-1.5	-2.1		-1.9					Thlg0034568
	AT4G34790				-1.0					nd
	AT3G61900		1.1		1.6					Thlg0049632
	AT4G12410	1.3	1.9		3.2					Thlg0117170
	AT5G03310			-1.1	-1.9					nd
	AT2G37030				-1.1					Thlg0137949
	AT3G09870	-2.8	-3.2	-1.7	-2.0					nd
	AT4G34810			-1.5	-1.8	-1.2	-1.1			Thlg0048584
	AT4G34760				-2.1					Thlg0151049
	AT5G50760				1.8					Thlg0041628
	AT1G76190	-1.5	-1.9	-1.4	-1.9					nd
	AT1G20470	-2.0	-2.8		-1.2	1.1				Thlg0221730
	AT1G29420	-1.0			-2.2		-1.1			Thlg0045309
	AT1G29440		-1.3		-3.5					Thlg0049223
	AT1G29450				-2.1					Thlg0025107
	AT1G29460		-1.2		-2.7					Thlg0023173
	AT1G29500				-2.0			-1.1		Thlg0025330
	AT1G29510				-2.8					nd
	AT1G56150			-1.0						nd
	AT3G12955	1.4	1.9	1.5	2.1					Thlg0277466
	AT5G27780				-2.8					nd
	AT5G20820		-2.1		-1.5					Thlg0075053
	AT1G72430				-1.4	-1.3	-1.8	-1.1	-1.3	Thlg0351268
	AT2G16580				1.4					nd
	AT4G36110	-1.6	-1.8	-1.0						nd

Table 8.12: ABA metabolism and transport

			A. thaliana				T. salsuginea				
			Light		Dark		Light		Dark		
	NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Biosynthesis	AAO3	AT2G27150				1.1					Thlg0191847
	AAO4	AT1G04580		2.3		3.4					nd
	NCED2	AT4G18350				1.8					nd
	NCED3	AT3G14440				1.0					nd
	NCED4	AT4G19170				-1.7			1.8	2.0	Thlg0019016
	BETA-OHASE 2	AT5G67080				2.7					
	CCD8	AT4G32810	1.3	1.2		2.2					nd
	HVA22F	AT2G42820				-1.1					Thlg0094028
	HVA22H	AT1G19950	-1.3	-2.4		-1.7					nd
	MAPKKK19	AT5G67080				2.5					Thlg0265940
Transport	ABCC2	AT2G34660				1.1					Thlg0161045
	ABCG30	AT4G15230		1.6		1.1					nd
	ABCG40	AT1G15520	-1.7	-1.4		1.8					Thlg0382189
	DTX50	AT5G52050				1.3					nd
Conversi	AtCYP707A-1	AT4G19230					-1.1		-1.0	-1.3	Thlg0117342
	AtCYP707A-3	AT5G45340	-2.1	-2.2		-1.6	-2.3	-2.4			Thlg0084259
	AtCYP707A-4	AT3G19270				1.1	1.2	2.0	1.9	2.2	Thlg0200278

Table 8.13: ABA signalling

			A. thaliana				T. salsuginea				
			Light		Dark		Light		Dark		
	NAME	At-ID	1x	3x	1x	3x	1x	3x	1x	3x	Th-ID
Receptors	PYL1	AT5G46790				-1.0			-1.1	-1.7	Thlg0016051
	PYL2	AT2G26040		-1.2		-1.4	-				nd
	PYL4	AT2G38310			-1.8	-2.5		-1.6	-1.1	-1.8	Thlg0019135
	PYL5	AT5G05440	2.9	2.1		-1.8	-1.4	-1.6	-2.0	-3.0	Thlg0263363
	PYL6	AT2G40330	1.4		-2.5	-3.6	-2.1	-2.4	-2.9	-4.3	Thlg0039173
	PYL8	AT5G53160				-1.2					Thlg0108605
	PYL9	AT1G01360		-1.1							Thlg0028568
Phosphatases	HAI1	AT5G59220	2.0	2.5	2.2	5.8		1.0	1.5		Thlg0083008
	HAI2	AT1G07430			1.7	3.5	-				nd
	PIA1	AT2G20630				2.7					Thlg0240857
	AHG1	AT5G51760	1.3	1.9		3.0					nd
	PP2CA	AT3G11410				1.7					Thlg0045726
	ABI1	AT4G26080				1.2					Thlg0040829
	ABI2	AT5G57050				2.7					Thlg0059943
	CIPK1	AT3G17510							1.1	1.2	Thlg0058211
	CIPK4	AT4G14580		1.2							nd
	CIPK5	AT5G10930			1.7	1.7					Thlg0163294
	CIPK10	AT5G58380				1.0					Thlg0376610
	CIPK15	AT5G01810	1.3	1.3							nd
	CIPK16	AT2G25090		1.4		1.2					Thlg0239773
	CIPK17	AT1G48260	-1.9	-2.5		-1.4					Thlg0122405
	CIPK18	AT1G29230				1.7					Thlg0399030
	CIPK19	AT5G45810	1.0	1.2		2.7					nd
	CIPK20	AT5G45820		-1.9		-4.2					nd
	CIPK21	AT5G57630				-1.0	-1.2				Thlg0220416
	CIPK22	AT2G38490		1.9		3.3					Thlg0324501
	CIPK25	AT5G25110				2.1					nd
	SNRK2.5	AT5G63650		-1.0							Thlg0396996
	SNRK2.8	AT1G78290	-1.5	-1.5							Thlg0381765
	SNRK2.9	AT2G23030		1.3		1.0		1.2	1.1		Thlg0168780
	CPK1	AT5G04870					-1.0				Thlg0376338
	CPK28	AT5G66210	-1.9	-2.0							Thlg0148851
	CPK31	AT4G04695				-1.1					Thlg0381916
	CPK32	AT3G57530	-1.3	-1.1				-1.0			Thlg0122440

9. Curriculum Vitae

10. Publication list

- Muhammad Nouman Sohail, **Sohail Mehmood Karimi**, Shaheen Asad, Shahid Mansoor, Yusuf Zafar and Zahid Mukhtar. (2012). Development of broad-spectrum insect resistant tobacco by expression of synthetic *cry1A*c and *cry2Ab* genes. Biotechnol. Lett. 34: 1553-1560.
- **Sohail Mehmood Karimi**, Muhammad Nouman Sohail, Imran Amin, Shahid Mansoor and Zahid Mukhtar. (2013). Molecular characterization of a new synthetic *cry2Ab* gene in *Nicotiana tabacum*. Biotechnol. Lett. 35: 969-974.

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12. Affidavit in English and German

I hereby confirm that my thesis entitled **A Comparative Study on Guard Cell Function of the Glycophyte *Arabidopsis thaliana* and the Halophyte *Thellungiella salsuginea* Under Saline Growth Conditions** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that the thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

…… ……

Place, date Signature (Sohail Karimi)

Hiermit erkläre ich an Eides statt, die **Eine vergleichende Studie zur Schließzellfunktion des Glycophyten Arabidopsis thaliana und des Halophyten Thellungiella salsuginea unter salinen Wachstumsbedingungen.** Eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Pruefungsverfahren vorgelegen hat.

…… …….

Ort, Datum Unterschrift (Sohail Karimi)

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14. List of figures