Salinity Tolerance of Tomato Plants: The Role of Jasmonic Acid and Root Ammonium Transporters

By

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ABSTRACT

Plant hormones and ion transporters are key elements for plant salt tolerance. To investigate the potential involvement of jasmonic acid (JA) and root ammonium transporters (*AMTs*) in salt tolerance, salt stress responses of tomato (*Solanum lycopersicon*) JA-deficient mutant *def-1* (*defenseless-1*) and two tomato species (*S. lycopersicon* and *S. pennellii*) varying in salt tolerance were analyzed, respectively. The physiological and biochemical analyses of *def-1* under salt stress showed a reduction in nitrogen (N) content and an increase in hydrogen peroxide and malondialdehyde compared to the wild type (WT) plants. The ROS (reactive oxygen species)-associated injury phenotype for *def-1* was associated with lower activity of both enzymatic antioxidants and non-enzymatic antioxidants. These findings suggest that JA plays a role in maintaining N and ROS homeostasis under salt stress. The results of the bioinformatics analysis for tomato *AMTs* indicated that the three known genes belong to the plant *AMT1* subfamily (electreogenic NH_4^+ transport system) and five new genes were found in the plant *AMT2* subfamily (electroneutral NH_3 transport system). Gene expression analysis revealed that tomato roots expressed two members of the *AMT1* subfamily (involved in NH_4^+ uptake) and one member of the *AMT2* subfamily (potentially involved in the efflux of the gaseous NH_3 species). The comparative analysis between *S. lycopersicon* and the wild species *S. pennellii* under salt stress, indicated that the latter is more salt tolerant. In root tissues of both species, the expression of key genes for NO_3^- uptake and assimilation were reduced under salt stress. However, salt tolerance of *S. pennellii* was coupled with higher relative mRNA levels of NH_4^+ uptake genes (*AMT1*-type transporters, *AMT1.1* and *AMT1.2*) and assimilation genes. These findings suggest that AMTs are involved in salt

tolerance by facilitating the NH_4^+ uptake and reducing the energy requirements for growth. Overall, the results of my research improve our understanding of salinity tolerance and will be beneficial to improve tomato growth in saline soil.

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LIST OF ABBREVIATIONS

ABA: abscisic acid

AMTs: ammonium transporters

AOC: allene oxide cyclase

APX: ascorbate peroxidase

AsA: ascorbic acid

BLAST: basic local alignment search tool

bp: base pair

CAT: catalase

def-1: defenseless-1

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

GR: glutathione reductase

GST: glutathione-s-transferase

H_2O_2: hydrogen peroxide

HATS: high affinity transporter system

HK1: histidine kinase receptor protein 1

HKT: high affinity K^+ transporter

JA: jasmonic acid

JA-Ile: jasmonoyl-l-isoleucine

JAR1: jasmonate-Resistant 1

JAZs: jasmonate zim-domain proteins

LATS: low-affinity transport system

LOX: lipoxygenase

MAPK: mitogen-activated protein kinase

MDA: malondialdehyde

MeJA: methyl jasmonate

MEPs: methyl ammonium permease or yeast transporters

mRNA: messenger RNA

MYC2/JIN1: jasmonate insensitive1

NHX: tonoplast Na^+/H^+ exchanger

NO: nitric oxide

NRTs: nitrate transporters

NSCCs: non-selective cation channels

$O_2^{\cdot -}$: superoxide radical

$OH^{\cdot}$: hydroxyl radical

ORF: open reading frame

PCR: polymerase chain reaction

Rh: rhesus proteins

ROS: reactive oxygen species

RT-PCR: reverse transcription polymerase chain reaction

SOD: superoxide dismutase

SOS1: plasma membrane Na^+/H^+ antiporter

SULTR: sulfate transporters

TMDs: trans-membrane domains

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CHAPTER 1. INTRODUCTION

Tomato (*Solanum lycopersicum)* is one of the most important vegetable crops worldwide. The total world production of tomatoes was estimated at 153 million tons in 2010, with value of $74 billion (FAO Database, 2010). For human health benefits, tomato provides high levels of antioxidants such as lycopene, which may has anti-cancer benefits (Sesso et al., 2003). In addition, tomato is an excellent source of vitamins C and E as well as minerals such as magnesium, potassium and phosphorous (Sesso et al., 2003). Almost 78% of the world production comes from five countries including China followed by the USA, India, Turkey and Egypt (FAOSTAT, 2010). The field production of tomato crop is mainly distributed in warm and dry regions (Cuartero and Fernández-Muñoz, 1998). During their production in such areas, tomato plants are vulnerable to a broad range of stresses, which can cause large reductions in plant growth and productivity. Stress is defined as an adverse condition or circumstance that disturbs the normal physiological functions of the plant (Munns and Tester, 2008), fall in two groups: 1) biotic stresses (resulting from biological factors such as insects and pathogens) and 2) abiotic stresses (originating from the non-living factors like water, temperature and nutrients resulting in drought, flooding, heat, cold and salinity).

Salinity stress is one of the major abiotic stresses that limits plant growth and crop yield (Parida and Das, 2005). Approximately 6% of the world's total land area and 20% of irrigated land (45 million ha) suffers from severe salinity problems (Pitman and Lauchli, 2002). According to the WORLD FOOD PROGRAM 2015, there are about 800 million people in the world who are suffering from hunger and malnutrition (FAO, 2015). The world population, approximately 7 billion people in 2014, is expected to be 9.1

billion by 2050. Thus, salinity can be a threat to the global food production and could likely contribute to food scarcity worldwide.

A better understanding of the physiological and molecular bases of how plants cope with the deleterious effects of salt stress is important for the establishment of high agricultural productivity and ensuring sustainability of food supply in saline soils. Therefore, in the last two decades, extensive research has been conducted to identify the key elements of plant salt tolerance, which include two major directions (Munns and Tester, 2008; Deinlein et al., 2014; Parihar et al., 2015; Dar et al., 2015). The first one is related to the signal transduction pathways for salt stress, involving signaling components such as calcium, free radicals and plant hormones. The second one focusses on the mechanisms of salt tolerance (osmotic adjustment through compatible solute formation, ion homoeostasis via membrane transporters and reactive oxygen species (ROS) scavenging by antioxidants), which are activated and regulated by salt stress signaling transduction pathways. More recently, the importance of plant hormones and the different plasma membrane transporters in salt stress has been highlighted (Kazan et al., 2014; Deinlein et al., 2014; Dar et al., 2015; Parihar et al., 2015). However, there are still missing parts for the complete picture regarding the roles of both plant hormones and plasma membrane transporters in plant salt tolerance.

Plant hormones play a central role in regulating plant responses to both biotic and abiotic stresses. Abscisic acid (ABA) is well known as an abiotic stress-related plant hormone with a major function in osmotic stress adjustment during salt and drought stresses via stomatal closure and osmolyte biosynthesis (Deinlein et al., 2014; Parihar et al., 2015). In comparison, jasmonic acid (JA) has been mostly studied in response to

biotic stresses such as herbivore attack (wounding) and pathogenic infection (Wasternack, 2007; Wasternack and Hause, 2013), but recently a growing body of evidence has confirmed that JA is also involved in salt stress. However, the physiological roles for JA in salt tolerance remain to be determined. The availability of plants bearing mutations within hormone biosynthesis, pathways such as the tomato JA-deficient mutant *def-1* (*defenseless-1*) (Howe et al., 1996) could be a powerful tool in advancing our understanding of how JA affects salt tolerance of tomato plants.

Salt stress recognition by signaling molecules (e.g. plant hormones) and mechanisms of tolerance are the cornerstone to the successful alleviation of salinity damage. Among the mechanisms of tolerance is the involvement of different transporters in ion homoeostasis during salt stress. Extensive studies have been conducted to identify the role of sodium (Na^+) transporters (SOS1, the plasma membrane Na^+/H^+ exchanger and NHX, the tonoplast Na^+/H^+ exchanger) to maintain plant Na^+ homeostasis (Deinlein et al., 2014; Parihar et al., 2015; Han et al., 2015). Although nitrogen (N) homeostasis under saline environments is also a key element for salt tolerance (Popova et al., 2003), less attention has been given to the role of N transporters in this regard. The two main sources of N available in soils for plants are nitrate (NO_3^-) and ammonium (NH_4^+) ions, which are absorbed by roots using nitrate transporters (NRTs) and ammonium transporters (AMTs), respectively. It has been shown that in tomato and other plant species such as barley (*Hordeum vulgare*), the preference for NH_4^+ uptake during salt stress reduces the energy requirements for growth and enhances salt tolerance (Flores et al., 2001; Kant et al., 2007; de Souza-Miranda et al., 2016). Hence, AMTs in tomato roots are likely to play a critical role in N homeostasis under saline conditions. To study

the role of AMTs in tomato salt tolerance there is a need to first identify the AMT members in this species. In plants, *AMT* gene family is divided into two subfamilies (*AMT1* and *AMT2*), which contribute to NH_4^+/ NH_3 root uptake and translocation (Koegel et al., 2013; Wu et al., 2015). To date, only the *AMT1* subfamily has been characterized in tomato (von Wirén et al., 2000), while little is known about *AMT2* genes in this species. Bioinformatics and molecular biology methods can be used to identify tomato *AMT2* genes and detect their tissue expression patterns. To address the role of root N transporters (*AMTs* and *NRTs*) in salt tolerance, comparative analysis with tomato species varying in salt tolerance could be employed. In spite of all the research conducted on salinity stress, there is still a need to fill a gap in our understanding of the roles of membrane transport proteins such as the ammonium transporters and plant growth regulators like jasmonic acid in plant salt tolerance.

Overall, the objectives of my study were: 1) to compare salinity stress responses at the physiological and biochemical levels of JA-deficient mutant *def-1* (*defenseless-1*) and its wild-type to elucidate the mechanisms by which JA regulates plant salt tolerance; 2) to identify tomato *AMT2* members and examine their tissue-specific expression patterns using bioinformatics and molecular biology methods ; 3) to compare salinity responses of the well-characterized salt-sensitive species, *S. lycopersicum* cultivar "Manitoba", and the halophyte wild species, *S. pennellii*, to identify the potential contribution of different root N transporter genes to salt tolerance. Such information will improve our understanding of salinity tolerance and will benefit tomato growth and production in saline soils.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Salinity stress is a major environmental factor limiting plant growth and productivity in agriculture. It is mainly caused by the presence of Na^+ and chloride (Cl^-) ions in the soil; however, calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^+) cations, as well as sulfate (SO_4^{2-}), carbonate (CO_3^{2-}) and bicarbonate (HCO_3^{2-}) anions could also contribute to saline soils in a lesser extent (Maggio et al., 2007; Manchanda and Garg, 2008). Salinity can be divided into primary salinity and secondary salinity (Munns, 2005; Yadav et al., 2011). Primary salinity results from rock weathering and the deposition of oceanic salts, while secondary salinity is caused by human activities, such as artificial irrigation and the overuse of fertilizers (Manchanda and Garg, 2008).

Plants are divided into glycophytes or halophytes according to their performance under salt stress (Sairam and Tyagi, 2004). Halophytes are the native flora of saline environments that have the ability to tolerate extreme salinity levels (ranging from 100 to 500 mM NaCl). The salt tolerance of halophyte plants is based on their morphological, biochemical and physiological features that are tightly regulated by their genetic background. On the other hand, glycophytes, which represent most of the commercial crops, are usually stressed under salt concentrations ranging from 10 to 100 mM (Flowers et al., 1997). In agriculture, salt stress is a major environmental factor that negatively affect plant growth and productivity. Under such conditions, plants must adjust their growth and development through ion fluxes across the plasma membrane and the integration of hormone signaling (Parida and Das, 2005; Munns and Tester, 2008).

Understanding the effects of salt stress on plants and the mechanisms of salt tolerance is highly important for food security worldwide.

The aims of this review are: 1) to discuss the deleterious effects of salinity stress on plants, including ionic stress, osmotic stress, oxidative stress and depletion of nutrients; 2) to describe the signal transduction pathway of salinity stress and 3) to examine the mechanisms of salt tolerance such as osmotic adjustment, ROS scavenging and ion homoeostasis via plasma membrane transporters.

2.2. Effect of salinity on plants

The soil solution of saline soils is composed of a range of salts which contribute to salt stress, but NaCl has been the focus of most of the research done on salt stress (Munns, 2005; Munns and Tester, 2008; Gupta and Huang, 2014). Salinity induces negative effects on plants that could lead to death. Salinity stress initially triggers two main stresses, namely, osmotic and ionic stress. Both types of stress cause formation of reactive oxygen species (ROS) and reduction in nutrient acquisition including nitrogen (Hasegawa et al., 2000). The differences between osmotic and ionic stress can be explained by the two-phase model (Munns, 2005; Munns and Tester, 2008; Gupta and Huang, 2014). In the first phase, the effects of salinity are due to rapid changes in plant water relations (osmotic stress) leading cell dehydration and shrinkage. In the second phase, starting with extended exposure to salt stress, the reduction in plant growth occurs as a result of a salt-specific effect. The detailed effect of salinity stress on plants will be discussed below.

2.2.1 Osmotic stress

Salt accumulation in the rhizosphere reduces the water potential for the soil solution that leads to reduction in water uptake by the roots; this subsequently reduces the relative water content of leaves and cell turgor (Manchanda and Garg, 2008). One of the first responses of plants to salt stress is stomatal closure in leaves to limit this loss of water (Chaves et al., 2009), but this also leads to reduction in the internal CO_2 level and thus indirectly affects photosynthesis (Taiz and Zeiger, 2002; Mahajan and Tuteja, 2005). In addition, osmotic stress may induces changes in plasma membrane properties and disturbs the normal bi-layer structure. These changes in the lipid bi-layer may result in displacement of membrane proteins and loss of membrane integrity and selectivity (Mahajan and Tuteja, 2005; Yadav et al., 2011).

2.2.2. Ionic stress

Ionic stress is a major challenge for plant growth and crop production, resulting from the accumulation of ions, especially Na^+ and Cl^-, in plant tissues. Sodium ions enter plant cells through the root plasma membrane via high affinity K^+ transporters (HKT) and non-selective cation channels (NSCCs); while the mechanism of Cl^- uptake systems are not known (Mian et al., 2011). The high concentration of Na^+ in the cytosol can alter biochemical reactions and affect most enzyme activities (Serrano, 1999). In addition, Na^+ can displace Ca^{2+} from membrane, leading to a disruption of cell membrane integrity and leakage of intracellular solutes (Cramer et al., 1985). For most plant species, it is believed that Na^+ ions are the main ions that contribute to the toxic effects of NaCl. However, in some woody species, Cl^- is the major ion inducing toxicity and controlling its transport has been associated with salt tolerance (Teakle and Tyerman, 2010). In general,

glycophyte cells cannot withstand more than 100 mM Na^+ in the soil (Mahajan et al., 2008; Munns and Tester, 2008), while some halophytes can withstand high Na^+ levels up to 500 mM without significant damage to the plant metabolism (Matoh et al., 1986).

2.2.3. Oxidative stress

Although under favorable growth conditions, reactive oxygen species (ROS) are continuously produced in plant tissues from aerobic metabolism such as photosynthesis, photorespiration and respiration to a certain extent (Abogadallah, 2010), under salt stress conditions, ROS cause oxidative stress in plants (Gill and Tuteja, 2010; Suzuki et al., 2012). Reactive oxygen species such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are induced during salt stress mainly by closure of stomata, which limits the influx of CO_2 in leaves (Türkan and Demiral, 2009 and Suzuki et al., 2012). As a result, carbon reduction and the consumption of the reducing agent NADPH in the Calvin cycle are decreased, resulting in a low amount of $NADP^+$ regenerated and leading to an excess of electrons in photosystem I. These changes result in electron leakage from the electron acceptor of photosystem I, ferredoxin, to O_2 as an alternative electron acceptor and initiate the Mehler reaction (Türkan and Demiral, 2009). This reaction produces $O_2^{\cdot -}$, which can consequently from other harmful ROS (H_2O_2 and $OH^{\cdot}$) through a series of reactions (Suzuki et al., 2012; Türkan and Demiral, 2009). Unlike atmospheric O_2, ROS (especially $OH^{\cdot}$) can oxidize multiple cellular components such as lipids, proteins and nucleic acids (DNA and RNA) (Abogadallah, 2010).

2.2.4. Nutrient depletion

Mineral nutrients are the main inputs for plant growth and are necessary to complete its life cycle. As plants acquire nutrients from soil, salinity causes a decrease in

the uptake of most of the macronutrients (potassium, calcium, magnesium and nitrogen) and micronutrients (iron, zinc and copper) (Shokri and Maadi, 2009; Shibli et al., 2007). Salinity reduces nutrient uptake by the direct competition between Cl^- and NO_3^- as well as Na^+ and K^+ (Hawkins et al., 1993; Sairam and Tyagi, 2004; Munns and Tester, 2008; Shokri and Maadi, 2009). In addition, salinity reduces the water potential of the soil solution, causing a reduction in water uptake and thus a reduction in nutrient uptake may occur (Ehlting et al., 2007; Shokri and Maadi, 2009). Frequently, salt-stressed plants exhibit severe symptoms including growth retardation, nutritional deficiency symptoms and leaf senescence. The first target of Na^+ toxicity is K^+ homeostasis. Potassium is an essential nutrient required for the proper functioning of plant cells, including turgor pressure adjustment, stomatal movement and enzyme activity (Szczerba et al., 2009). Therefore, the maintenance of a high K^+/ Na^+ ratio in the cytosol of plant root cells is critical during salt stress (Kronzucker and Britto, 2011). Under non-stress conditions, plants maintain relatively high levels of K^+ (100-200 mM) and low Na^+ concentrations (1-10 mM) in the cytosol. During salt stress, a rise in extracellular Na^+ level will initiate a Na^+ electrochemical potential gradient that will promote the passive transport of Na^+ from the soil into the cytosol (Blumwald et al., 2000).

Regardless of saline or non-saline soils, nitrogen is the most limiting nutrient for plant growth; it is required for many cell components, such as amino and nucleic acids (Hu and Schmidhalter, 2005; Ehlting et al., 2007). High NaCl concentration may also depolarize root cells and reduce ammonium uptake, which occurs by membrane potential-driven uniport (Ludewig et al., 2007). In tomato and other plant species such as pea (*Pisum sativum),* the negative effect of salinity on N uptake has often been reported

to result in reduction of plant growth and development (Cantrell and Linderman, 2001; Frechilla et al., 2001; Yao et al., 2008).

2.3. Salt stress signal transduction

Under salt stress, plants are able to recognize both osmotic and ionic stresses. In *Arabidopsis thaliana*, the histidine kinase receptor protein (HK1) has been proposed to be involved in osmotic stress sensing (Tran et al., 2007; Wohlbach et al., 2008). In addition, other components, such as Ca^{2+} channels, have also been proposed as sensors for osmotic stress (Kurusu et al., 2015; Deinlein et al., 2014). On the other hand, ionic stress is suggested to be perceived via Na^+-sensitive enzymes in the cytoplasm or via membrane proteins (Türkan and Demiral, 2009). The plasma membrane Na^+/H^+ antiporter (SOS1), is another suggested Na^+ sensor (Zhu, 2003). Following salinity stress perception, secondary messengers, such as Ca^{2+} and nitric oxide (NO), as well as plant hormones, can alter global transcriptional profiles to enhance multiple salt tolerance mechanisms (Munns and Tester, 2008; Deinlein et al., 2014; Dar et al., 2015).

2.3.1. Secondary messengers and salt stress

Secondary messengers such as Ca^{2+} and NO are key molecules that mediate multiple aspects of salt stress. Calcium is released from the intracellular compartments, mainly from vacuoles, in response to biotic and abiotic stresses and plant hormones (Hirschi, 2001; Lecourieux et al., 2006; Kurusu et al., 2015). Upon salinity stress, Ca^{2+} activates Ca-dependent proteins such as the salt OVERLY SENSITIVE 3 (SOS3), also known as CBL4 (CALCINEURIN B-LIKE 4), which interacts with SOS2 a CBL-interacting protein kinase. Then the SOS3/SOS2 complex activates SOS1 (a plasma membrane Na^+/H^+ antiporter) via phosphorylation, resulting in the removal of excess Na^+

from plant cells and thus reduction Na^+ toxicity (Zhu, 2003; Munnus and Tester, 2008). In addition, the role of Ca^{2+} as a signal molecule in stomatal closure is well documented (Suhita et al., 2004). During osmotic stress, ABA triggers cytosolic calcium elevation, which activates anion channels to release anions from guard cells (anion efflux), causing membrane depolarization. This change in membrane potential activates the outward-rectifying K^+ (K^+_{out}) channels, located in the plasma membrane of guard cells, resulting in K^+ efflux, while the inward-rectifying K^+ channels are inhibited to prevent further entrance of K^+. The resulting water loss from the guard cells causes loss of turgor, leading to stomatal closure (Tuteja, 2007; Kurusu et al., 2015).

Nitric oxide (NO) is also an important second messenger involved in both biotic and abiotic stresses as well as plant development (e.g. germination, root growth and stomatal closure) (Trapet et al., 2015). During salt stress, exogenous NO treatment enhanced protection against oxidative stress in rice (*Oryza sativa*) and cucumber (*Cucumis sativus*) (Uchida et al., 2002; Fan et al., 2013). These studies suggest that NO, itself, has antioxidant properties and could act as a signal in activating ROS scavenging enzymes under salt stress. However, further investigation is required to confirm if endogenous NO has also a protection role against oxidative stress.

2.3.2. Roles of plant hormones

Phytohormones such as auxin, cytokinin (CK), gibberellin (GA), brassinosteroid (BRs), abscisic acid (ABA), jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) can work together or independently to regulate almost all physiological responses of plants (Wasternack, 2007; Davies, 2010). Among these plant hormones, ABA and JA play crucial roles in plant adaptation to multiple stresses. The function of ABA in salt

tolerance is well documented, while the role JA in salt stress tolerance is not totally understood. The roles of ABA and JA during salt stress will be discussed in the following pages.

2.3.2.1. Abscisic acid (ABA)

Abscisic acid belongs to a group of secondary metabolites known as terpenoids or isprenoids (Nambara and Marion-poll, 2005; Taylor et al., 2000). In the plastid, ABA is synthesized from phytoene, a carotenoid derived from pyruvate and glyceraldehydes-3-posphate (Davies, 2010; Arve et al., 2013). Abscisic acid, known as a stress hormone, seems to have a major function in salt tolerance of plants. It also plays a critical role during multiple stages of the plant's life cycle such as seed development, bud dormancy and leaf senescence (Hsu and Kao, 2003; Devinar et al., 2013; Fahad et al., 2015). During salt stress, ABA synthesis is triggered rapidly during the osmotic stress phase, inducing stomatal closure in order to diminish the water loss from the leaves through transpiration (Hassine and Lutts, 2010). In addition, ABA activates multiple stress-responsive genes that encode enzymes involved in compatible osmolyte biosynthesis, such as dehydrins (Gao et al., 2004; Fahad et al., 2015). ABA is also crucial for synthesis of osmoprotectants such as proline during stress (Iqbal et al., 2014). Zhang et al. (2007) and Lu et al. (2009) reported that ABA mediated salt tolerance by accumulation of NO, which in turn activated mitogen-activated protein kinases (MAPK) and genes encoding antioxidant enzymes to scavenge ROS. The importance of ABA as a vital cellular signal during stress could be explained by the overlap between ABA and other signal molecules. Several studies reported that multiple secondary messengers such as ROS, Ca^{2+}, NO and

phospholipids have been observed as downstream targets for ABA signaling (Pei et al., 2000; Suhita et al., 2004; Cutler et al., 2010; Fahad et al., 2015).

2.3.2.2. Jasmonates (JAs)

Jasmonates (JAs), including jasmonic acid (JA) and its related compounds, are ubiquitous lipid-derived molecules that function as master switchs in several responses (Wasternack, 2007; Wasternack and Hause, 2013). In chloroplasts, the biosynthesis of jasmonates (JAs) begins with the peroxidation of α-linolenic acid (fatty acid released from galactolipids of chloroplast membranes) by lipoxygenase (LOX) to form a fatty acid hydroperoxide, 13(S)-hydroperoxylinolenic acid (HPLA) (Fig. 2.1). This intermediate compound is dehydrated by an allene oxide synthase (AOS) to form 12, 13(S)-epoxy-octadecatrienoic acid (12, 13-EOT), which can be cyclized to 12-oxo-phytodienoic acid (OPDA) by the action of an allene oxide cyclase (AOC). In peroxisomes, *cis* (+)-OPDA is further converted into (+)-7-*iso*-JA by three beta oxidation steps and 12-oxo-phytodienoic acid reductase (OPR). (+)-7-*iso*-JA is converted into a stable *trans* configuration, (-)-JA (generally known as jasmonic acid) or is submitted to further modifications to produce JA derivatives including (+)-7-*iso*-Jasmonoyl-l-isoleucine (JA-Ile) and methyl jasmonate (MeJA) (Fig. 2.1). JA-Ile is the bioactive from of JA that is formed by the conjugation between JA and isoleucine in the presence of the enzyme Jasmonate-Resistant 1 (JAR1) (Wasternack and Hause, 2013; Yan et al., 2013).

In the recent years, JA signaling pathways have been elucidated in *Arabidopsis thaliana* (Fig. 2.2) (Chico et al., 2008; Chung et al., 2009; Wasternack and Hause, 2013). Similar to IAA signaling, JA-dependent gene induction involves hormone-activated

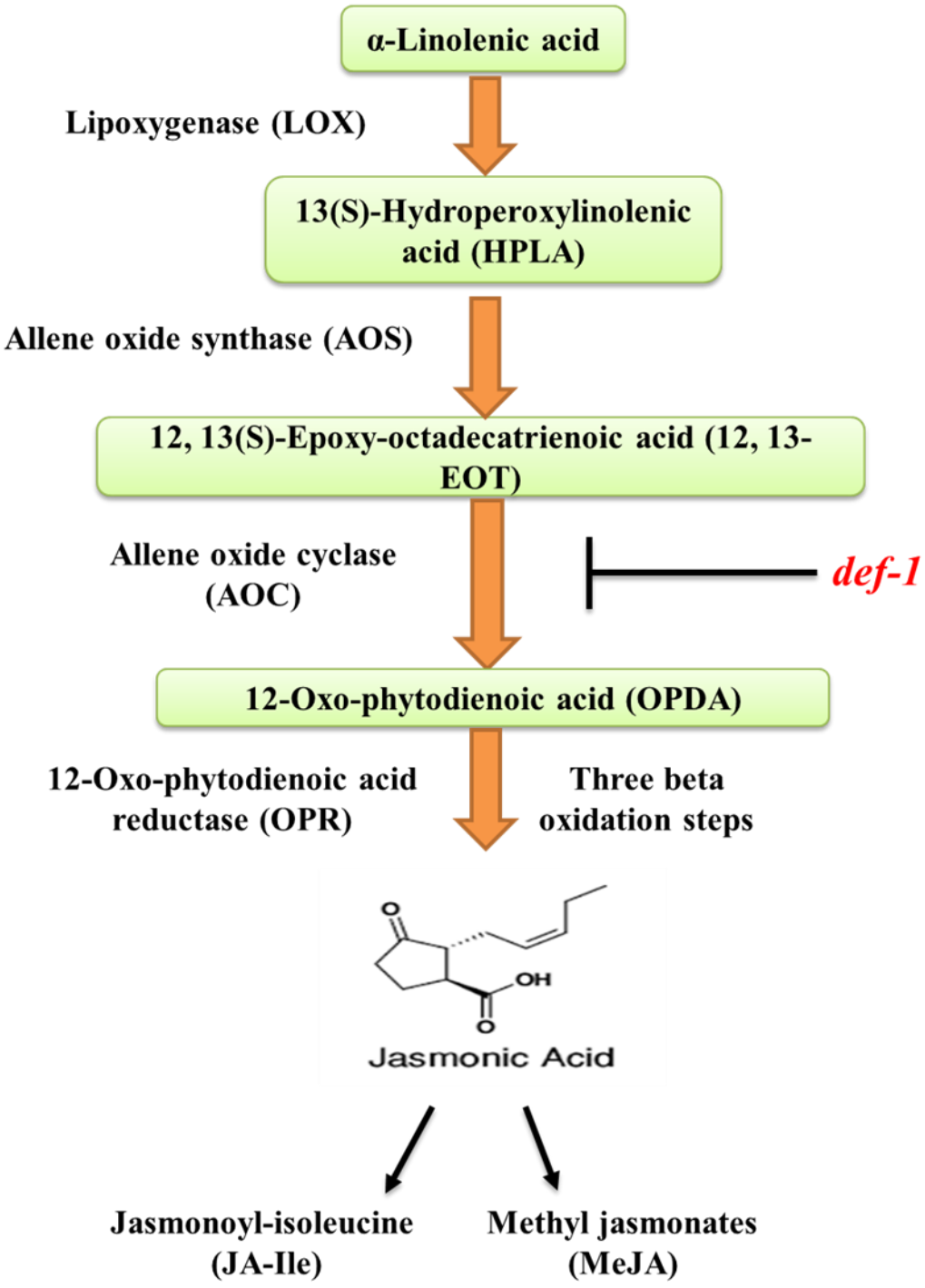

Fig. 2.1. Jasmonic acid (JA) biosynthesis pathway and *def-1* mutant with a deficiency in AOC activity.

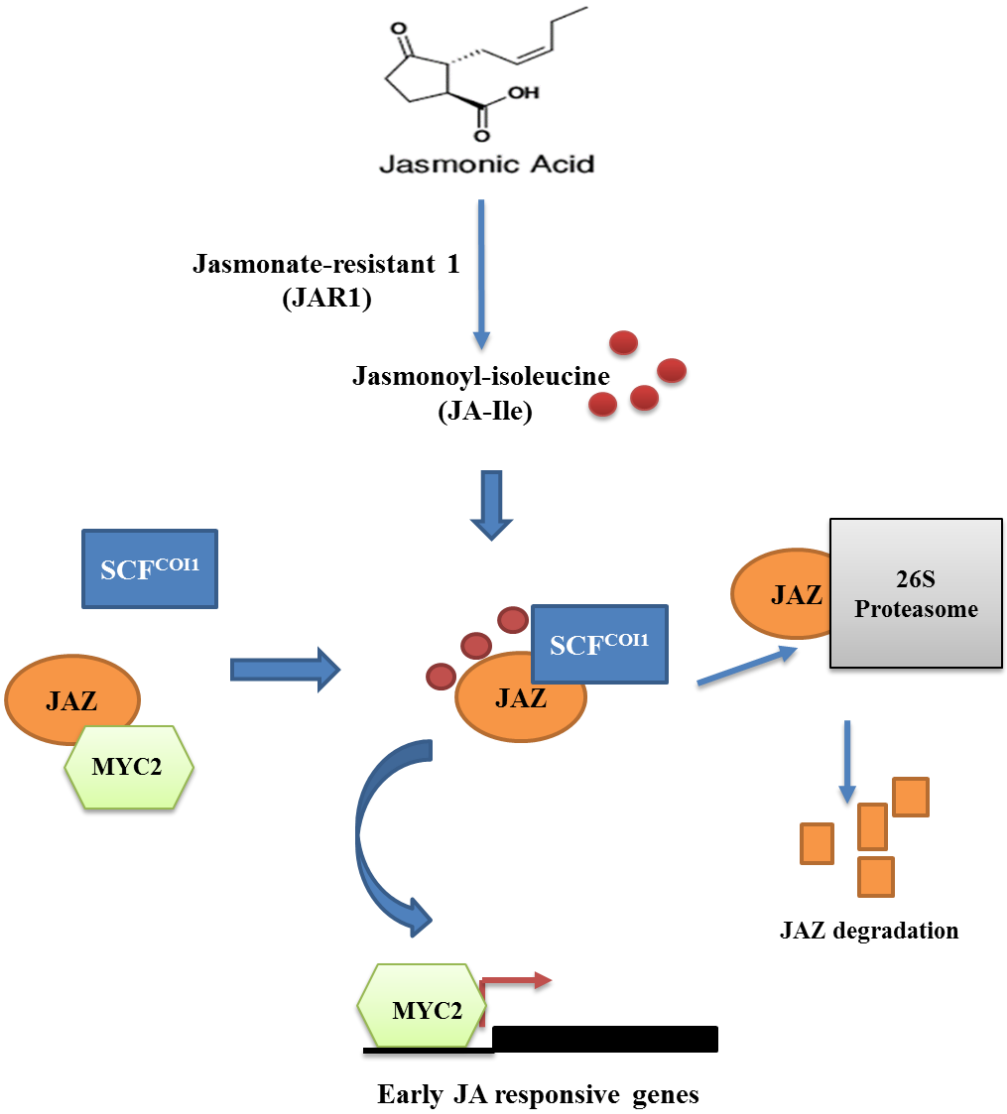

Fig. 2.2. Molecular mechanisms of JA signaling pathway.

degradation of transcriptional repressors, the jasmonate zim-domain (JAZ) proteins (Chini et al., 2007; Chung et al., 2009). In *Arabidopsis*, 12 *JAZ* genes have been identified (Chini et al., 2007). These *JAZ* genes possess a Jas (Jasmonate-associated) domain, which mediates hormone-dependent interactions of JAZ proteins with a basic helix-loop-helix (bHLH) transcription factor, MYC2/JIN1 (jasmonate insensitive1) as well as the Skp1–Cul1–Fbox protein COI1 (SCF^{COI1}) ubiquitin E3 ligase complex (Chini et al., 2007; Thines et al., 2007). In response to JA biosynthesis, the formation of JA-Ile promotes the SCF^{COI1}- mediated ubiquitination of JAZs (repressor for MYC2), followed by the degradation of JAZ protein by the 26S proteasome and release of the transcription factors, e.g., MYC2 (Fig. 2.2). The released MYC2 promotes the expression of early JA-responsive genes, including the *JAZ* genes themselves (Chico et al., 2008; Chung et al., 2009; Wasternack and Hause, 2013). Since MYC2 up-regulates its own repressor (JAZs), the suppression of MYC2 by JAZs triggers the late JA response (Chini et al., 2007; Chung et al., 2009; Mira et al., 2016).

For several decades, JAs have become well accepted as key cellular signals for defense against biotic stresses such as pathogens, insects and wounding (Farmer and Rayan, 1990; Browse, 2009; Ballaré, 2011). Moreover, JAs mediate several aspects of plant development, including root growth, seed germination, pollen viability, stomatal closure and senescence (Cheong and Choi, 2003; Haga and Iino, 2004; Wasternack and Hause, 2013; Riemann et al., 2013). Over the last decade, a growing body of evidence suggests that JA is also involved in salt stress (see introduction of chapter 3 for details). However, the mechanisms by which JA activates salt tolerance are still unknown. JA synthesis mutants, mainly from tomato and *Arabidopsis*, have provided the tools to refine

our understanding of the functions of JA in plant biology, such as defense against insects and fungal pathogens (Vijayan et al., 1998; How et al., 1999; Glazebrook, 2005), and pollen viability and development (Li et al., 2004). Therefore, analyses of these mutants could be key elements in the identification of the role of JA in salt tolerance.

Both forward and reverse genetic approaches have been applied to elucidate the central role of JA in plants (Browse, 2009). Lightner et al. (1993) employed a screen of mutant populations (forward genetics) to study wound-inducible signaling in tomato based on the deficiency in a wound response phenotype. The screen for mutants that are defective in the wound response led to the isolation of two non-allelic recessive mutants (Jl1 and Jl5), which were deficient in the activation of proteinase inhibitors after wounding stress (Lightner et al., 1993). Both Jl1 and Jl5 mutants showed increased activity of proteinase inhibitors after methyl jasmonate treatment, suggesting that both mutants exhibit disruption in the signaling cascade between the wound event and the downstream induction of JA (Lightner et al., 1993). A further study showed that the tomato mutant Jl5 was also more susceptible to the lepidopteran predator *Manduca sexta* (tobacco hornworm), therefore the name *defenseless-1* (*def-1*) was assigned (Howe et al., 1996). In addition, it was shown that *def-1* is deficient in JA accumulation in response to wounding and elicitation by systemin (wound signaling peptide). Moreover, the *def-1* mutant is defective in the activation of proteinase inhibitors (defense proteins) in response to elicitors (chitosan and systemin) of the wound response or the upstream precursors linolenic acid and 13(S)-hydroperoxylinolenic acid (HPLA). In contrast, downstream metabolites of the octadecanoid pathway, such as 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA), promote the accumulation of proteinase inhibitors

(Howe et al., 1996; Howe and Ryan, 1999). These results suggested that in *def-1* the octadecanoid pathway is blocked between HPLA and OPDA (Howe et al., 1996). As a step to identify the putative enzyme for JA biosynthesis that may be encoded by *Def-1*, genetic mapping studies have been attempted and showed that the known genes encoding JA biosynthetic enzymes (*LOXc*, *LOXd*, *AOS1*, *AOS2*, *AOC* and *OPR3*) do not co-map with the *Def-1* locus (Ziegler et al., 2000; Li et al., 2002). This finding suggested that *Def-1* may correspond to JA biosynthetic gene that has not been cloned (Li et al., 2002). Stenzel et al. (2003) reported that although *def-1* plants possess an AOC cDNA sequence and protein levels identical to the WT plants, the mutant exhibits lower AOC activity as indicated by *in vitro* enzyme assays. Thus, the specific factor affecting AOC activity in *def-1* remains to be determined (Fig. 2.1). The *def-1* mutant has been used extensively in many studies to illustrate how JA influences tomato development and seed germination (Wu et al., 2003), defense against herbivorous insects (Li et al., 2002; Ament et al., 2004; Kant et al., 2008; Scott et al., 2010; Grinberg-Yaari et al., 2015), defense against fungal pathogens (AbuQamar et al., 2008; El Oirdi et al., 2011; Mehari et al., 2015), arbuscular mycorrhizal symbiotic interaction (León-Morcillo, 2012) and lycopene biosynthesis in tomato fruits (Liu et al., 2012). Clearly, the *def-1* mutant could be also useful tool to determine the role of JA in salt tolerance.

2.4. Mechanisms of salt tolerance

Plants have evolved multiple adaptive strategies to withstand environmental stresses. During these hostile conditions such as salt stress, plants trigger salt tolerance mechanisms that aim to reestablish cell homeostasis. Plant salt tolerance is affected by many factors including plant genotype, provenance and developmental stage as well as

the intensity and duration of the salt stress, surrounding environmental conditions, and how the stress was applied to the plants (Hasegawa et al., 2000; Munns and Tester, 2008). As plant salt tolerance is a genetically complex trait, activation of multiple genes encoding plasma membrane transporters, antioxidant enzymes and compatible solutes are required to establish this process (Hasegawa et al., 2000; Munns and Tester, 2008; Türkan and Demiral, 2009). Collectively, salt tolerance mechanisms fall into four categories: homoeostasis and alleviation of Na^+ toxicity, osmotic adjustment, ROS scavenging and ions homoeostasis via plasma membrane transporters. The detailed mechanisms for salt-tolerance in plants are discussed below.

2.4.1. Alleviation of Na^+ toxicity

Salt stress increases the levels of toxic ions, especially Na^+, in plant cells. To prevent the negative effects of excessive Na^+ on plant cells, Na^+ can be moved out of the cytoplasm by multiple strategies including transport to old leaves or woody tissues, exclusion, compartmentalization in the vacuoles and/or accumulation in special organs. The ability of plant cells to avoid Na^+ toxicity is a crucial step for salt tolerance (Hasegawa et al., 2000).

2.4.1.1. Na^+ transport and retention in specific tissues

As a strategy to protect the growing leaves from Na^+ toxicity, Na^+ can accumulate preferentially in the old leaves in some species such as rice (*Oryza sativa*) (Wang et al., 2012). As a result, the old leaves (sink leaves) are dropped down when the salt has reached toxic levels (Hasegawa et al., 2000). In some woody plant species such as plum (*Prunus salicina*), salt is also accumulated in the woody tissues of roots and twigs, suggesting that these tissues act as storage compartments to help removing salt from the

plant leaves (Ziska et al., 1991). In addition, Na^+ retention in root tissues is another mechanisms that limits its transport to leaves in order to reduce injury symptoms (Munns et al., 2005).

2.4.1.2. Na^+ exclusion

Active transport of Na^+ through the plasma membrane is mediated by antiporters against the electrochemical potential gradient (Zhu, 2003; Munns, 2005). The plasma membrane Na^+/H^+ antiporter (SOS1) effluxes the excess Na^+ from the cytosol to the external medium (Munns and Tester, 2008). Moreover, SOS1 has been suggested to function as a Na^+ sensor in plants (Zhu, 2002).

2.4.1.3. Na^+ compartmentalization

The intracellular vacuolar sequestration of Na^+ is another mechanism employed by plants to avoid Na^+ toxicity in the cytoplasm (Zhu, 2003; Munns, 2005). The sequestration of Na^+ into the vacuolar lumen against its electrochemical gradient is mediated by the Na^+/H^+ exchanger (NHX), which is located in the tonoplast (Blumwald et al., 2000; Türkan and Demiral, 2009). The overexpression of the tonoplast *NHX1* antiporter in *Arabidopsis*, tomato and canola (*Brassica napus*) plants increased the salt-tolerance by reducing Na^+ toxicity (Apse et al., 1999; Blumwald, 2000; Chinnusamy et al., 2006). Halophytes and glycophytes differ in their capacity to sequester Na^+ into the vacuole, while halophytes can use Na^+ as osmoticum in order to maintain turgor pressure and osmotic potential, glycophytes have limited capacity for this mechanism (Blumwald et al., 2000).

2.4.1.4. Special organs or structures to minimize salt toxicity

Some halophyte species, possess special organs, including salt glands or salt bladders, which limit Na^+ accumulation in leaves. Salt glands secrete the excess salts from leaves to form salt crystals, which could be visible on the leaves surface of those halophyte species such as black mangroves (*Avicennia germinans*) (Flowers et al., 2008). Salt bladders, present on leaf surface of some halophyte species such as ice plants (*Aizoaceae*), do not secrete salts but act as storage compartment (Flowers et al., 2008). Among the halophytes, some succulent plants have fleshy stems or leaves that can store water to reduce salt toxicity (Flowers et al., 2008).

2.4.2. Osmotic adjustment

During salt stress, in response to the imbalance in water relations and the increased water loss, many plants species have the ability to change their intracellular osmotic potential. Those plants, especially salt-tolerant species, achieve this process by increasing the accumulation of compatible solutes in the cytoplasm and/or by having tight control of stomatal closure. The increase in compatible solute levels enables the maintenance of the turgor pressure and a water potential gradient to allow water uptake by lowering the osmotic potential (Sairam and Tyagi, 2004; Sanchez et al., 2008).

The mechanisms to adjust the osmotic potential (osmoregulation) are different between halophytes and glycophytes. Halophytes can use both organic and inorganic solutes such as Na^+ and Cl^- for osmotic adjustment, while glycophytes use mainly organic solutes (Manchanda and Grag, 2008). The synthesis of osmolytes requires energy resulting in a reduction of plant growth. However, osmolytes increase the capacity of plants to survive under non-favorable conditions (Munns and Tester, 2008). Although

osmolytes (compatible solutes) accumulated at high concentration during salinity stress, they do not interfere with the function and structure of macromolecules such as proteins (Burg and Ferrais, 2008). These compatible solutes include a broad range of compounds such as amino acids (e.g. proline), sugars (e.g. raffinose, glucose, sucrose and fructose), sugar alcohols (e.g. glycerol and mannitol), betaines (e.g. glycine betaine) and cyclic polyhydric alcohols (e.g. myoinositol and pinitol) (Munns 2005, Türkan and Demiral, 2009). In plants, proline is synthesized from two different precursors, glutamate and ornithine (Verbruggen and Hermans, 2008). The glutamate pathway catalyzed by Δ1-pyrroline-5-carboxylate synthetase (P5CS) represents the main pathway for proline synthesis in salt-stressed plants (Stines et al., 1999). Proline is considered to be one of the major osmoticum that accumulates during salinity stress and has been associated with salt-tolerance in many plant studies (Ashraf and Harris, 2004). The phenotypic characterization of the *Arabidopsis* proline deficient mutant s*p5cs1*as well as *aba2-1* (ABA-deficient mutant) and *p5cs1/aba2-1* (double mutants) suggests that ABA mediates proline synthesis during osmotic stress (Sharma et al., 2011). However, salt sensitive genotypes can synthesize more proline during salt stress than the salt-tolerant genotypes (Sun et al., 2010), indicating that proline accumulation in this case could be an indicator for stress rather than for acclimation (Sun et al., 2010). In addition to their role in osmotic adjustment, osmolytes can also improve salinity tolerance by protecting and stabilizing membranes and enzymes during stress conditions (Misra and Gupta, 2005). Moreover, the role of osmolytes such as proline in ROS scavenging has been suggested (Parida and Das, 2005; Türkan and Demiral, 2009).

2.4.3. ROS scavenging

To maintain ROS homeostasis and mitigate their toxicity during salt stress, plants have evolved ROS scavenging mechanisms using enzymatic and non-enzymatic antioxidants (Apel and Hirt, 2004; Gill and Tuteja, 2010). The enzymatic antioxidant system includes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), dehydroascrobate reductase (DHAR), monodehydroascrobate reductase (MDHAR), guacicol peroxidase (GOPX) and glutathione-s-transferase (GST). Most of those antioxidant enzymes can detoxify the ROS effectively via the ascorbate-glutathione (AsA/GSH) cycle, also known as the Halliwell-Asada pathway. In this cycle, SOD first catalyzes the conversion of $O_2^{\cdot -}$, the first ROS to be produced, to O_2 and H_2O_2 (Bowler et al., 1992; Apel and Hirt, 2004). The resulting H_2O_2 is then scavenged to H_2O via CAT or APX (Asada 1997; Willekens et al., 1997). The scavenging reaction for CAT does not need a reductant, while APX need a reductant such as ascorbate. Regeneration of ascorbate involves the activity of other enzymes including MDHAR, DHAR and GR (Gill and Tuteja, 2010). Various comparative studies have demonstrated a strong correlation between salt-tolerant genotypes and high antioxidant activities in different plant species (Sreenivasulu et al., 2000; Mittova et al., 2002). In addition, during salt stress, transgenic plants overexpressing SOD, GR and APX exhibit tolerance to oxidative stress (Vinocur and Altman 2005; Türkan and Demiral, 2009; Miller et al., 2010). The other category of antioxidants is non-enzymatic ones such as ascorbate (AsA), reduced glutathione (GSH), carotenoids, vitamins, phenols and flavonoids. Ascorbate and GSH have the ability to scavenge multiple ROS such as $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ (Gill and Tuteja, 2010). Reduced

glutathione is involved in the ascorbate-glutathione cycle and has a protective role for the thiol group of proteins during oxidative stress (Mullineaux and Rausch, 2005; Yusuf et al., 2012). Several lines of evidence suggest that vitamins such as tocopherols and ascorbic acid have a role in keeping an adequate redox state and preventing cellular lipid peroxidation (Munne-Bosch, 2005; Dellapenna and Pogson, 2006). Ascorbic acid is an important antioxidant that reacts with H_2O_2 and $OH^{\cdot}$ to produce less toxic compounds (Reddy et al., 2004; Ahmad et al., 2010). Carotenoids, synthesized naturally as pigments, such as β-carotenes, xanthophylls and lycopenes, also have antioxidant activities via quenching the singlet oxygen (Paiva and Russell, 1999).

Phenolic compounds, relatively abundant in different plant tissues, have *in vitro* ROS scavenging activities higher than tocopherols and ascorbate (Blokhina et al., 2003). This high ROS scavenging activity for phenolic compounds is due to the unique chemical properties, such as the large number of OH groups and their high activity as electron donors (Blokhina et al., 2003). Phenolic compounds have a great diversity in their carbon skeleton, which reflects the broad diversity of these compounds such as flavonoids, tannins and lignin (Jaganath and Crozier, 2010). Overall, the roles of enzymatic and non-enzymatic antioxidants in salt-induced oxidative stress are well documented. However, how plants regulate antioxidant systems during salinity stress is not fully understood.

2.4.4. Ion homoeostasis and plasma membrane transporters

The high level of NaCl in saline soils disturbs cellular ion homeostasis (e. g. K^+, Ca^{2+}, SO_4^{2-}, NO_3^- and NH_4^+) (Sairam and Tyagi, 2004; Munns and Tester, 2008; Shokri and Maadi, 2009). This disturbance of ion homeostasis can result in cellular damage and plant growth cessation or even plant death. Thus, restoring ion homeostasis after

imbalance caused by salt stress is a critical mechanism for salt tolerance (Hauser and Horie, 2010; Hasegawa, 2013). Major advances have been made to identify the mechanisms, which are achieved by different protein transporters involved in ion uptake, distribution or efflux, to re-establish the homeostasis of ions including K^+, Ca^{2+} and SO_4^{2-} during salt stress (Hasegawa, 2013; Hauser and Horie, 2010; Cao et al., 2013).

Potassium is required for diverse cellular processes such as enzyme activity, osmotic regulation and maintenance of membrane potential (Hasegawa, 2013; Munns and Tester, 2008). Various studies have indicated that increasing cytosolic K^+ levels relative to Na^+ (high K^+/Na^+ ratio) in leaves is a critical strategy for salinity tolerance (Ren et al., 2005; Hauser and Horie, 2010; Hasegawa, 2013). In *Arabidopsis* and rice (*Oryza sativa*), the high affinity potassium transporters (HKTs) play a vital role in maintaining a high K^+/Na^+ ratio in shoots under salt stress (Ren et al., 2005; Hauser and Horie., 2010). AtHKT1.1 and OsHKT1.5 transporters mediate Na^+ transport from the stem xylem vessels to xylem parenchyma cells. Sodium exclusion from xylem vessels causes membrane depolarization of xylem parenchyma cells and promotes K^+ secretion into xylem vessels by K^+ outward-rectifying (KOR) channels (Hauser and Horie, 2010). The reduction in Na^+ and the increase in K^+ contents of xylem vessels, which carry water and ions to shoots, help to maintain a high K^+/Na^+ ratio in shoots (Ren et al., 2005; Hauser and Horie, 2010). Whether the excluded Na^+ from xylem vessels is transported back to the roots by loading into the phloem remains a question to be addressed (Hauser and Horie, 2010).

Calcium plays critical roles in salt-stressed cells such as second messenger for stress signal transduction, maintenance of the functional integrity of the plasma

membrane and regulation of ion transport and selectivity (Tuna et al., 2007). Salt stress can also lead to reduced Ca^{2+} uptake and xylem loading in the roots, creating a deficiency symptom in leaves (Halperin et al., 1997). Under this condition, the Ca^{2+} pump (ACA), located on the root cell membrane, can show a significant contribution to Ca^{2+} efflux from root cells into the root apoplast to facilitate the xylem loading of Ca^{2+} and translocation to the growing leaf tissues (Maathuis, 2006).

More recently, the contribution of sulfate transporters (SULTR) to salt tolerance has been reported (Gallardo et al., 2014; Cao et al., 2013). Cao et al. (2013) suggested a role for the SULTR3.1 transporter in providing sulfate for cysteine synthesis under salt stress. Cysteine is required for the biosynthesis of stress-defense compounds such as ABA and glutathione (Cao et al., 2013).

Overall, the former studies have identified the contribution of plasma membrane transporters to restoring the homeostasis of some major ions under salt stress. However, nitrogen, the most demanded nutrient for plant growth (Flores et al., 2001; Popova et al., 2003), is also affected by salt stress (Fig. 2.3), but the contribution of N transporters in salt tolerance still remains unclear. Several studies indicate that the application of N fertilizer for salt-stressed plants usually enhances plant growth and salt tolerance (Flores et al., 2001; Popova et al., 2003; Ehlting et al., 2007). In plants, NO_3^- and NH_4^+ are the main sources for inorganic N uptake by roots (Tsay and Hsu, 2011). The root uptake of NO_3^- and NH_4^+ is mediated by various transporter genes for nitrate (*NRTs*) and ammonium (*AMTs*) (Rennenberg et al., 2010). In salt stressed plants, the preference of

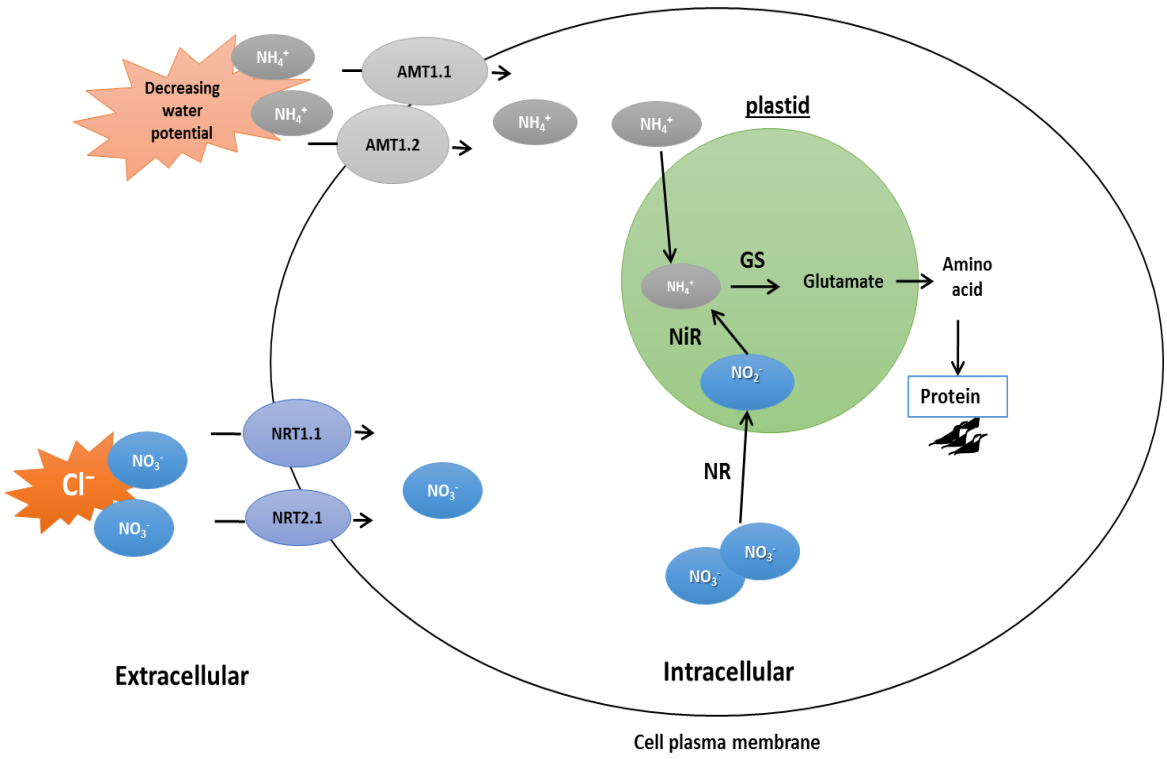

Fig. 2.3. Putative nitrogen (N) uptake and assimilation during salt stress conditions in the plant cell. The decrease in N uptake by plant roots exposed to salt stress results mainly from both the competition between NO_3^- and Cl^- (Botella et al., 1994; Yao et al., 2008) and the reduction in the water potential of the soil solution, causing a limitation in water uptake and thus a reduction in nutrient uptake (Ehlting et al., 2007). Nitrate (NO_3^-) assimilation in root cells includes two distinct steps: nitrate is first reduced to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR) and then reduced to ammonium (NH_4^+) in the plastids by nitrite reductase (NiR). In contrast, NH_4^+ is incorporated into organic form (glutamate) in one step via the glutamine synthetase (GS). Therefore, the preference of NH_4^+ uptake during salt stress is associated with energy saving and improved salt tolerance (Bloom et al., 1992; Flores et al., 2001).

NH_4^+ uptake over NO_3^- uptake by the roots has been associated with energy savings for N assimilation (Fig. 2.3) (Bloom et al., 1992; Flores et al., 2001; Zhang et al., 2014). Thus, salt-stressed plants do not have to spend extra energy reducing NO_3^- into NH_4^+. Such a saving of energy with NH_4^+ uptake versus NO_3^- uptake might lead to a better allocation of energy required for salt tolerance mechanisms and promote plant growth during salt stress (Kant et al., 2007). These results highlight the importance of N transporters in salt tolerance, especially ammonium transporters.

2.4.4.1. Nitrate transporters (NRTs) and salt stress

Nitrate is susceptible to leaching in the soil if it is not absorbed by microorganisms or plant roots, as NO_3^- is unable to form complexes with soil particles (Strahm and Harrison, 2006). In the soil solution, NO_3^- concentrations are variable, ranging from 100 µM to 7 mM (Dechorgnat et al., 2011). Due this fluctuation in NO_3^- levels, plants have evolved and developed different uptake systems to adjust for this variability. Two NO_3^- transporter subfamilies (NRT1 and NRT2) have been reported to act coordinately for both NO_3^- uptake from the soil solution and NO_3^- movement throughout plant organs (Tsay et al., 2007). In *Arabidopsis*, the *NRT1* subfamily includes 53 members and the *NRT2* consists of 7 members. When the external NO_3^- level is high the low-affinity transport system (LATS) functions and is constitutively expressed. On the other hand, at low external level of NO_3^-, the high affinity transporter system (HATS) operates for NO_3^- uptake (Crawford and Glass, 1998; Tsay et al., 2007). The low affinity transport system is mediated by the *NRT1* gene subfamily (Tsay et al., 1993). This

transporter is mainly located in cortical and endodermal cells of mature roots and in the epidermis cells of root tips, suggesting an important role in NO_3^- uptake (Huang et al., 1999). The NRT1.1, involved in NO_3^- uptake, could also regulate multiple developmental processes such as releasing from seed dormancy, supporting root proliferation and stimulating stomatal opening (Remans et al., 2006; Krouk et al., 2010). Unlike *NRT1.1*, *NRT1.2* is involved in NO_3^- uptake without a signaling role and is mainly expressed in the epidermal cells of both mature and young roots (Huang et al., 1999; Tsay et al., 2007). The high affinity transport system in plants is mediated by the *NRT2* gene subfamily (Tsay et al., 2007; Li et al., 2015). This was demonstrated in the *Arabidopsi*s double mutant (disrupted in *nrt2.1* and *nrt2.2*), which shows a large reduction of NO_3^- influx during high external NO_3^- concentration (Li et al., 2007). Similar to the *NRT1* subfamily, *NRT2* can also act either as a NO_3^- sensor or a signal transducer (Gojon et al., 2011). For example, *AMT2.1* acts as repressor of lateral root initiation (Little et al., 2005).

Some previous studies have shown that salt stress has negative effects on both the transcriptional levels of NO_3^- transporters (*NRT1* and *NRT2* subfamilies) in roots and NO_3^- uptake for different plant species (Yao et al., 2008; Zhang et al., 2014). In poplar (*Populus*), a moderate salt treatment (75 mM NaCl) for 21 days reduced the mRNA levels of most of the *NRT* genes in roots, which was also associated with reduction in NO_3^- uptake (Zhang et al., 2014). The reduction in NO_3^- uptake was also observed in tomato after exposure to similar level of salt (75 mM NaCl) but for a short exposure time (72h) (Yao et al., 2008). In the same study, down-regulation in mRNA expression levels of key genes involved in NO_3^- uptake such as *NRT1.1*, *NRT1.2* and *NRT2.2* were also reported. In addition, a very short exposure time (6h) to relatively high salt stress level

(200 mM NaCl) reduced the transcriptional levels of *NRT1* in *Arabidopsi*s roots (Chen et al., 2002). These findings suggest that *NRTs* may not function as a key component in N homeostasis or salt tolerance, since they are downregulated by salt stress, and open the possibility that *AMTs* could play a role in salt tolerance.

2.4.4.2. Ammonium transporters (AMTs) and salt stress

Although the NH_4^+ concentration (1-25 µM) is often lower than that of NO_3^- in soil, NH_4^+ is the preferable source of N in some plant species (Couturier et al., 2007; Tsay and Hsu, 2011). The first ammonium transporter was isolated from yeast (*Saccharomyces cerevisiae*) by complementation analysis of a yeast mutant that was defective in NH_4^+/ NH_3 uptake (Marini et al., 1994). Later, it was shown that most prokaryotes and eukaryotes have AMT, MEP and/or Rh (Ammonium transporter, Methyl ammonium permease and/or Rhesus, respectively) proteins. AMT-type proteins have been identified in plants, while rhesus proteins (NH_3 channels, present in algae and mammals) have not so far been found in plants (Ludewig et al., 2007). Plant roots have two ammonium uptake systems: 1) HATS (high-affinity transport system), which contributes to ammonium uptake in the low external concentration range (< 1 mM) and 2) LATS (low-affinity transport system) that operates at higher external concentrations (>1 mM) (Glass et al., 2002). Increasing evidence shows that root AMTs are responsible for the major high-affinity uptake of ammonium (Loqué and von Wirén, 2004; Couturier et al., 2007; Straub et al., 2014). The function of AMTs in plants is not only restricted to the root nitrogen uptake from soil, but they are also involved in NH_4^+/ NH_3 recycling during leaf senescence or photorespiration in leaves (von Wirén et al., 2000; Couturier et al., 2007). In plants, the ammonium transporter family (AMT) is divided into two subfamilies

(AMT1 and AMT2) (Loqué and von Wirén, 2004). AMT subfamilies apparently differ in their transported substrates. While AMT1 transports the charged ion NH_4^+, AMT2 seems to transfer the neutral molecule NH_3 (Couturier et al., 2007; Straub et al., 2014; Wu et al., 2015). Different plant species have unequal number of AMTs in their genome. For example, 6 (5 for AMT1 and 1 for AMT2), 14 (6 for AMT1 and 8 for AMT2) and 8 (3 for AMT1 and 5 for AMT2) members of the AMT family have been found in *Arabidopsis thaliana*, *Populus trichocarpa* and *Lotus japonicus* respectively (Couturier et al., 2007; Tsay and Hsu, 2011). This suggests that plants from different species or environments as well as at different stages of their life cycle mediate NH_4^+/ NH_3 uptake and translocation with unequal number of AMTs (Couturier et al., 2007). Only one recent study highlighted the role of AMTs in salt tolerance (Zhang et al., 2014). In this study, the salt-stressed root of a woody species, *Populus simonii* (Chinese poplar) showed up-regulation of mRNA levels for *AMT* genes that was associated with higher NH_4^+ uptake compared to control conditions. At the same time, the mRNA levels of most *NRTs* genes and NO_3^- uptake were reduced by salt stress. This suggests a role of NH_4^+ transporters in NH_4^+ flux during acclimation to salinity stress (Zhang et al., 2014). However, tomato, as herbal annual plant, is different in its structure from the woody perennial trees such as poplar. In addition, both species may have unequal number of AMTs. Therefore, the involvement of N transporters in salt tolerance in non woody plant species also need to be tested.

2.5. Conclusion

Salinity is a worldwide threat that causes a large reduction in the global yield of crops including tomato plants. The literature reviewed indicates that the adverse effects

of salinity on plants result from ionic stress, osmotic stress, oxidative stress and nutrients depletion. To survive in saline environments, plants have to adjust their growth and development under such conditions through the integration of both salt stress signaling (e. g. plant hormones) and mechanisms of salt tolerance (including ion homoeostasis by changes in plasma membrane transporters). Although a large number of studies have been conducted, several aspects of salinity tolerance remain to be explored. Potential contributions for JA and *AMTs* were highlighted in the literature review. However, their roles in plant salt tolerance are not fully understood. Studying the response of the JA biosynthesis tomato mutant (*def-1*) to salt stress at the physiological and biochemical levels should be a useful tool to elucidate the mechanisms by which JA regulates plant salt tolerance. On the other hand, in order to investigate the contribution of *AMTs* to salt tolerance, there is a need to identify the *AMT2* subfamily in the genome of tomato. This will help obtain a more complete picture of the entire set of *AMTs* in plant species and their role in transporting NH_4^+/ NH_3 in roots. The diversity in salt tolerance between the wild and domesticated genotypes could be used to further investigate the contribution of *AMTs* and *NRTs* to salt tolerance.

CHAPTER 3. ENHANCED OXIDATIVE STRESS IN THE JASMONIC ACID-DEFICIENT TOMATO MUTANT *DEF-1* EXPOSED TO SALT STRESS*

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My contribution:

I conducted all experiments, analyzed the data and wrote the manuscript.

3.1. Abstract

Jasmonic acid (JA) has been mostly studied in response to biotic stresses such as herbivore attack (wounding) and pathogenic infection. More recently, the involvement of JA in abiotic stresses including salinity was highlighted; yet, its role in salt stress remained much less explored. In the current study, we compared the physiological and biochemical responses of wild-type (WT) tomato (*Solanum lycopersicum*) cv Castlemart and its JA-deficient mutant *defenseless-1* (*def-1*) under salt stress to investigate the role of JA. Plant growth, photosynthetic pigment content, ion accumulation, oxidative stress-related parameters, proline accumulation and total phenolic compounds, in addition to both enzymatic and non-enzymatic antioxidant activities, were measured in both genotypes after 14 days of 100 mM NaCl treatment. Although we observed in both genotypes similar growth pattern and foliar sodium, calcium and potassium levels after salt stress, *def-1* plants exhibited a more pronounced decrease of nitrogen content in both leaves and roots and a slightly higher level of sodium in roots compared to WT plants. In addition, *def-1* plants exposed to salt stress showed reactive oxygen species (ROS)-associated injury phenotypes as indicated by the higher level of both hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) as well as a slight decrease of photosynthetic pigments compared to WT plants. These oxidative stress symptoms in *def-1* were associated with lower activity of both enzymatic antioxidants including glutathione reductase (GR), glutathione-s-transferase (GST) and superoxide dismutase (SOD), and non-enzymatic antioxidants as indicated by the lower scavenging activity for 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Furthermore, the levels of the non-enzymatic ROS scavengers proline and total phenolic compounds increased in both genotypes exposed to salt stress, while only proline level increased in the WT plants. Overall the results of this

study suggest that endogenous JA mainly enhanced tomato salt tolerance by maintaining ROS homeostasis.

Keywords: *Solanum lycopersicum*; salt stress; jasmonic acid; oxidative stress; ROS homeostasis.

3.2. Introduction

Salinity is considered to be a global threat that decreases the yield of commercial crops worldwide (Parida and Das, 2005). Tomato (*Solanum lycopersicum*), as a glycophyte, is relatively sensitive to salinity stress mainly at the seedling stage (Sun et al., 2010). Salt stress induces both osmotic and ionic stresses resulting in the disturbance of multiple physiological processes such as ionic homeostasis, water relations and gas exchanges (Hasegawa et al., 2000; Parida and Das, 2005). As a result of limited CO_2 fixation in salt-stressed plants, the over-production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) can be induced in the chloroplasts and other organelles (Parida and Das, 2005; Miller et al., 2010). The resulting ROS-associated injury typically includes oxidative damage to proteins, lipids, DNA and photosynthetic pigments (Apel and Hirt, 2004). To survive under saline environments, plants have evolved mechanisms of salt tolerance such as exclusion or selective accumulation of toxic ions, compatible solute synthesis and ROS homeostasis by the activation of both enzymatic and non-enzymatic antioxidants (Hasegawa et al., 2000; Parida and Das, 2005).

Plant hormones play a crucial role in plant defense against both biotic and abiotic stresses (Wasternack, 2007; Peleg and Blumwald, 2011). Jasmonic acid (JA) is one of the hormones that acts as a vital signaling molecule in biotic stress responses (herbivores and

necrotrophic pathogens) and development (root growth and fertility) (Wasternack, 2015). Furthermore, increasing evidence has shown that JA is also involved in the plant responses to many abiotic stresses such as UV and cold (Dar et al., 2015). The biosynthesis of JA begins with the peroxidation of α-linolenic acid by lipoxygenase (LOX) leading to the formation of JA and its derivatives, including (+)-7-iso-Jasmonoyl-l-isoleucine (JA-Ile) and methyle jasmonate (MeJA), collectively known as jasmonates (JAs). JA-Ile is synthesized in the presence of the JA amino acid synthetase enzyme JAR1 (Jasmonate-Resistant 1) by the conjugation of JA and isoleucine (Yan et al., 2013). JA-Ile promotes the interaction of JAZs (repressor for Jasmonate insensitive1, MYC2/JIN1) with the SCF^{COI1} E3 ubiquitin ligase, resulting in JAZ ubiquitination, which is followed by the degradation of the JAZ protein by the 26S proteasome and the release of the transcription factor MYC2. The released MYC2 then promotes the expression of early JA-responsive genes (Dombrecht et al., 2007; Chico et al., 2008; Chung et al., 2009; Mira et al., 2016).

While JA has been mostly studied in biotic stress such as herbivore attack (wounding) and pathogenic infection (Wasternack, 2007), a growing body of evidence suggests that JA is also involved in salt stress. Salinity stress activates genes involved in the JA synthesis branch of α-linolenic acid metabolism (Pedranzani, 2003). In addition, JA has been shown to accumulate in response to salt stress in different plant species (Moons et al., 1997; Pedranzani, 2003). For example, in *Solanum lycopersicum*, a correlation between salt tolerance and high levels of JA has been reported (Pedranzani, 2003). Moreover, exogenous JA treatments alleviated salt-induced injury in a variety of plants by increasing the photosynthesis rate (Walia et al., 2007), proline content (Fedina

and Tsonev, 1997), ABA level (Kang et al., 2005), activity of antioxidant enzymes (Qiu et al., 2014) and/or decreasing Na^+ accumulation in the shoots (Fedina and Tsonev, 1997). Hence, it is likely that exogenous JA does play a positive role in salinity tolerance. However, it is difficult to draw similar conclusions for the role of endogenous JA in salt tolerance, as while exogenous JA is present constitutively in the plant tissues, endogenous JA signaling is tightly controlled, resulting in fluctuations in endogenous JA level under stress conditions (Ismail et al., 2014).

A few recent studies have focused on the use of mutants and transgenic *Arabidopsis thaliana* plants altered in the JA pathway to study the role of JA in salt-stressed plants (Zhao et al., 2014; Ding et al., 2016; Lim et al., 2015). The overexpression of two genes of the JA biosynthesis pathway, *TaAOC1* (gene encoding the enzyme allene oxide cyclase, Zhao et al., 2014) and *CaLOX1* (Lim et al., 2015) resulted in improved salt tolerance. These plants exhibited either a high survival rate, elevated superoxide dismutase (SOD) activity and an increased level of JA (Zhao et al., 2014) or an improved germination rate, biomass and chlorophyll content (Lim et al., 2015) during salt stress. In addition, a *lox3* (*lipoxygenase3*) mutant (impaired in JA and JA-Il accumulation) showed hypersensitivity to salinity (associated with a reduction in survival rate, germination and lateral root number). This salt sensitive phenotype was rescued by exogenous treatment with MeJA (Ding et al., 2016). Although these studies suggest that endogenous JA can positively regulate salt tolerance; the mechanisms by which JA regulates salt tolerance still need to be further investigated. Studies using plants bearing mutations within a hormone-biosynthetic pathway have been instrumental in advancing our understanding of plant responses to unfavorable environmental conditions (Peleg and Blumwald, 2011).

Therefore, the objective of our study was to determine the involvement of JA in both ROS and ion homeostasis, as major aspects of salt tolerance, by comparatively analyzing the stress responses of JA-deficient mutant *def-1* (*defenseless-1*, with a defective JA synthesis pathway) (Howe et al., 1996) and its wild-type. We hypothesize that salt tolerance of tomato JA-deficient mutant *def-1* will be lower than in the wild type plants, exhibiting oxidative stress symptoms and ionic imbalance.

3.3. Materials and methods

3.3.1. Plant growth and salt treatment

Tomato (*Solanum lycopersicum*) seeds of the *def-1* mutant and wild-type (WT) cv Castlemart were kindly provided by Dr. Gregg Howe (Michigan State University, USA). Seeds of both genotypes were surface sterilized for 10 min with 5% sodium hypochlorite. The seeds were then rinsed several times with distilled water and germinated in 9-cm Petri dishes containing two filter papers (Whatman No.1) moistened with 5 ml of half strength modified Hoagland nutrient solution (see composition below). The Petri dishes were incubated at 24 °C for 2 days in the dark and then transferred for 5 days at the following greenhouse conditions: 25/19 °C day/night temperatures, 18h/6h photoperiod with natural light supplemented by sodium lamps (P.L. light systems, Beamsville, ON, Canada). The well-grown seedling were transferred for 12 days to an aerated hydroponic solution (half strength modified Hoagland solution containing 2 mM NH_4NO_3; 1 mM KH_2PO_4; 1.5 mM $CaCl_2$; 0.5 mM KCl_2; 1 mM $MgSO_4$; 23 µM H_3BO_3; 5 µM $MnCl_2$; 0.4 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.07 µM H_2MoO_4 in addition to 7 µM Fe-EDTA).

The nutrient solution was changed every 4 days to aviod nutrient deficiency. Five plants from each genotype were placed in 10-L containers (each container was considered as one replicate), in a completely random block design with four replicates. Seedlings from both genotypes were exposed to salinity stress at the stage of two-three true leaves by adding 100 mM NaCl to the nutrient solution for 14 days. To avoid NaCl-induced osmotic shock, NaCl was added to the nutrient solution in a stepwise method by 25 mM increments a day until reaching the final concertation (100 mM). After 14 days of salt treatment, leaves were collected, immediately frozen in liquid nitrogen and stored at -80°C for subsequent determination of lipid peroxidation, H_2O_2 level, proline content and enzymatic antioxidants analysis. The remaining plants were harvested, washed three times with distilled water and the tissues were lyophilized to determine dry weights, total phenolic content, DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity as well as ion accumulation in shoots and roots.

3.3.2. Photosynthetic pigments

To determine leaf chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids, 30 mg of freeze-dried tissues were homogenized with 10 mL of acetone (80%) and kept in dark for 24h. The absorbance was measured at 480, 645, and 663 nm. Quantifications of Chl a, Chl b and carotenoids in the extracts were calculated using MacKinney equations (Sestak et al., 1971):

Chl a (mg g^{-1} DW) = (12.72×A663) − (2.58×A645)

Chl b (mg g^{-1} DW) = (22.87×A645) − (4.67×A663)

Carotenoids (mg g^{-1} DW) = (0.114×A663) + A480 − (0.638×A645)

3.3.3. Elemental analysis

Lyophilized shoots and roots were ground separately using a coffee grinder (Black & Decker, Brockville, ON Canada). The sodium, potassium and calcium contents of plant tissues were analyzed using a Direct Current Plasma (DCP) spectrophotometer. For N analysis, a CHNOS elemental analyser 'vario Micro' (Elementar, Hanau, Germany) was used. The Elemental analysis was performed by Stratford Agri. Analysis (Stratford, ON, Canada).

3.3.4. Free proline content

In the leaf tissue, free proline content was determined according to method described by Bates et al. (1973). One gram frozen tissue was homogenized with 10 ml sulphosalicylic acid (3%) using a mortar and pestle. The homogenate was then centrifuged at 4900 x g for 5 min. One ml supernatant was mixed with both 1 ml ninhydrin reagent (3% (w/v) ninhydrin in 60% (v/v) 6 M phosphoric acid) and 1 ml of glacial acetic acid. The mixture was heated at 100 °C for 60 min and the reaction was stopped by transferring the samples to an ice bath for 10 min. The absorbance was recorded at 520 nm using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, England). Proline content was expressed as µmol proline g^{-1} FW using a calibration curve (0- 0.250 µmol/ml proline).

3.3.5. Total phenolic content

Total phenolic content was measured using Folin-Ciocalteu's reagent (Sigma-Aldrich, Canada) according to Singleton and Rossi (1965). The total phenolic content of leaves was expressed in mg of gallic acid equivalents (GAE)/g dry weight.

3.3.6. Determination of lipid peroxidation

Lipid peroxidation of leaves was measured as the amount of malondialdehyde (MDA) produced in leaves using the 2-thiobarbituric acid (TBA) method as described by Heath and Packer (1968). Half a gram of leaf tissues was ground with 0.25 % TBA in 10 % trichloroacetic acid (TCA) using a mortar and pestle. The homogenate was then centrifuged at 10,000 x g for 20 min. The supernatant was heated at 95 °C for 30 min and the reaction was stopped by transferring the samples to an ice bath for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The levels of MDA–TBA complex (red pigment) was calculated from the extinction coefficient equal to 155 mM^{-1} cm^{-1}. The level of MDA–TBA was expressed as µmol g^{-1} fresh weight.

3.3.7. Hydrogen peroxide (H_2O_2) measurement

The level of H_2O_2 was measured in leaves of both genotypes using the FOX-1 method (ferrous oxidation with Xylenol Orange) described by Wolf (1994). Frozen tissues (100 mg) were homogenized using a mortar and pestle in 5 ml of 5% TCA containing 100 µg of active charcoal. The mixture was filtered using filter paper (Whatman N^o 1) and then incubated for 30 min at room temperature with the FOX-1 reagent (100 µM Xylenol Orange, 100 mM sorbitol, 25 mM H_2SO_4 and 250 µM ammonium sulphate). The absorbance was recorded at 560 nm and the levels of H_2O_2 were expressed as µmol g^{-1} fresh weight using a standard curve.

3.3.8. Extraction of antioxidant enzymes

Antioxidant enzymes and total protein were extracted from frozen leaf tissues of both genotypes according to Agarwal et al. (2005). One gram of frozen tissues was

homogenized with 10 mL of 100 mM K-phosphate extraction buffer (pH 7.0) containing 1% polyvinylpyrrolidone (PVP), 5 mM ascorbic acid (AsA) and 1 mM ethylenediaminetetraacetic acid (EDTA) using a pestle and a mortar. The homogenate was centrifuged at 19,000 x *g* (4 °C) for 20 min, and the supernatant was stored at -20 °C for enzyme analysis. Total protein contents were recorded in the crude extract based on the method of Bradford (1976) using bovine serum albumin as a standard.

3.3.9. Antioxidant enzyme assays

Catalase (EC 1.11.1.6) activity was estimated based on the method of Aebi (1984) by observing the disappearance of H_2O_2. In 2 ml of 15 mM freshly prepared H_2O_2 and 50 mM K-phosphate buffer (pH 7.0), 50 µl of enzyme extract was added. The decrease in absorbance was measured for 1 min at 240 nm. The degradation of 1 µmol H_2O_2 per min was defined as one unit of CAT. Superoxide dismutase (EC 1.15.1.1) activity was recorded based on the method described by Dionisio-Sese and Tobita (1998). The enzyme activity was determined in a 3 mL of reaction mixture containing 50 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 2 µM riboflavin, 75 µM nitroblue tetrazolium (NBT) and 13 mM L-methionine. A set of test tubes (without enzyme extract) was incubated in light (300 µmol m^{-2} s^{-1} for 20 min) and used as light blank. Following the light treatment, the reaction was stopped by switching the light off, and the absorbance value was recorded spectrophotometrically at 560 nm and subtracted from the absorbance value of the light blank. One unit of SOD activity was defined as the volume of enzyme extract required to cause 50% inhibition in the photochemical reduction of NBT. For ascorbate peroxidase (EC 1.11.1.11) activity, the assay was initiated by adding 100 µL of enzyme extract to 2 ml of reaction mixture containing 100 mM K-phosphate buffer (pH

7.0), 1.2 mM H_2O_2, 0.1 mM EDTA and 0.5 mM ascorbic acid (AsA) as described by Nakano and Asada (1981). The decrease in the absorbance of AsA at 290 nm was recorded spectrophotometrically every 10 s for 2 min. The amount of enzyme required for the oxidation of 1 µmol ascorbate per min was defined as one unit of APX. Glutathione reductase (EC 1.6.4.2) activity was assayed based on the method described by Fryer et al. (1998). The reaction was started by the addition of 70 µL of enzyme extract to 1 mL of reaction mixture containing 100 mM K-phosphate buffer (pH 7.0), 1 mM oxidized glutathione (GSSG), 1 mM EDTA and 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in the absorbance that resulted from NADPH oxidation to NADP was observed spectrophotometrically at 340 nm for 2 min at 10 s intervals. The amount of enzyme that oxidizes 1 µmol NADPH per min was defined as one unit of GR. For glutathione-S-transferase (EC 2.5.1.18) activity, the reaction was based on the formation of the conjugate reaction product using 1-chloro-2, 4-dinitrobenzene and glutathione as substrates (Mauch and Dudler, 1993). The reaction mixture consisted of 0.1 M potassium phosphate (pH 6.5), 1 mM 1-chloro-2, 4-dinitrobenzene, 3.6 mM reduced glutathione and 50 µl of enzyme extract. GST activity was determined spectrophotometrically at 340 nm. For CAT, APX, GR and GST the activities were calculated using the extinction coefficients of H_2O_2 (39.4 mM^{-1} cm^{-1}), ascorbate (2.8 mM^{-1} cm^{-1}), NADPH (6.22 mM^{-1} cm^{-1}) and conjugate formation (9.6 mM^{-1} cm^{-1}), respectively.

3.3.10. DPPH radical scavenging activity

The free radical-scavenging ability of leaf tissues was tested according to Goffman and Bergman (2004) using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). To get a methanolic leaf extract of both genotypes, 0.5 g of freeze-dried tissue was incubated in 80% methanol in the dark for 4 hours. The crude methanolic leaf extract (0.83-4.1 mg) were added to freshly prepared DPPH methanolic solution (0.2 mM) and incubated in darkness for 30 min. The absorbance of the reaction mixture was measured spectrophotometrically at 515 nm. The DPPH % inhibition was calculated based on the following formula: $(A_{cont}–A_{sample}) /A_{cont}) \times 100$. Where A_{cont} is the absorbance of the DPPH solution without any leaf extract and A_{sample} is the absorbance of the DPPH solution with the leaf extract. For each sample, the IC_{50} value was calculated to determine the amount (in mg) of leaf extract required to scavenge 50% of the DPPH substance. Ascorbic acid was used as an internal control with an IC_{50} value equal to 7.35 µg ml^{-1}.

3.3.11. RNA extraction and gene expression analysis

Total RNA was isolated with TRI reagent (Sigma- Aldrich Canada) under RNase-free conditions. Total RNA samples were checked for both quality and concentration using a NanoDrop 2000C spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To eliminate genomic DNA (gDNA), total RNA samples were submitted to DNase (DNase 1, Invitrogen, Carlsbad, CA, USA) treatment. After DNase treatment, total RNA samples were investigated for genomic DNA contamination by polymerase chain reaction (PCR) using the primer pair actinF/actinR (Table 3.1) for the *actin* gene. *Actin* primers were selected to have the forward primer located within the exon 1 of the gene and the reverse primer in the exon 2. The cDNA fragment size was 160 bp;

however, the corresponding gDNA sequence was 280 bp. The absence of gDNA fragment of *actin* after the PCR analysis, indicated that total RNA sample was free of gDNA contamination. First- strand cDNA was synthesized using iScript cDNA synthesis kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions. Appropriate annealing temperature for all primers were investigated. Table 3.1 presenting the product size and the respective employed annealing temperature for each primer pairs. For quantitative real-time PCR (qPCR) (Miniopticon, Biorad, Mississauga, Ontario, Canada) assay, a standard curve was performed, with a dilution series of known quantities (100 fg- 0.1 fg cDNA). For the standard curve, a minimum R^2 value of 0.98 was required for each gene. Quantitative real-time PCR (Bio-Rad, Mississauga, ON, Canada) analysis was performed using the SSO FastEvaGreen Supermix (Biorad) with cDNA transcribed from 50 ng total DNased RNA and 1 µmol L^{-1} of each primer. To confirm that the primers amplified a single product (one band), melting curve assay of the amplification products was conducted for each gene. *Actin* was used as an internal reference gene (Ghanem et al., 2011).

3.3.12. Data analysis

All data were submitted to a two-way ANOVA analysis (NaCl treatments and genotypes). When a significant interaction between NaCl and genotypes was found a Tukey's HSD test was used to determine the significant differences between means at a probability level of ≤ 0.05.

3.4. Results

3.4.1. RNA level of JA responsive genes in def-1 and WT

The effect of impairment of JA synthesis in *def-1* plants on the transcription of JA responsive genes was investigated in leaves of five week old seedlings (Fig. 3.1). The tested JA responsive genes were *JAR1* (Jasmonate-Resistant 1), *JAZ1* (jasmonate ZIM-domain1), *JAZ3* (jasmonate ZIM-domain 3), and the gene coding for plant defensin1 (*PDEF1*). In *def-1*, the mRNA levels of the genes in leaves were lower than in WT plants (Fig. 3.1).

3.4.2. Growth and pigments

In the control condtions, *def-1* showed smaller biomass (shoot and root dry weights, shoot length and number of leaves) than the WT plants (Table 3.2). The small biomass, observed under control condtions, for *def-1* was associated with higher leaf Chl a and carotenoids contents (Table 3.2). After 14 days of exposure to 100 mM NaCl, the growth parameters (shoot and root dry weights, shoot length and number of leaves) of *def-1* and its wild type were reduced, without any difference between genotypes (Table 3.2). In *def-1*, both leaf Chl a and carotenoids were decreased (14.5 % and 14 %, respectively) by salt treatment with no significant change in Chl b; whereas, in wild-type plants, none of the photosynthetic pigments were affected by salt treatment (Table 3.2).

3.4.3. Elemental analysis

At the end of the salt treatment, Na concentration increased in the shoots and roots of both genotypes compared to the control plants (Tables 3.3 and 3.4). In salt-treated leaves, Na accumulation was similar in *def-1* and in their WT plants, while in roots, *def-1* accumulated higher levels of Na than the WT plants. Macronutrients, like K

and Ca showed similar levels of reduction in salt-treated leaves and roots in both genotypes. *Def-1* showed higher N contents in both shoots and roots compared to the WT plants under control condtions. However, salt stress caused a significant decrease in N concentration in shoots and roots of *def-1,* whereas no change was detected in WT plants in the same tissues.

3.4.4. Biochemical markers for oxidative stress

In *def-1* leaves, salt-treated plants showed higher level of MDA (almost 2 fold) compared with the plants grown under control conditions, while in the wild type plants MDA level was not affected by the salt treatment (Fig. 3.2 A). The levels of H_2O_2 was increased by almost 2 fold in *def-1* leaves exposed to salt treatment compared to the control plants; whereas in WT plants the levels of H_2O_2 were similar in salt–treated plants and the control ones (Fig. 3.2 B).

3.4.5. Changes in the antioxidant activities after salt treatment

Catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathion-s-transferase (GST) and superoxide dismutase (SOD) activities in the leaves of *def-1* and wild type plants grown under control and salt stress conditions are presented in Fig. 3.3. CAT and APX activities were not affected by salt treatment in any genotypes (Fig. 3.3). While in the wild type plants, salt stress increased the activity of GR, GST and SOD by 74%, 35% and 34%, respectively; in *def-1,* the activities of these enzymes were not affected by the salt treatment. Salt stress also decreased the protein levels in both the wild type and *def-1,* with no significant differences between genotypes. In order to determine the non-enzymatic antioxidant activity of *def-1* and WT leaves, the amount of leaf extract (expressed in mg) sufficient to scavenge 50% of DPPH was plotted as a

measure of the scavenging activity (Fig. 3.4). The salt-stressed plants from both genotypes showed higher ability to detoxify the DPPH solution than the control plants. In addition, the WT plants had a high antioxidant activity with an IC_{50} value of 2.06 mg ml^{-1} compared to the JA mutant that showed an IC_{50} value of 2.52 mg ml^{-1} (Fig. 3.4). The level of proline was increased by salt stress in the leaves of both genotypes (Fig. 3.5A); however the increase was more pronounced in the wild type (5 fold) than in *def-1* (4 fold). Salt stress also increased the level of the total polyphenolic compounds of salt-stressed plants of both the wild type and *def-1*, with no significant differences between genotypes (Fig. 3.5B).

3.5. Discussion

3.5.1. def-1 plants exhibit reduced mRNA levels of JA-responsive genes

Analysis of mutants deficient in JA biosynthesis or signaling, mainly from tomato and *Arabidopsis*, has been extensively used for the dissection of JA function in plant development and biotic stress (Wasternack and Hause, 2015; Ding et al., 2016; Zhao et al., 2016). To study the function of endogenous JA during salt stress, the responses of JA synthesis tomato mutant (*def-1*) (Howe et al., 1996) and the corresponding wild type Castlemart variety were analyzed in this study. The impairment of JA production in *def-1* plants is well supported in the literature (Howe et al., 1996; Dombrowski et al., 2003). Stenzel et al. (2003) suggested that *def-1* carries a mutation in an unidentified gene that affects AOC (involved in JA biosynthesis) activity. To further investigate the key genes downstream of JA biosynthesis in *def-1*, we examined the transcript levels of *JAR1* (required to synthesize the bioactive form of JA, JA-Ile); *JAZ1* and *JAZ3* (crucial components in JA singling); and *PDEF1* (encoding cationic peptides for plant defense

against pathogens and also used as a marker gene for JA biosynthesis) (Mira et al., 2016). In our study, the reduced levels of those genes in *def-1* compared to the WT plants, supports that the impairment of JA biosynthesis in *def-1* (Howe et al., 1996) is associated with defects in events downstream of the JA biosynthesis pathway.

3.5.2. Salt-stressed def-1 and WT showed similar reduction in growth

Plant biomass is commonly used to compare salt tolerance between different genotypes (Ashraf and Harris, 2004). In our study, the relative reduction in growth parameters (shoot and root DW, shoot length and number of leaves) was similar in *def-1* and WT plants exposed to salinity. In a previous study, although an *Arabidopsis* ethylene-insensitive mutant grew similarly to the WT plants under salt stress, the mutant showed higher oxidative stress than the WT (Asensi-Fabado et al., 2012). Therefore, in relatively short-term experiments, plant biomass may not be the most reliable criteria to compare salt tolerance of different genotypes.

3.5.3. Salt-stressed def-1 and WT accumulated different levels of Na and N

During salt stress, uptake and translocation of elements such as Na, Ca, K and N have been proposed as physiological indicators for salinity tolerance (Munns and Tester, 2008). Although exogenous application of JA effectively reduced the accumulation of Na in salt-stressed leaves of barley (*Hordeum vulgare L.*) and pea *(Pisum sativum*) (Fedina and Tsonev, 1997; Walia et al., 2007), a similar result was not observed in this study, where Na accumulated in salt-stressed leaves to a similar level in the both *def-1* and WT genotypes. These results suggest that exogenous and endogenous JA may have different effects in salt stress responses (Ismail et al., 2014). However, *def-1* accumulated significantly higher levels of Na in the roots than the WT. This could mean that either Na

uptake have been increased through the non-selective cation channels (NSCCs), and/or that the extrusion of Na from root cells via the SOS1 antiporter (Shi et al., 2002; Munnus and Tester, 2008) is reduced in this mutant. Therefore, further studies are required to illustrate how JA regulates Na transport in salt-stressed roots of tomato.

Excessive levels of Na in the nutrient growing media can lead to competition with other ions, such as Ca^{+} and K^{+}, resulting in ion deficiency and/or ion imbalance in the plants (Hu and Schmidhalter, 2005). This was also well illustrated in this study, where levels of Ca and K where reduced by salt stress in both shoots and roots of the two genotypes. These findings suggest that salt sensitivity of *def-1* does not appear to be related to changes in Ca and K levels.

Another critical element for salt tolerance is nitrogen, as many of the salt protective compounds such as compatible solutes (e.g. proline) and antioxidant enzymes (e.g. SOD, GR and GST) require N for their biosynthesis (Munns and Tester, 2008). In addition, it has been suggested that JA signaling could mediate N uptake and translocation in plants (Cho et al., 2007; Zhang and Gong, 2014). Therefore, the observed salt-induced reduction in N content of both leaves and roots in *def-1* plants, which did not occur in WT, may reflect the role of JA in N homeostasis during salt stress.

3.5.4. Enhanced oxidative stress in the def-1 mutant

The level of ROS increases in plants in response to abiotic stresses such as salinity and drought (Parida and Das, 2005; Miller et al., 2010). Parameters such as photosynthetic pigment content and accumulation of MDA and H_2O_2 during salt stress have been widely used as indicators for oxidative stress (Apel and Hirt, 2004; Asensi-Fabado et al., 2012; Lim et al., 2015). In our study, the *def-1* mutant showed a higher

level of H_2O_2 compared to WT plants after exposure to salt stress. Hydrogen peroxide is not a free radical as it does not have unpaired electrons, therefore it is comparatively innocuous (Halliwell et al., 2000). However, H_2O_2 may be converted non-enzymatically to the extremely noxious $OH^{\bullet}$, causing cell damage (Ishida et al., 1999). In addition, oxidative damage of cell plasma membranes (measured by the MDA accumulation) and reduction in photosynthetic pigments were observed only in *def-1* plants. These results confirmed that the *def-1* mutant, which is deficient in JA production (Howe et al., 1996), clearly displayed oxidative stress symptoms as characterized by ROS-associated injury phenotypes during salt stress. The involvement of endogenous JA in alleviating ROS-associated injury and reducing the accumulation of MDA and H_2O_2 was also suggested in tomato exposed to other abiotic stimuli such as cadmium stress (Zhao et al., 2016).

3.5.5. The def-1 mutant showed lower antioxidant activities

To alleviate cellular damage caused by ROS, plants need to maintain an equilibrium between the ROS production rate and scavenging rate by an array of enzymatic and non-enzymatic antioxidants (Apel and Hirt, 2004; Gill and Tuteja, 2010). The major antioxidant enzymes include SOD, CAT, GPX, GST and GR, which detoxify ROS in different sites in plant tissues (Gill and Tuteja, 2010). In the current study, the enhanced activity of SOD, the most efficient enzyme mediating the scavenging of $O_2^{\bullet-}$ radical to H_2O_2 (Gill and Tuteja, 2010; Zhao et al., 2014), in wild type salt-stressed plants compared to *def-1* implies that the JA mutant (*def-1*) had a lower capacity for $O_2^{\bullet-}$ scavenging. The involvement of SOD in tomato salt tolerance was previously reported in studies where a salt-tolerant genotype showed a high activity of SOD that was associated with less oxidative stress compared to a salt-sensitive genotypes (Mittova et al., 2002;

Mittova et al., 2004). Once superoxide has been converted to H_2O_2, this ROS can be scavenged by a broad range of enzymes (Blokhina et al., 2003).

Ascorbate peroxidase (APX) and CAT represent the major enzymes responsible for H_2O_2 degradation (Miller et al., 2010). Ascorbate peroxidase plays a key role for quenching H_2O_2 in the cytosol and the chloroplast through the ascorbate-glutathione cycle, and its activation during salt stress has been associated with tolerance to oxidative injury in various plant species (Amor et al., 2006; Miller et al., 2010; Sabra et al., 2012). In *def-1* leaves, although the H_2O_2 content was higher than in the wild type plants when exposed to salt stress; APX and CAT activities were not affected by salt, suggesting lower scavenging activity for H_2O_2 in *def-1*. On the other hand, however, in the salt-stressed WT plants, the unchanged level of APX and CAT activities likely reflects little need to rely on enzymatic scavengers as they accumulated lower level of H_2O_2.

Two antioxidants, glutathione *S*-transferase (GST) and GR are among the enzymes that are related directly to GSH (glutathione) metabolism (Hasanuzzaman et al., 2012). Glutathione, a non-enzymatic antioxidant, is involved directly in the scavenging of most ROS and regenerating other water-soluble antioxidants such as AsA (ascorbic acid) through AsA-GSH cycle (Halliwell and Foyer, 1976; Hasanuzzaman et al., 2012). Consistent with the function of GST in ROS scavenging, the lower activity level of GST and GR in salt-stressed *def-1* compared with wild type plants might reflect lower GSH content. Overall, our results showed that the plants deficient in JA production (*def-1* plants) have lower scavenging ability under salt stress than the plants that can produce JA (WT plants). Several lines of evidence have illustrated the involvement of JA in enzymatic antioxidants enhancement in response to other abiotic stresses such as

cadmium and drought (Anjum et al., 2011; Zhao et al., 2016). All these results seem to confirm the hypothesis that JA plays a key role in the regulation of enzymatic antioxidant activities to protect plants from oxidative damage induced by stresses such as salinity.

In addition to enzymatic antioxidant, plants are equipped with non-enzymatic antioxidants such as proline and phenolic compounds (Verbruggen and Hermans, 2008; Petridis et al., 2012). One of method extensively used to estimate the total amount of non-enzymatic antioxidants activities in plants is to quantify the scavenging activity of the stable radical DPPH (Xie et al., 2008). Our results show that *def-1* plants, not only had lower enzymatic antioxidant activities compared to WT under salt stress, but they also had lower levels of non-enzymatic antioxidants, as indicated by the DPPH assay, resulting in the ROS-damage observed in *def-1* plants. Proline accumulation during salt stress has been commonly associated with the alleviation of osmotic stress (Verbruggen and Hermans, 2008). However, proline can also act as a ROS scavenger, as indicated by early *in vitro* studies (Smirnoff and Cumbes, 1989) and by the analysis of an *Arabidopsis* proline biosynthesis mutant, which showed that proline is involved in the control of either the activity or the stability of the antioxidant enzymes in the glutathione-ascorbate cycle under salt stress (Verbruggen and Hermans, 2008; Székely et al., 2008). In our study, salt-stressed *def-1* plants accumulated a lower level of proline, associated with higher ROS-damage, compared to the WT, suggesting that proline could be employed in salt tolerance of WT plants as ROS scavenger. The involvement of endogenous JA in salt tolerance via proline production was also reported in rice (*Oryza sativa*) seedlings expressing the JA biosynthetic key gene *AOC* of peanut (*Arachis hypogaea*) (Liu et al., 2015).

The biosynthesis of phenolic compounds, which also act as ROS scavengers, can be stimulated by salinity (Petridis et al., 2012). In the current study, the similar increase in total phenolic compounds in both genotypes during salt stress could be a part of the plant defense. However, the observed ROS-associated injury in *def-1* during salt stress suggests that the increase in total phenolics is not the main antioxidant for ROS scavenging for this genotype. Overall, the ROS-associated injury in the JA mutant during salt stress can be mostly explained by the lower antioxidant capacity of this mutant.

3.6. Conclusion

Our results, using the tomato JA mutant *def-1*, suggest that endogenous JA plays a significant role in protecting plants from salt-induced oxidative stress through the activation of both enzymatic antioxidants, including GR, GST and SOD, and non-enzymatic antioxidants such as proline. Overall, the present study provides new insight into the role of endogenous JA in tomato salt tolerance via ROS homeostasis. Future studies, will be required to identify the underlying molecular mechanism responsible for the reduced N in JA mutant under salt stress.

Table 3.1. Primer sequences employed in qPCR. F: forward primer, R: reverse primer.

Gene name	GenBank accession no.	Nucleotide sequence (5′ ➔ 3′)	Annealing T (°C)	Source of primers	Product size (bp)
JAR1	XM_010316757	*JAR1* F: TGTCTTCACCAATTTCGCAGGTT *JAR1* R: CCTGCAGCTTCCACGGCTAGT	60	Garcia-Abellan et al. 2015	150
JAZ1	NM_001247954	*JAZ1* F: CTGATCAATCTGGTGTGAGTTTT *JAZ1* R: CAGAAGGCTGTGGCATTGAC	60	Mielke et al. 2011	80
JAZ3	NM_001247444	*JAZ3* F: GATGGTCAATGTCTTCGAGG *JAZ3* R: CAAACACCATCTGCAGCAG	60	This study	150
PDE	NC_015438.2	*PDEF1* F: CTCTTTGTTACCTATGAGGTAG *PDEF1* R: CTTCCTCACCCAAAGTTGCT	60	Stotz et al. 2009	200
Actin	AB695290	*Actin* F: GAAATAGCATAAGATGGCAGA *Actin* R: ATACCCACCATCACACCAGTA	55	This study	160

Table 3.2. Growth parameters and pigments content (mg g^{-1} DW) of WT and *def-1* treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter. Parameters including shoot DW, root DW, shoot length, number of leaves and Chl b do not show significant interaction between genotypes and salt.

	WT			*def-1*		
	0 mM NaCl	100 mM NaCl	Change (%)	0 mM NaCl	100 mM NaCl	Change (%)
Shoot DW (g)	8.11 ± 0.45 [a]	5.34 ± 0.32 [b]	-34.15	6.58 ± 0.36 [b]	3.28 ± 0.32 [c]	-50.15
Root DW (g)	1.53 ± 0.10 [a]	1.14 ± 0.06 [b]	-25.49	1.01 ± 0.06 [b]	0.72 ± 0.076 [c]	-28.71
Shoot length (cm)	15.5 ± 0.6 [a]	11.5 ± 0.4 [b]	-25.80	11.4 ± 0.6 [b]	9.04 ± 0.63 [c]	-20.70
Leaf number	10.7 ± 0.5 [a]	7.16 ± 0.40 [b]	-33.08	7.66 ± 0.20 [b]	5.83 ± 0.40 [c]	-23.89
Chl a	6.63 ± 0.16 [b]	6.56 ± 0.26 [b]	-1.05	7.49 ± 0.14 [a]	6.40 ± 0.11 [b]	-14.55
Chl b	2.01 ± 0.06 [a]	2.15 ± 0.35 [a]	+6.96	2.46 ± 0.01 [a]	1.97 ± 0.16 [a]	-19.91
Carotenoids	0.280 ± 0.001 [b]	0.32 ± 0.01 [ab]	+14.2	0.360 ± 0.003 [a]	0.31 ± 0.01 [b]	-13.9

Table 3.3. Leaf elemental content (%) of WT and *def-1* treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter. Parameters including Na, K and Ca contents do not show significant interaction between genotypes and salt.

	WT		*def-1*	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Na	0.110 ± 0.004 [b]	2.03 ± 0.01 [a]	0.085 ± 0.01 [b]	1.93 ± 0.01 [a]
K	3.66 ± 0.26 [a]	2.52 ± 0.211 [b]	3.54 ± 0.01 [a]	2.22 ± 0.08 [b]
Ca	2.77 ± 0.12 [a]	2.41 ± 0.01 [b]	2.85 ± 0.02 [a]	2.46 ± 0.01 [b]
N	4.06 ± 0.14 [b]	4.00 ± 0.22 [b]	4.49 ± 0.04 [a]	3.88 ± 0.15 [b]

Table 3.4. Root elemental content (%) of WT and *def-1* treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter. Parameters including K and Ca contents do not show significant interaction between genotypes and salt.

	WT		*def1*	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Na	0.10 ± 0.09 [c]	3.41 ± 0.07 [b]	0.08 ± 0.01 [c]	3.75 ± 0.05 [a]
K	5.02 ± 0.07 [a]	3.00 ± 0.08 [b]	5.40 ± 0.08 [a]	2.95 ± 0.07 [b]
Ca	0.660 ± 0.004 [a]	0.44 ± 0.01 [b]	0.63 ± 0.01 [a]	0.43 ± 0.01 [b]
N	3.800 ± 0.003 [b]	3.61 ± 0.09 [b]	4.43 ± 0.15 [a]	3.63 ± 0.13 [b]

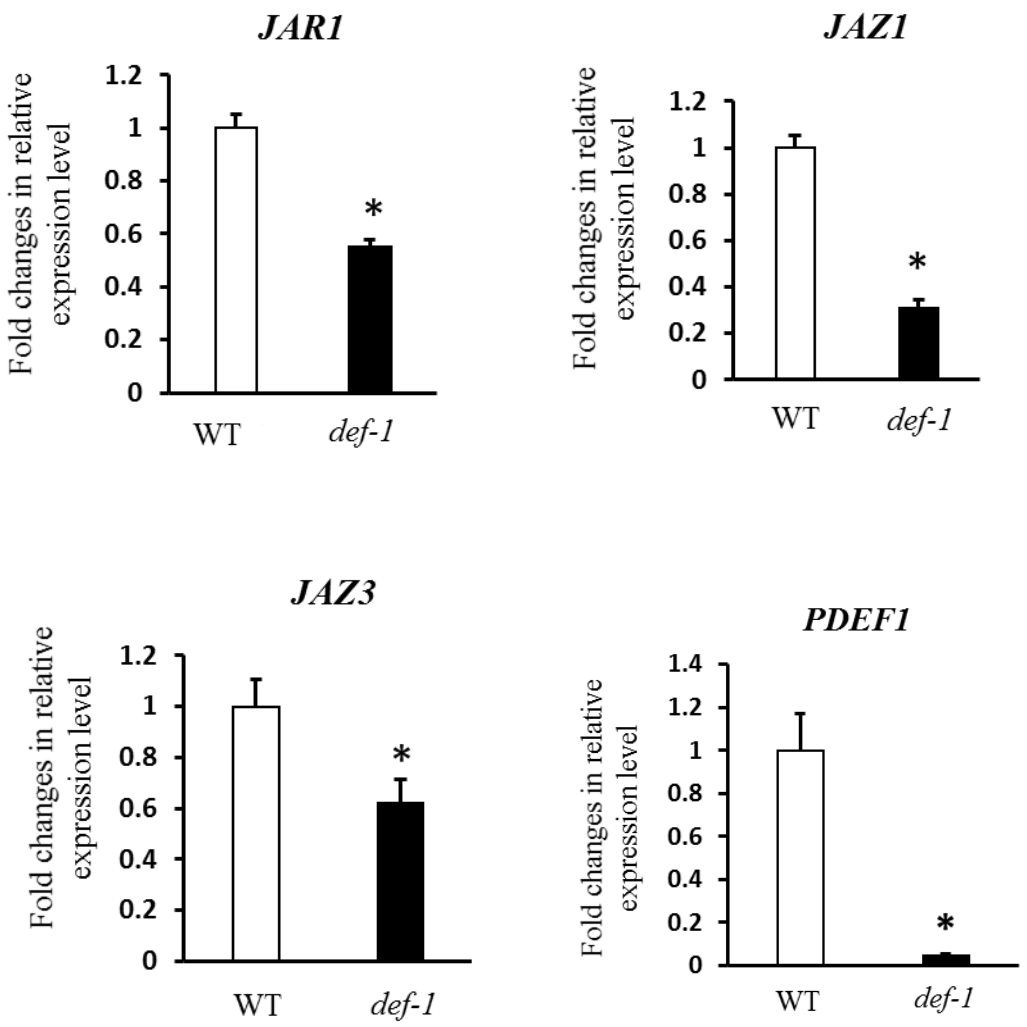

Fig. 3.1. Differences in relative mRNA levels of *JAR1* (Jasmonate-Resistant 1), *JAZ1* (jasmonate ZIM-domain1), *JAZ3* (jasmonate ZIM-domain 3), and the gene coding for plant defensin1 (*PDEF1*) in leaf tissues of WT and *def-1*. The mRNA levels of the target genes were normalized relative to the housekeeping gene *actin* and relative mRNA levels of WT plants were set as 1. * denotes significant differences in mRNA levels between WT and *def-1* plants (t-test with $p < 0.05$). Data represent means (± SE) of 4 biological replicates.

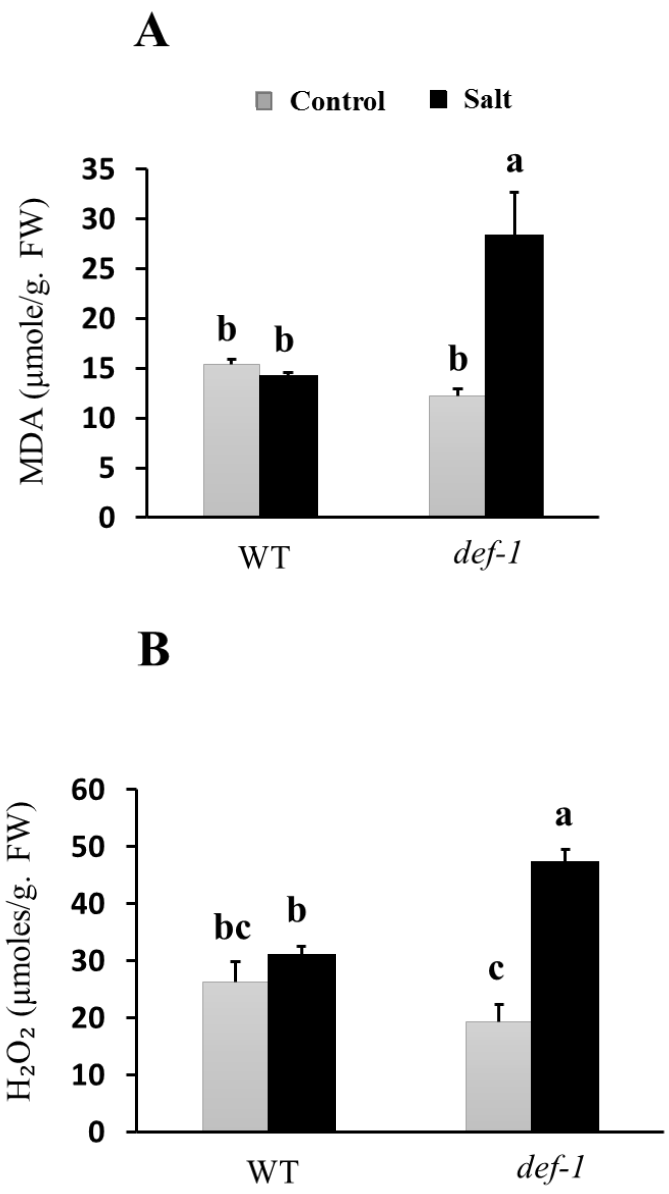

Fig. 3.2. Malondialdehyde (MDA) (A) and hydrogen peroxide (H_2O_2) (B) levels in WT and *def-1* leaves treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± SE. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter.

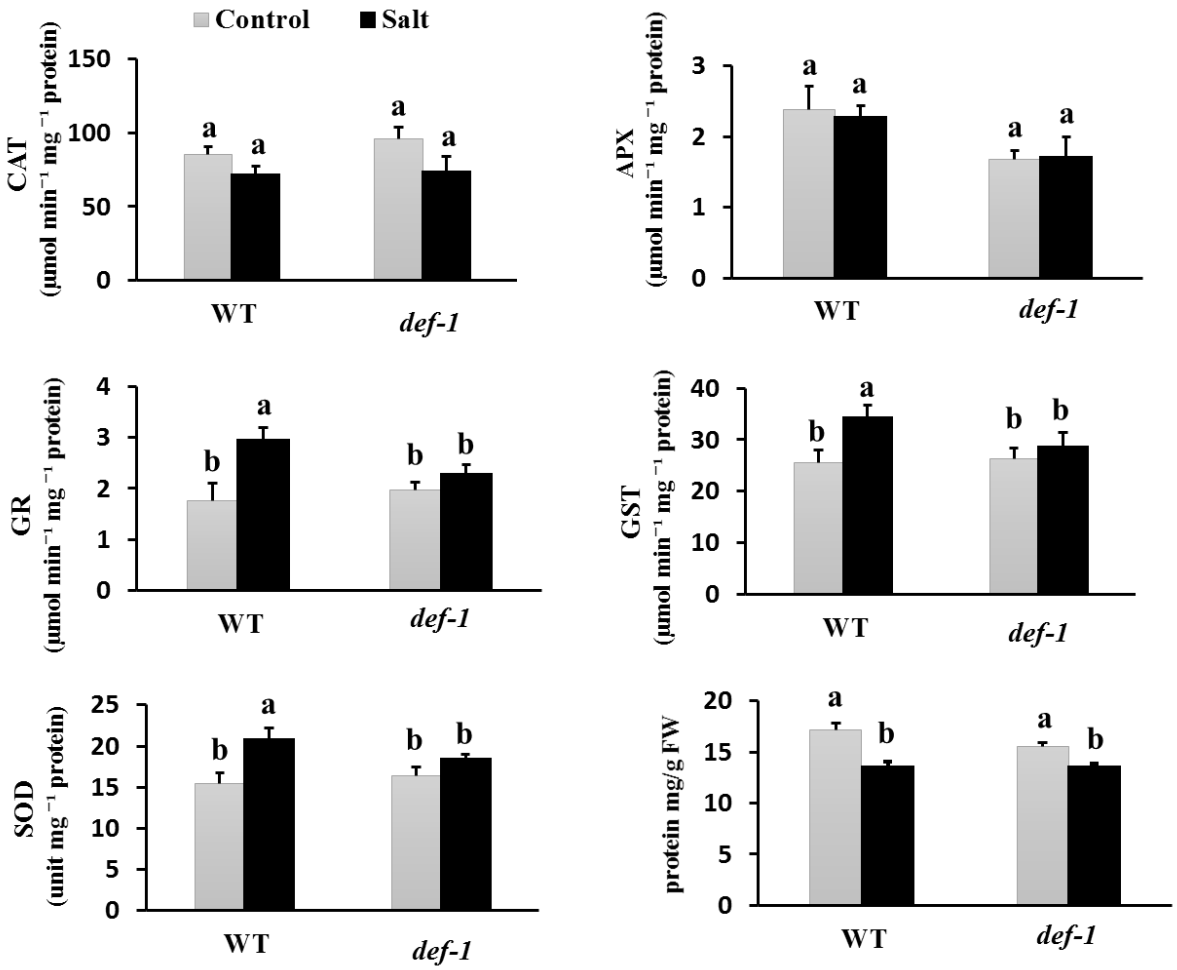

Fig. 3.3. Total protein content and activities of CAT (Catalase), APX (Ascorbate peroxidase), GR (Glutathione reductase), GST (Glutathione reductase) and SOD (Glutathione-s-transferase) in the leaves of WT and *def-1* leaves treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± SE. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter.

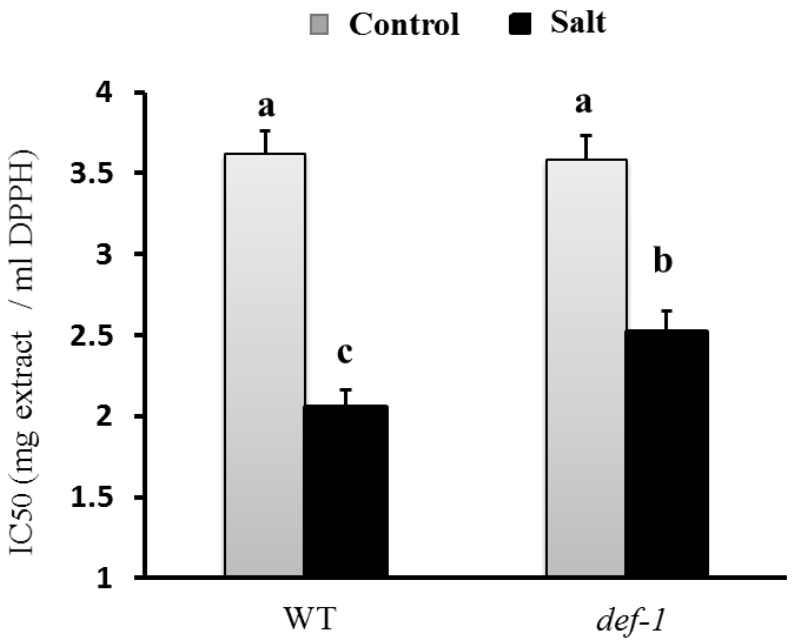

Fig. 3.4. Leaf extract amount necessary for 50% inhibition (IC50) of the free radical activity of DPPH in WT and *def-1* leaves treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± SE. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter.

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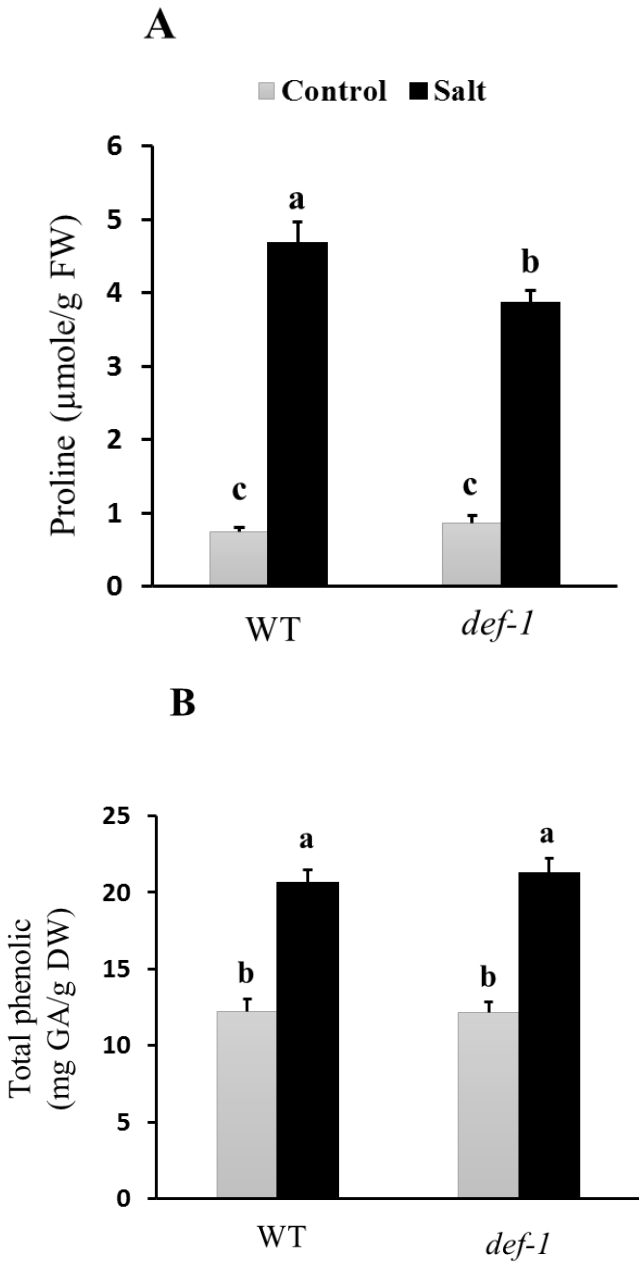

Fig. 3.5. Proline (A) and total phenolics (B) levels of WT and *def-1* leaves treated with 0 and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± SE. Means are considered different at $p \leq 0.05$ when they are followed by different letters within the same parameter.

CHAPTER 4. TISSUE EXPRESSION AND SEQUENCE ANALYSIS OF AMT2-TYPE AMMONIUM TRANSPORTERS IN TOMATO*

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* A part of this chapter will be submitted to Biotechnology Reports

My contribution:

I conducted all experiments, analyzed the data and wrote the manuscript.

4.1. Abstract

Ammonium transporters (AMTs) are critical for the translocation and uptake of ammonium/ammonia (NH_4^+/NH_3) in plants. These proteins are divided into AMT1 and AMT2 subfamilies, which may transport NH_4^+ and NH_3, respectively. In tomato (*Solanum lycopersicum*), a model species for vegetable crops, only the *AMT1* subfamily has been characterized in a previous study; less information is available about the AMT2 subfamily. The objective of this study was to identify tomato *AMT2* subfamily members and characterize their sequence structures and their expression patterns. In this study, eight AMT-type ammonium transporters were identified in the tomato genome. All of tomato AMTs possess 11 putative trans-membrane domains (TMDs) in their amino acid sequences. Tomato AMTs formed two distinct related subfamilies. The SlAMT1 subfamily includes three members (SlAMT1.1, SlAMT1.2 and SlAMT1.3) and the SlAMT2 subfamily includes five members (SlAMT2.1, SlAMT4.1, SlAMT4.2, SlAMT4.4 and SlAMT4.5). The genetic tree analysis showed that the SlAMT2 branch is more closely related to AMTs from prokaryotes (bacteria) and MEP-type ammonium transporters from yeast than SlAMT1. Although the amino acid identity between the *Escherichia coli* (the well characterized model) and tomato AMT proteins is below 22 %, the proteins from both organisms showed similar structures (homo-trimer). This observation suggests the importance of the conserved residues in N transport by tomato AMTs. In this study, only four genes (*SlAMT1.1*, *SlAMT1.2*, *SlAMT1.3* and *SlAMT2.1*) were found to be expressed in tomato seedlings grown in a hydroponic system. The newly identified *SlAMT2.1* showed root specific expression and its transcript level was highly regulated by nitrogen availability, suggesting that this transporter likely plays a

role in NH_3 transport in root cells. To confirm that tomato AMT2.1 encodes a functional transporter, the ability of *SlAMT2.1* cDNA to complement the growth of the yeast mutant Triple-mepΔ (unable to grow in the presence of ammonium as the sole nitrogen source) was tested. The difficulties to achieve this assay and the possible alternative methods to study the gene of interest are described. The current study provides basic genomic and transcriptomic information for tomato AMTs and will guide future studies for understanding the function of AMTs in tomato physiology.

4.2. Introduction

Ammonium and nitrate are the main sources of nitrogen for most plant species (Couturier et al., 2007). It has been shown that plant ammonium transporters (AMTs) are responsible for the high-affinity (K_m in the micromolar range) NH_4^+/NH_3 transport system in plants (Couturier et al., 2007; Tsay and Hsu, 2011; Wu et al., 2015). Although plant AMTs share similarities in their sequences, they can vary in their affinity and tissue expression patterns (Straub et al., 2014). Plant AMTs consist of two distinct subfamilies: the AMT1 and AMT2 (Koegel et al., 2013; Wu et al., 2015). Three major differences exist between these families. First, most plants (8 plant species with identified AMTs) have more *AMT2* genes than *AMT1* in their genome, with the exception of *Arabidopsis thaliana* (Li et al., 2015). Secondly, the gene structure varies. In general *AMT1* genes lack introns, whereas *AMT2* genes have several introns (Couturier et al., 2007). Finally, members of the AMT1 subfamily are electrogenic transport systems that likely transport the charged molecule NH_4^+, whereas AMT2 subfamily members are electroneutral transport systems that may transport only the gaseous molecule NH_3 (Ludewig et al., 2007; Guether et al., 2009; Straub et al., 2014).

In plants, *AtAMT2.1* was the first *AMT2* gene identified in *Arabidopsis* (Sohlenkamp et al., 2002). The slight reduction in the mRNA level of this gene in the presence of high CO_2 concentration in leaves (reducing the rate of photorespiration) suggested a partial role of *AtAMT2.1* in recycling the NH_3 released during photorespiration (Sohlenkamp et al., 2002). Subsequently, *AMT2* genes have been identified in other plant species with different physiological roles. For example, in *Lotus japonicus* (birdsfoot trefoil), the transfer of NH_4^+/NH_3 from the fungal arbuscular hyphae to the cortical cells of roots is mediated by *AMT2* (Guether et al., 2009). In *Medicago truncatula* (barrelclover), the abundant expression of *AMT2.1* in the root tissues as well as its high affinity for ammonium in the oocytes functional expression system, revealed that *MtAMT2.1* has a role in NH_4^+ acquisition from soil by the NH_4^+ recruiting/ NH_3 translocation mechanism (Straub et al., 2014). Overall, plant *AMT2s* seem to have multiple physiological roles (Li et al., 2015).

Tomato is a model system for vegetable crops with a major commercial value (Sun et al., 2010). Abiotic stresses such as salinity disturbs N uptake and assimilation, resulting in a reduction of plant growth and yield (Flores et al., 2001). The importance of AMTs in salt tolerance was supported by the fact that ammonium is the preferred source of nitrogen for some plants under salt stress (Flores et al., 2001; Kant et al., 2007). Plant roots possess both *AMT* subfamilies to facilitate NH_4^+/NH_3 uptake and/or translocation (Straub et al., 2014; Li et al., 2015). In a previous study, three high affinity functional ammonium transporters (*AMTs*) have been identified in the tomato genome (von Wirén et al., 2000). Two ammonium transporters, *AMT1.1* and *AMT1.2*, are involved in NH_4^+ uptake in roots, while the expression of *AMT1.3* was detected in leaves, suggesting a role

in N metabolism (von Wirén et al., 2000). However, in tomato, little is known about the *AMT2* subfamily. It is necessary to have a better understanding of *AMT2* members in tomato prior to study their potential role in salt tolerance. The objective of this study was to identify *AMT2* subfamily members in tomato genome and determine their sequence structures and their tissue expression patterns. I hypothesized that *AMT2* members are present in the tomato genome and have distinct sequence structures and tissue expression patterns compared to *AMT1*.

In this study, the tomato AMT family was described; gene structure, phylogeny and conserved residues were analyzed. In addition, the expression pattern of tomato *AMTs* in different tissues was analyzed at the seedling stage to obtain preliminary information on their physiological role. Moreover, the transcript levels for *SlAMT2.1* in roots was tested under different N levels to evaluate the response of this gene to external N availability. In an attempt to test the function of SlAMT2.1, a yeast (*Saccharomyces cerevisiae*) complementation assay was used.

4.3. Materials and methods

4.3.1. Plant growth conditions

Seeds of tomato, *Solanum lycopersicum* (cv. Manitoba, obtained from T&T seeds, Winnipeg, Canada), were surface sterilized for 15 min with 5% sodium hypochlorite. The seeds were then rinsed with distilled fresh water and germinated in Petri dishes (9-cm diameter). Each dish (containing two filter papers, Whatman No.1) was moistened with 5 ml of nutrient solution (as described below). After 7 days, tomato seedlings were transferred for two weeks to an aerated half strength modified Hoagland solution containing 2 mM NH_4NO_3; 1 mM KH_2PO_4; 1.5 mM $CaCl_2$; 0.5 mM KCl; 1 mM

$MgSO_4$; 23 µM H_3BO_3; 5 µM $MnCl_2$; 0.4 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.07 µM H_2MoO_4 in addition to 0.007 µM Fe-EDTA. Three tomato seedlings were planted in 10-L plastic containers, with a total of four replicates (four containers). To avoid nutrient depletion, the nutrient solution was changed every 5 days. The hydroponically cultivated seedlings were grown in a greenhouse under 25/19 °C day/night temperatures, 18h/6h photoperiod with natural light supplemented by sodium lamps (P.L. light systems, Beamsville, ON, Canada). Once seedlings were 3 weeks old, tissues from root, petiole, stem and leaf were collected in liquid nitrogen and stored at -80°C for subsequent *AMT* expression analysis. To test the transcription patterns of *AMT2.1* in response to N availability, three week old tomato seedlings growing on nutrient solution as described above were submitted to N starvation by removing the NH_4NO_3 from the nutrient solution. After 3 days of N deficiency treatment, the nutrient solution was replaced by a solution containing 2 mM NH_4NO_3 for 2 h. Root tissues were collected after 0, 1 and 3 days of N deficiency and after 2h of N resupply and stored at -80°C for the mRNA analysis.

4.3.2. Bioinformatics analysis and gene identification of tomato AMTs

The known amino acid sequences of AMTs from *Populus termula x alba* and *Arabidopsis* (Sohlenkamp et al., 2002; Couturier et al., 2007) were employed to identify the putative AMT domain-containing protein sequences of tomato in the Sol Genomics Network database (https://solgenomics.net/) using a BLAST search (Altschul et al., 1997). Eight sequences encoding tomato AMT proteins were identified and their open reading frame regions (ORFs) were detected using the open reading frame (ORF) finder online server program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) (Rajput and Upadhyaya, 2007). For each tomato *AMT* gene, the chromosomal localization was

procured from the Sol Genomics Network database. Multiple protein sequence alignment of AMTs from tomato and *Arabidopsis* was conducted using PSI-Praline multiple sequence alignment tool (www.ibi.vu.nl/programs/pralinewww) (Feenstra et al., 2007). For the genetic tree analysis, amino acid sequences of AMTs from tomato and different organisms were analyzed using the software Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/index.cgi) (Pauchet et al., 2010) with ML (Maximum Likelihood) method and 1000 bootstrap replicates.

4.3.3. RNA extraction and cDNA synthesis from tomato tissues

Total RNA was extracted under RNase-free conditions using TRI reagent (Sigma-Aldrich Canada). Both quality and concentration of total RNA of the samples were tested by using a NanoDrop 2000C spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). An OD 260/280 ratio greater than 1.9 was considered an acceptable indicator of good RNA quality.

To remove genomic DNA, total RNA samples were submitted to DNase (DNase 1, Invitrogen, Carlsbad, CA, USA) treatment. Following this treatment, total RNA was tested for genomic DNA contamination by polymerase chain reaction (37 cycles) using the primer pair actinF/actinR (Table 4.1) for the *actin*. No detected amplification indicated that total RNA samples were not contaminated with genomic DNA. One µg of total DNased RNA was used to synthesize the first-strand cDNA using an iScript cDNA synthesis kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions.

4.3.4. Reverse transcription-PCR (RT-PCR)

To study the expression pattern of *AMTs* (8 genes) in different tomato tissues, each of the *AMT* primers (Table 4.1) were employed in the RT-PCR analysis. The PCR program for all *AMTs* was as follows: 94°C for 3 min and 36 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The endogenous *actin* gene (amplified for 26 cycles) was used as an internal control. The PCR products were separated using agarose gel electrophoresis containing ethidium bromide (Sigma-Aldrich Canada) for bands visualization. Images of the gels were observed using the imager Biorad Versadoc 4000 Mp and Image Lab^{TM} 3.0 software. PCR products for each employed primer pair produced single amplicon of the predicted size (Table 4.1). Every reaction was set up at least in three biological replicates.

4.3.5. Quantitative real−time PCR (qPCR)

To study the expression pattern of *SlAMT2.1* under N availability, quantitative real-time PCR (qPCR) analysis was performed. In order to determine the absolute mRNA quantity of *SlAMT2.1,* a standard curve was used (100 fg- 0.1 fg cDNA). The reaction mixture (15 µL) was containing 1 µmol L^{-1} of *SlAMT2.1* primers and cDNA transcribed from 50 ng total DNased RNA in SSO FastEvaGreen Supermix (Biorad). For normalizing the qPCR reaction, the tomato *actin* was used as internal control according to Ghanem et al. (2011) and Li et al. (2015).

4.3.6. Yeast complementation assay for tomato AMT2.1

For *SlAMT2.1* isolation, the predicted open reading frame (ORF) corresponding to *SlAMT2.1* was amplified by PCR reaction using Phusion high-fidelity DNA polymerase (Thermo Scientific, Ottawa, ON, Canada), cDNA from root tissues and primers (*AMT2.1-*

SpeI- F, ATA AACTAGTATGTCCGTACCACCAGGA and *AMT2.1 XhoI R*, GTATACTCGAGTTATAAATCAAATGTCAATACTGTAGTT) flanking the entire ORF. These specific primers carried restriction sites for *Spe*I on the 5' end and *Xho*I on the 3' end. The PCR products were observed on 1% agarose gel electrophoresis using the imager Biorad Versadoc 4000 Mp and Image Lab^{TM} 3.0 software. The designed primers successfully isolated the target gene (*SlAMT2.1*) at the predicted size (1450 bp) as shown in Fig. 4.1A. The target band was cut from the agarose gel and *SlAMT2.1* cDNA was extracted using gel purified PCR product (QlAquick Gel Extraction Kit, Qiagen).

To clone *SlAMT2.1* into the yeast expression vector (pRS426-MET25), both the purified *SlAMT2.1* and the yeast vector were digested with *Spe*I and *Xho*I using the FastDigest kit (Thermo Scientific, Ottawa, ON, Canada) and then were gel purified (QIAquick Gel Extraction Kit, Qiagen Inc, Mississauga, ON, Canada). Subsequently, the ligation reaction was incubated at 4°C for 12 h after treatment with DNA ligase kit (Promega, Madison, WI, USA). The yeast expression vector harboring *SlAMT2.1* cDNA was transformed to the competent *E. coli* DH5α cells and grown overnight cultures at 37°C. The *E. coli* colonies were screened for the correct insertion (expression vector harboring *SlAMT2.1* cDNA) using PCR with primers (M13-puc-F, 5'-CACGACGTTGTAAAACGAC-3' and M13-puc-R, 5'-GGATAACAATTTCACACAGG-3') flanking the insertion site on the vector backbone. The PCR products were separated using 1% agarose gel electrophoresis for bands visualization. The *E. coli* colonies that containing empty pRS426-MET25 vector showed bands of size 800 bp, while the bacterial colonies carried pRS426-MET25 vector and *SlAMT2.1* cDNA showed bands of size 2250 bp (Fig. 4.1B). After plasmid construct

extraction from *E. coli* by the Qiaprep Spin Miniprep kit (Qiagen, Mississauga, ON, Canada), 1 µg of the plasmid construct was digested with *Spe*I and *Xho*I using the FastDigest kit (Thermo Scientific, Ottawa, ON, Canada) and then the digested products were separated using 1% agarose gel electrophoresis. *SlAMT2.1* cDNA was successfully cleaved from the digested plasmid construct at 1450 bp (Fig. 4.1C), confirming the insertion of *SlAMT2.1* cDNA in this vector. Furthermore, the orientation and accuracy of the inserted *SlAMT2.1* cDNA was confirmed by sequencing (Robarts Research Institute, London, Ontario, Canada). The empty pRS426-MET25 vector and the same vector harboring *SlAMT2.1* cDNA were transformed to *Saccharomyces cerevisiae* strain 31019b, *mep1*Δ *mep2*Δ *mep3*Δ (Marini et al., 1997) using the lithium acetate protocol (Gietz et al., 1995). Yeast transformants were selected on YNB-N minimal media containing (A) 1 mM $(NH_4)_2SO_4$, 3% glucose (w/v) and 2% agar (w/v) (B) 0.1% glutamine, 3% glucose (w/v) and 2% agar (w/v).

4.3.7. Gene expression of MPEs in yeast

Total RNA was extracted by vortexing the yeast cells with iron beads in the presence of TRI reagent (Sigma-Aldrich Canada). The concentration of total RNA of the samples was checked using a NanoDrop 2000C spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA integrity was confirmed using 1% agarose gel stained with ethidium bromide (Sigma- Aldrich Canada) that showing clear bands for 28S and 18S ribosomal RNAs. First-strand cDNA was synthesized from 1 µg of total DNased RNA using iScript cDNA synthesis kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions. To investigate the expression of *MPEs* in yeast, each of the *MPE* primers (Table 4.2) were employed in RT-PCR analysis.

The PCR program for all *MPEs* was as follows: 94°C for 3 min and 33 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The endogenous *actin* gene (amplified at 26 cycles) was used as internal control. For bands visualization, the PCR products were separated using agarose gel electrophoresis containing ethidium bromide (Sigma- Aldrich Canada).

4.4. Results and discussion

4.4.1. Identification of AMT genes in tomato

Based on blast searches using the available sequences of *Populus termula x alba* and *Arabidopsis thaliana* AMT proteins (Sohlenkamp et al., 2002; Couturier et al., 2007), 8 putative AMT proteins and their encoding genes (Appendix A) were identified in the tomato genome database (https://solgenomics.net/). For the 5 *AMT* genes that had not been identified previously, names were assigned (Table 4.3) according to the names of closely related homologues from other plant species as indicated by the genetic tree analysis (Fig. 4.2). The length of tomato AMT proteins ranged from 461 to 515 amino acids (Table 4.3). In previous studies, analysis of the genomes of poplar and *Arabidopsis* revealed the presence of 14 and 6 *AMT* genes, respectively (Tsay and Hsu, 2011; Couturier et al., 2007). The tomato genome has a greater number of *AMT* genes than in *Arabidopsis* and lower number than in poplar. Tomato, as an annual plant with fleshy fruits, differ fundamentally from perennial plant species (poplar) and has a longer life cycle than *Arabidopsis.* Therefore, plant species with different life cycles or different structure may transport NH_4^+/NH_3 with an unequal number of AMTs (Couturier et al., 2007).

4.4.2. Tomato AMT possess putative trans-membrane domains (TMDs)

To identify the distinct domains of the tomato AMT family, the 8 AMT protein sequences from tomato were aligned with the characterized AMT proteins from *Arabidopsis* (Pantoja, 2012). The results showed that all the tomato AMT proteins contain 11 putative trans-membrane domains (TMDs) with additional N-terminal and C-terminal domains (Fig. 4.3). The majority of those putative trans-membrane domains (helices) may facilitate binding to membrane phospholipids (Thomas et al., 2000; Howitt and Udvardi, 2000). The N-terminus has been proposed to stabilize the AMT trimeric structure, which led to intermolecular interaction between subunits (monomers) to regulate plant AMT activity (Graff et al., 2011). Truncation of the N-terminus in SlAMT1.1 led to loss of functionality when expressed in yeast (Graff et al., 2011). The C-terminal (carboxy terminal) regulates the activity of plant AMTs via specific residues (Pantoja, 2012).

4.4.3. Gene structural and genetic tree analyses of tomato AMTs

To estimate the evolutionary relationships between the predicted tomato AMT proteins and other known AMTs from plants as well as diverse other organisms (prokaryotes and eukaryotes), a genetic tree analysis was conducted (Fig. 4.2). The genetic tree analysis showed two clear protein clusters (AMT1 and AMT2 subfamilies) for plant AMTs, as reported before by Suenaga et al. (2003). For tomato, three proteins (AMT1.1, AMT1.2 and AMT1.3) were in the AMT1 cluster, and the remaining AMT proteins (AMT2.1, AMT4.1, AMT4.2, AMT4.4 and AMT4.5) were in the AMT2 cluster (Fig. 4.2). In addition, in this tree, the AMT1 subfamily is closely related to AMTs in green algae, nematode and insects, while the AMT2 subfamily is more related to the

Amtbs in prokaryotes (bacteria) and MEPs

(methylammonium and ammonium permease)-type transporters in yeast (Fig. 4.2). Interestingly, the plant AMT1 subfamily is more related to AgAMT (*Anopheles gambiae* ammonium transporter) than the AMT2 subfamily. *Anopheles gambiae* AMT is the only animal AMT with confirmed NH_4^+ transport capability (Pitts et al., 2014). Overall, the genetic tree analysis revealed that tomato AMT formed two distinct related subfamilies.

Gene structure analysis indicated that genes in the tomato AMT1 subfamily had 1 exon, with the exception of AMT1.2, which had 2 exons. On the other hand, genes that belong to AMT2 subfamily had 3 exons, with the exception of AMT2.1and AMT4.1 that had 4 exons (Fig. 4.4B). Similar results were reported in poplar and *Arabidopsis*, where AMT2 subfamily members had larger number of exons than in the AMT1 subfamily (Sohlenkamp et al., 2002; Couturier et al., 2007). As shown in Fig. 4.4B and Table 4.3, although the size of exons (or ORF) were generally well conversed between tomato AMT4 members (around 1400 bp), the total gene length for the genes varied (1783-3112 bP) due to the presence of variable number and length of introns. In addition, AMT1.2 had a long intron, while its ORF (bp) length was similar to AMT1.1 (which had no intron). Therefore, the main differences in gene structure between different tomato AMTs seem due to the size of their introns.

4.4.4. Expression of tomato AMT genes in various tissues

To investigate the expression patterns of tomato *AMTs* at the seedling stage, RT-PCR was used to analyze the expression of *AMT1.1*, *AMT1.2*, *AMT1.3*, *AMT2.1*, *AMT4.1*, *AMT4.2*, *AMT4.4* and *AMT4.5* in roots, stems, petioles and leaves (Fig. 4.5). The expression analysis of *AMTs* in tomato tissues showed that four *AMT* genes (*AMT1.1*,

AMT1.2, *AMT1.3* and *AMT2.1*) were expressed at the seedling stage (Fig. 4.5), while none of the *AMT4* genes (*AMT4.1*, *AMT4.2*, *AMT4.4* and *AMT4.5)* were detected in any organs examined under our experimental conditions (data not shown). It is worth mentioning that multiple members of the *AMT2* subfamily (*GmAMT4.1, GmAMT4.3* and *GmAMT4.4)* in soybean (*Glycine max*) showed specific expression in roots after arbuscular mycorrhizal fungi inoculation, suggesting that they may play a critical role in the transfer of NH_3 from the peri-arbuscular space into the plant root cortical cells (Kobae et al., 2010). Tomato plants are among the wide range of host plants for arbuscular mycorrhizal fungi. Therefore, the level of involvement of tomato *AMT4* genes in this symbiotic relationship could be an interesting point for future research.

In this study, the four detected *SlAMT* genes showed different expression patterns in shoots and roots (Fig. 4.5). The distinct expression patterns of *AMTs* in tomato seedlings could support the idea that the AMT family functions not only in roots for ammonium translocation, but also in leaves for ammonium recycling during photorespiration and catabolism of protein (Couturier et al., 2007). Multiple studies have found that the plant *AMT1* subfamily is expressed in roots (Suenaga et al., 2003; Couturier et al., 2007; Li et al., 2015), with the exception of *LjAMT1.1, LjAMT1.2* (D'Apuzzo et al., 2004) and *SlAMT1.3,* according to my data (Fig. 4.5). On the other hand, the plant *AMT2* subfamily seems to be expressed in both shoots and roots as shown in *Arabidopsis*, rice (*Oryza sativa)* and poplar (Suenaga et al., 2003; Couturier et al., 2007; Shohlenkamp et al., 2002). Unlike in the other plant species, our expression analysis in tomato showed that *SlAMT2.1* is the only tomato *AMT* root specific

transporter. This result suggests a potential role of *SlAMT2.1* in transporting NH_3 in root cells.

The *SlAMT1* subfamily genes (with the expectation of *SlAMT1.1*) were expressed in both the stems and petioles (Fig. 4.5). In fact, the role of plant *AMTs* in those tissues has been widely neglected (Couturier et al., 2007). In *Arabidopsis*, high levels of NH_4^+ have been detected in the stem xylem, suggesting that a considerable amount of NH_4^+ might be translocated to the shoots (Rawat et al., 1999; Couturier et al., 2007). In addition, in NH_4^+-fed tobacco (*Nicotiana tabacum)* plants, significant NH_4^+ levels were detected in stem phloem sap (Tercé-Laforgue et al., 2004). Therefore, the contribution of AMTs to NH_4^+ allocation between shoots and roots in plants needs further investigation.

4.4.5. The root specific AMT2.1 is highly regulated by N availability

Von Wirén et al. (2000) reported that the transcript level of *SlAMT1.1* and *SlAMT1.2* in tomato root is affected by the changes in N levels, suggesting that both transporters may be involved in NH_4^+ uptake from soil. To investigate the effect of N availability on mRNA level of *AMT2.1* in roots, tomato seedlings grown hydroponically were submitted to N starvation for 72h, then resupplied with N for 2h. Our gene qPCR analysis showed that transcripts of *SlAMT2.1* were induced strongly by N starvation after 72h and decline with N resupply (Fig. 4.6). A similar trend has been previously observed for *AtAMT2.1* in *Arabidopsis* roots (Sohlenkamp et al., 2002). Overall, these results show that *SlAMT2.1* is also responding to N cues.

4.4.6. Molecular mechanism of tomato AMTs

In this study, the well characterized EcAmtB (*Escherichia coli* AMT) (Khademi et al., 2004; Zheng et al., 2004) was used as a model to understand the molecular mechanism of tomato AMTs (Ludewig et al., 2007). It has been shown that the protein structure of AmtB from *E. coli* is arranged as homo-trimers (3 subunits), with each monomer (subunit) containing a hydrophobic pore (channel) in its center, proposed to transfer uncharged NH_3 (Khademi et al., 2004; Zheng et al., 2004). In EcAmtB, NH_4^+ is recruited by the selective NH_4^+ binding (recruitment) sites, which is located on the periplasmic/ extracellular side of the plasma membrane (Khademi et al., 2004; Zheng et al., 2004). Subsequently, the recruited NH_4^+ is deprotonated (removing of H^+) in the central section of EcAmtB channel by a deprotonation region, formed by His168 and His318. It has been suggested that this pair of His deprotonates NH_4^+ and facilitates the movement of NH_3 through the hydrophobic pore. At the cytoplasmic pore exit, NH_3 would be finally re-deprotonated (adding H^+) in the compartment with neutral pH (Fig. 4.7A) (Khademi et al., 2004; Zheng et al., 2004; Pantoja, 2012).

Tomato AMT1 proteins showed high amino acid identity between them, ranging from 57-73% (Fig. 4.8). In comparison, identity between tomato AMT1 and AMT2 subfamilies is low with values between 16-21% (Fig. 4.8). In addition, identity between tomato AMTs and the EcAmtB homologue from bacteria (*E. coli*) is also low (21%) (Fig. 4.8). Despite these differences between the AMTs from tomato and bacteria, the protein structures of tomato AMTs (Fig. 4.9A) are overall similar to the homo-trimers structure previously detected in EcAmtB (Khademi et al., 2004; Zheng et al., 2004), which

suggests that the conserved residues must establish a common mechanism of transport (Pantoja, 2012).

The amino acids EcD160 and EcW148, proposed to form the high affinity NH_4^+ binding sites in EcAmtB (Pantoja, 2012), are well conserved in both SlAMT1 and SlAMT2 subfamilies (Fig. 4.3). Some previous studies have demonstrated the role of those NH_4^+ binding sites in plant AMT activity. For example, in the tomato AMT1.1, the mutation SlW178L (EcW148) converted the transporter into an inactive system, unable to transport NH_4^+ or MeA (Mayer et al., 2006). In other studies, mutations in the equivalent residue for EcD160 in *Arabidopsis* (AtAMT1.1 D198) and yeast (ScMEP2 D186N) inhibited ammonium transport (Marini et al., 2006; Loqué et al., 2007). Although the presence of the conserved residues has been confirmed in all tomato AMT members, differences in the putative NH_4^+ binding sites were observed between the AMT1 and AMT2 subfamilies in tomato. For example, the high affinity NH_4^+ binding site equivalent to EcS219 in EcAmtB was detected only in the tomato AMT1 subfamily (Fig. 4.3). The pair of His (His168 and His318), proposed to deprotonate NH_4^+ and facilitate NH_3 movement in EcAmtB, is also well conserved in tomato AMTs (Fig. 4.3). Overall, protein structure, high affinity NH_4^+ binding sites, as well as deprotonation regions of tomato AMTs are similar to those of EcAmtB. This suggests that similar NH_4^+ recruiting/ NH_3 translocation mechanism may occur in tomato AMTs and EcAmtB (Ludewig et al., 2007).

However, plant AMT1 could function differently from AMT2, as AMT1 is an electrogenic transport system (transporting one charge across the membrane) (Wood et al., 2006; Straub et al., 2014; Neuhäuser and Ludewig, 2014). Therefore,

it could be hypothesized that AMT1 transports NH_3 through the hydrophobic channels in addition to NH_4^+ or H^+ through an unknown pathway (Fig. 4.7B). This model is supported by the identification of point mutations in AtAMT1.2 (resulting in exchanges of residues, namely AtQ67K, AtM72I and AtW145S) which convert the wild-type of AtAMT1.2 (electrogenic transport system) into an electroneutral ammonium transporter (Neuhäuser and Ludewig, 2014). These mutants did not show amino acid changes in the hydrophobic channel region but in the more peripheral regions involved in the subunits (monomers) contact (Neuhäuser and Ludewig, 2014). Hence, NH_3 must be translocated in the hydrophobic channel and NH_4^+ or H^+ are translocated by a different pathway affected by the mutations (Neuhäuser and Ludewig, 2014). Interestingly, my amino acid sequence alignment showed that the mutations in the equivalent residue AtM72I in *Arabidopsis* was replaced by residue I44 in the tomato AMT2 subfamily (Fig. 4.3). Plant AMT2 is an electroneutral transport system (no charge is transported across the membrane) and may transport NH_3 only (Fig. 4.7C) (Ludewig et al., 2007; Guether et al., 2009; Straub et al., 2014). Therefore, the predicted residue I44 in AMT2 could be essential for the electroneutral transport system in this subfamily.

Whether AMTs are electrogenic or electroneutral greatly affects their physiological role. Electrogenic transporters (translocating net charges across the membrane) are function in NH_4^+ cell uptake, as cation uptake is driven by the transmembrane electrochemical gradient (McDonald and Ward, 2016). In *Arabidopsis*, AtAMT1 members were localized in the plasma membrane of the outer cell layers (epidermis, cortex and endodermis), suggesting a role for those transporters in NH_4^+ uptake from soil or the root apoplast (Yuan et al., 2007).

In contrast, electroneutral transporters are primarily driven by the transmembrane NH_3 partial pressure gradient (Fig. 4.7C) (McDonald and Ward, 2016). Ammonia is a weak base with a pKa of 9.25 (Sohlenkamp et al., 2002). Therefore, given the pH of the root cell cytoplasm (neutral) and the pH of the root cell apoplast (acidic), the concentration of NH_3 is expected to be higher in the cytoplasm than in the apoplast (Wood et al., 2006). Hence, the role of the *Medicago truncatula* AMT2.1 homolog in NH_3 root uptake as suggested earlier by Straub et al. (2014) may be questionable. In their study, this gene was shown to be localized in the plasma membrane of the outer cell layers (epidermis and cortex). If so, the NH_3 concentration gradient would suggest that this gene could be involved in NH_3 efflux from those outer cell layers. On the other hand, a previous study has shown that the expression of the *Arabidopsis* AMT2.1 homolog was localized in the root vascular tissues (Sohlenkamp et al., 2002). This could suggest another potential function for the plant AMT2.1 in roots. The plant AMT2.1 transporter may play a role in NH_3 loading into the xylem. This function for AMT2.1 is also supported by the fact that NH_4^+ /NH_3 has to be loaded into the xylem vessels of the root to be transported then assimilated in the aerial parts of plant (Yuan et al., 2007). Overall, those proposed functions for plant AMT2.1 (NH_3 efflux from root cells and/or NH_3 loading into xylem) in roots should be tested in future studies.

4.4.7. Functional expression of SlAMT2.1 in a yeast mutant defective in ammonium uptake

Heterologous expression includes transferring a fragment of DNA from a specific organism to hosts (expression systems) for synthesis of the encoded proteins. This method allows the production of plant proteins in hosts to investigate their function as

well as their biochemical features (Yesilirmak and Sayers, 2009). Multiple factors influencing the choice of hosts include stability of the foreign protein, requirement for posttranslational modifications, efficiency of the expression system, as well as cost and simplicity (Yesilirmak and Sayers, 2009). Yeast (*Saccharomyces cerevisiae*), a single celled eukaryotic organism, is a powerful heterologous expression systems for proteins. Yeast cells are easy to manipulate, and yeast cultures can grow rapidly with relatively low cost (Bassham and Raikhel, 2000). Genetic studies have led to the identification of three ammonium transporter genes (*MEP1*, *MEP2* and *MEP3*) in yeast (Marini et al., 1997). MEP2 displays the highest affinity for ammonium (Km = 1–2 μM), followed by MEP1 (Km = 5–10 μM), and MEP3 shows the lowest affinity for ammonium (Km = 1.4-2.1 mM) (Marini et al., 1997). The yeast mutant strain 31019b (*mep1*Δ *mep2*Δ *mep3*Δ) is a null mutant of the endogenous *MEP* genes. Therefore, this mutant cannot grow on a medium supplemented with ammonium concentration lower than 5 mM as a sole source of nitrogen (Marini et al., 1997).

To confirm that tomato *AMT2.1* encodes a functional transporter, the ability of the *SlAMT2.1* cDNA to complement the growth of yeast mutant strain 31019b was tested on solid YNB-N (yeast nitrogen base without amino acids) minimal medium (obtained from Amresco, USA). In addition, the yeast mutant strain 31019b6 transformed with empty pRS426-MET25 vector was used as a negative control on the same medium (Fig. 4.10). After 5 days of incubation (37° C), yeast growth was detected for the negative control treatment (Fig. 4.10). This observation suggested that contamination with the wild-type yeast cells might have occurred through the different culture steps, or the growing medium has an additional source of nitrogen. In the positive growth control, the yeast

mutant strain 31019b also showed growth with 0.1% glutamine as a nitrogen source (Fig. 4.10).

To test the possibility that the observed growth in the negative control treatment resulted from the contamination with wild-type yeast cells, both wild-type strain 23344c and yeast mutant strain 31019b (without any insertion) were tested for their ability to grow on the same medium as described above. After 5 days of incubation (37° C), growth of the yeast mutant strain 31019b was again observed (Fig. 4.11A). Subsequently, yeast cells of both wild-type and mutant were collected and the deletion of all *MEPs* in yeast mutant strain 31019b was confirmed by the *MEP* expression analysis (Fig. 4.11C). This suggests that contamination with the wild-type yeast cells was not the reason for the observed yeast growth for negative control treatment and raises the possibility that the medium used had an external source of nitrogen.

To obtain a growing medium without nitrogen contamination, another yeast growing medium was tested. This medium contained vitamins, micro and macro elements required for yeast growth according to the formula by Acumedia, (USA) (Appendix B, Table 8.1) supplemented with 2% agar (w/v), 3% glucose (w/v) and 1 mM $(NH_4)_2SO_4$ as the sole nitrogen source. Both wild-type strain 23344c and yeast mutant strain 31019b (without any insertion) were tested for their ability to grow on this medium (Fig. 4.12). In addition, YPD (Yeast Peptone Dextrose) medium was used as a positive control for yeast growth. After 8 days of incubation, growth of the yeast mutant strain 31019b (triple-mepΔ) was observed (Fig. 4.12). This suggested that this medium may have also been contaminated with a source of nitrogen. Multiple components were used to prepare the yeast growth media, which increase the chances to get nitrogen traces

such as amino acid in this medium. Discovering the source of external nitrogen in those media would be a challenge for this assay, since this would require to perform nitrogen analysis for each of the medium components.

The *Xenopus* oocyte, as a host for heterologous expression, could provide a convenient system to study the function of SlAMT2.1. In the oocyte expression system, the mRNA for the gene of interest, introduced by direct injection into the *Xenopus* oocyte cytoplasm, is translated into protein (Yesilirmak and Sayers, 2009). The function of the plant ammonium transporter in oocytes could also be directly tested by ^{15}N-labeled ammonium uptake assay (Straub et al., 2015). The advantage for this system is that it does not required a growth assay on a nitrogen-based medium and the ^{15}N isotope uptake could be performed in a few minutes (Straub et al., 2015).

4.5. Conclusion

Ammonium is a primary source of N for plants, so knowing how it is transported in plant cells is critical to maximize N uptake and increase agricultural production. The data presented here suggests that tomato roots possess two distinct types of *AMT* (*SlAMT1* and *SlAMT2* subfamilies). The possible reason for retaining both types of transporters is that they may function differently. Tomato *AMT1* members are more likely to function in NH_4^+ root uptake, while *SlAMT2.1* might be involved in NH_3 efflux from root cells or NH_3 loading into the xylem vessels. The proposed functions for SlAMT2.1 in this study should be tested in future research.

Table 4.1. Primer sequences employed in PCR for tomato (*S. lycopersicum*). F: forward primer, R: reverse primer.

Gene name	Accession number of Sol Genomics Network	Nucleotide sequence (5′ ➔ 3′)	Annealing T (°C)	Product size (bp)
AMT1.1	Solyc09g090730	*AMT1.1* F: TGGTCAATGGAGCGCTGTAG *AMT1.1* R: TATCCCCCATGCACCACAAC	60	313
AMT1.2	Solyc04g050440	*AMT1.2* F :CAAACGTGCTTGATGCTGCT *AMT1.2* R :CAACCATCACCGGACCAGAA	60	304
AMT1.3	Solyc03g045070	*AMT1.3* F : TTTGCCGGTAGTGGTGTTGT *AMT1.3* R :TAACAATCCCCGCGGTTGAA	60	304
AMT2.1	Solyc10g076480	*AMT2.1* F :AGAATGCCAGAGACAACGCA *AMT2.1* R : AGCAGCGGTGAATCCAGAAA	60	324
AMT4.1	Solyc09g065740	*AMT4.1* F : ACGCATGTTTGTGCTGCAAT *AMT4.1* R : CCTGCTAGAATCGCGCCTAA	60	302
AMT4.2	Solyc01g097370	*AMT4.2* F: GTAGCAGCCACCTTAGTGGG *AMT4.2* R :CAAGCAATGCACCCGCTATC	60	325
AMT4.4	Solyc08g067080	*AMT4.4* F: AGGAGGAATCCTGTCTGGTCT *AMT4.4* R: TCTCCATCACCCCACAAAGC	60	303
AMT4.5	Solyc03g033300	*AMT4.5* F :TTGTTTGCATCACCCCTGGT *AMT4.5* R: TGAGCTCCCATTTGCCGTAG	60	450
Actin	AB695290	*Actin* F:GAAATAGCATAAGATGGCAGACG *Actin* R: ATACCCACCATCACACCAGTA	55	160

Table 4.2. Primer sequences employed in PCR for yeast (*S. cerevisiae*). F: forward primer, R: reverse primer.

Gene name	Accession no. for genes	Nucleotide sequence (5′ ➔ 3′)	Annealing T (°C)	Product size (bp)
MEP1	536818	*MEP1 F*: AAGGCCCTGGTTGCTTATCT *MEP1 R*: GTGCAGACTTCCTTCTTGCC	60	534
MEP2	330443715	*MEP2 F*: GGTATCATCGCTGGCCTAGT *MEP2 R*: CAATTTGAGCAGCGTCGGTA	60	436
MEP3	330443753	*MEP3* F: GCAAGCTCACTGTCACCAAA *MEP3* R: GCCGTCCATTCCAATAACCC	60	372
Actin	330443543	*Actin* F: GTTACGTCGCCTTGGACTTC *Actin* R: R:CGGCAGATTCCAAACCCAAA	60	160

Table 4.3. *AMT* gene family in tomato.

S. no	Gene name	Accession number of Sol Genomics Network	Chromosome location	ORF (bp)	Protein size (amino acids)	Exon number	Intron number	Gene length (DNA-bp)
1	*AMT1.1*	Solyc09g090730	SL2.50ch09:70165531..70167004	1473	491	1	0	1473
2	*AMT1.2*	Solyc04g050440	SL2.50ch04:47231399..47234372	1545	515	2	1	2974
3	*AMT1.3*	Solyc04g045070	SL2.50ch03:11495587..11496969	1383	461	1	0	1383
4	*AMT2.1**	Solyc10g076480	SL2.50ch10:59472160..59474959	1452	484	4	3	2800
5	*AMT4.1**	Solyc09g065740	SL2.50ch09:64021974..64025085	1428	476	4	3	3112
6	*AMT4.2**	Solyc01g097370	SL2.50ch01:88259856..88261638	1431	477	3	2	1783
7	*AMT4.4**	Solyc08g067080	SL2.50ch08:55994765..55996963	1458	486	3	2	2199
8	*AMT4.5**	Solyc03g033300	SL2.50ch03:4881297..4883838	1407	469	3	2	2542

**AMT* genes of tomato identified in this study

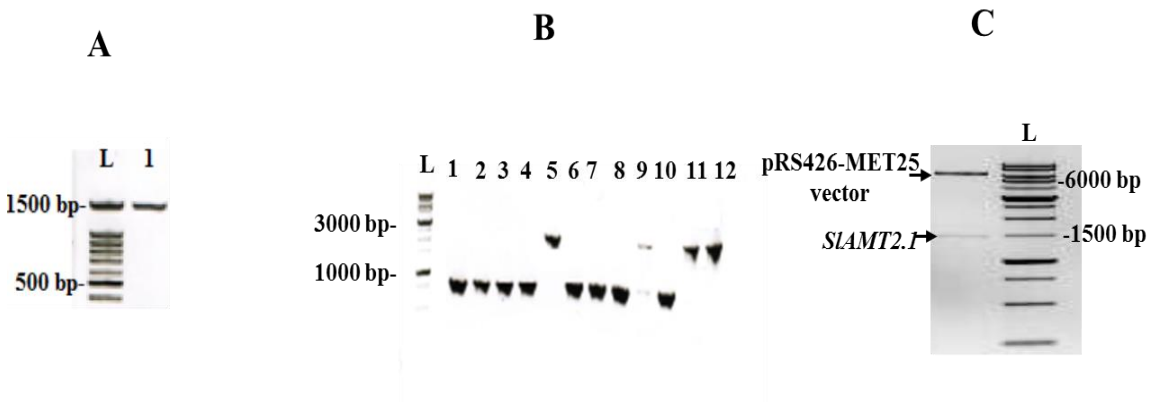

Fig. 4.1. Agarose gel electrophoresis. (A) Agarose gel electrophoresis showing the amplification of the whole open reading frame of *SlAMT2.1* from tomato root cDNA at the predicated size (1450 bp) (lane 1). (B) PCR products for the *E. coli* colonies. Lane 1, 2, 3, 4, 6, 7, 8 and 10 (800 bp) showing *E. coli* colonies containing empty pRS426-MET25 vector. Lane 5, 9, 11 and 12 (2250 bp) showing *E. coli* colonies carrying the plasmid construct (pRS426-MET25 vector and *SlAMT2.1* cDNA). (C) Gel image showing the correct size (1450 bp) of the *SlAMT2.1* after enzyme digestion of pRS426-MET25 vector containing *SlAMT2.1* cDNA by *Spe*I and *Xho*I enzymes. Lane L: DNA ladder.

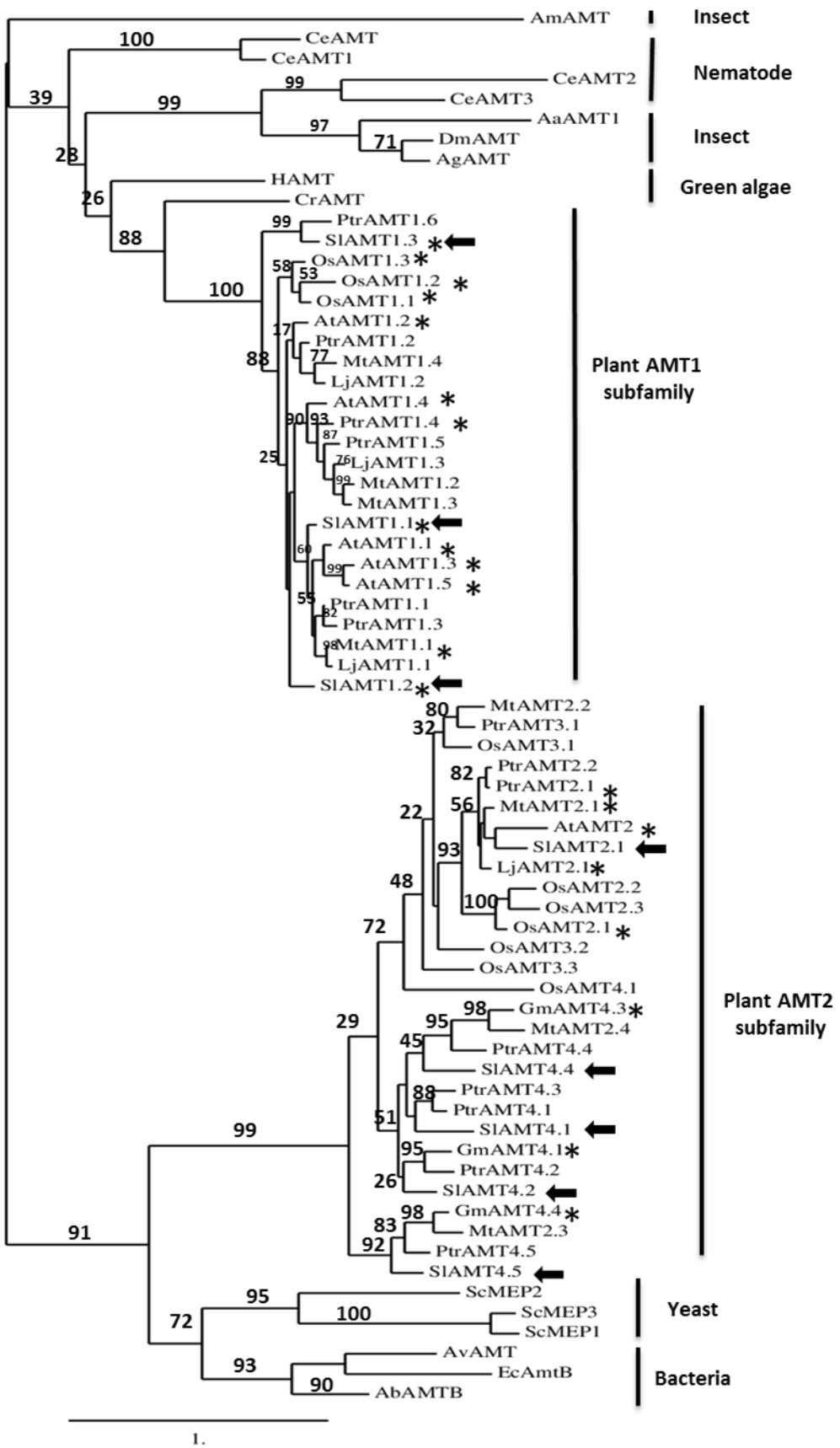
AmAMT
Insect
CeAMT
CeAMT1
CeAMT2
CeAMT3
Nematode
AaAMT1
DmAMT
AgAMT
Insect
HAMT
CrAMT
Green algae
PtrAMT1.6
SlAMT1.3
OsAMT1.3
OsAMT1.2
OsAMT1.1
AtAMT1.2
PtrAMT1.2
MtAMT1.4
LjAMT1.2
AtAMT1.4
PtrAMT1.4
PtrAMT1.5
LjAMT1.3
MtAMT1.2
MtAMT1.3
SlAMT1.1
AtAMT1.1
AtAMT1.3
AtAMT1.5
PtrAMT1.1
PtrAMT1.3
MtAMT1.1
LjAMT1.1
SlAMT1.2
Plant AMT1 subfamily
MtAMT2.2
PtrAMT3.1
OsAMT3.1
PtrAMT2.2
PtrAMT2.1
MtAMT2.1
AtAMT2
SlAMT2.1
LjAMT2.1
OsAMT2.2
OsAMT2.3
OsAMT2.1
OsAMT3.2
OsAMT3.3
OsAMT4.1
GmAMT4.3
MtAMT2.4
PtrAMT4.4
SlAMT4.4
PtrAMT4.3
PtrAMT4.1
SlAMT4.1
GmAMT4.1
PtrAMT4.2
SlAMT4.2
GmAMT4.4
MtAMT2.3
PtrAMT4.5
SlAMT4.5
Plant AMT2 subfamily
ScMEP2
ScMEP3
ScMEP1
Yeast
AvAMT
EcAmtB
AbAMTB
Bacteria
1.

Fig. 4.2. Genetic tree showing the relationship between AMTs from tomato and different organisms. The accession numbers of the AMTs are given as follows: AtAMT1.1 (At4g13510), AtAMT1.2 (At1g64780), AtAMT1.3 (At3g24300), AtAMT1.4 (At4g28700), AtAMT1.5 (At3g24290), AtAMT2.1 (At2g38290) from *A. thaliana*; PtrAMT1.1 (Poptr1_1 : 804848), PtrAMT1.2 (Poptr1_1 : 665333), PtrAMT1.3 (Poptr1_1 : 565016), PtrAMT1.4 (Poptr1_1 : 799507), PtrAMT1.5 (Poptr1_1 :645545), PtrAMT1.6 (Poptr1_1 : 804509), PtrAMT2.1 (Poptr1_1 : 802015), PtrAMT2.2 (Poptr1_1 : 808726), PtrAMT3.1 (Poptr1_1 : 175190), PtrAMT4.1 (Poptr1_1 :754260), PtrAMT4.2 (Poptr1_1 : 260884), PtrAMT4.3 (Poptr1_1 : 205629), PtrAMT4.4 (Poptr1_1 : 806873), PtrAMT4.5 (Poptr1_1 : 424130) from *Populus trichocarpa*; OsAMT1.1 (Os04g43070), OsAMT1.2 (Os02g40710), OsAMT1.3 (Os02g40730),OsAMT2.1 (Os05g39240), OsAMT2.2 (Os01g61550), OsAMT2.3 (Os01g61510),OsAMT3.1 (Os01g65000), OsAMT3.2 (Os02g34580), OsAMT3.3 (Os03g62200), OsAMT4.1 (Os12g01420) from *Oryza sativa*; MtAMT1.1 (MTR_1g045550), MtAMT1.2 (MTR_7g113340), MtAMT1.3 (MTR_7g098930), MtAMT1.4 (MTR_1g079760), MtAMT2.1 (MTR_7g069640), MtAMT2.2 (MTR_8g095040), MtAMT2.3 (MTR_8g074750) MtAMT2.4 (MTR_7g115050) from *Medicago truncatula*; LjAMT1.1 (Q9FSH3), LjAMT1.2 (Q7Y1B9), LjAMT1.3 (Q70KK9), LjAMT2.1 (AF187962) from *Lotus japonicas*; SlAMT1.1 (Solyc09g090730), SlAMT1.2 (Solyc04g050440), SlAMT1.3 (Solyc03g045070), SlAMT2.1 (Solyc10g076480), SlAMT4.1 (Solyc09g065740), SlAMT4.2 (Solyc01g097370), SlAMT4.4 (Solyc08g067080), SlAMT4.5 (Solyc03g033300) from *Solanum lycopersicum*; GmAMT4.1 (Glyma09g41810.1), GmAMT4.3 (Glyma19g43380.1), GmAMT4.4 (Glyma02g04960.1) from (*Glycine max*); MEP1 (730015), MEP2 (1302091), MEP3 (6325396) from *Saccharomyces cerevisiae*; AbAMTB (AAC38548.1) from *Azospirillum brasilense*; AvAMT (ACM38068.1) from *Agrobacterium vitis*; AmtB (1103924) from *Escherichia coli*; HAMT (KDD76492.1) from *Helicosporidium sp*.; CrAMT (AAL85345.1) from *Chlamydomonas reinhardtii*; CeAMT (NP_508783.2), CeMAT2 (3023293), CeMAT1 (NP_508784.1), CeMAT13 (NP_495761.2) from *Caenorhabditis elegans*; AaAMT (DQ011229.1) from *Aedes aegypti*; AmAMT (NM_001077818.1) from *Apis mellifera*; DmAMT (NP_001097800.1) from *Drosophila melanogaster*; AgAMT (AGAP003989) from *Anopheles gambiae*. Protein sequences were aligned by MUSCLE and tree was constructed by Phylogeny.fr using ML (Maximum Likelihood) method with 1000 bootstrap replicates. Asterisks indicate plant AMT with verified ammonium/ammonia transport ability (von Wirén et al., 2000; Sohlenkamp et al., 2002; Sonoda et al., 2003; Suenaga et al., 2003; Couturier et al., 2007; Guether et al., 2009; Kobae et al., 2010; Straub et al., 2014). Black arrows show tomato AMTs.

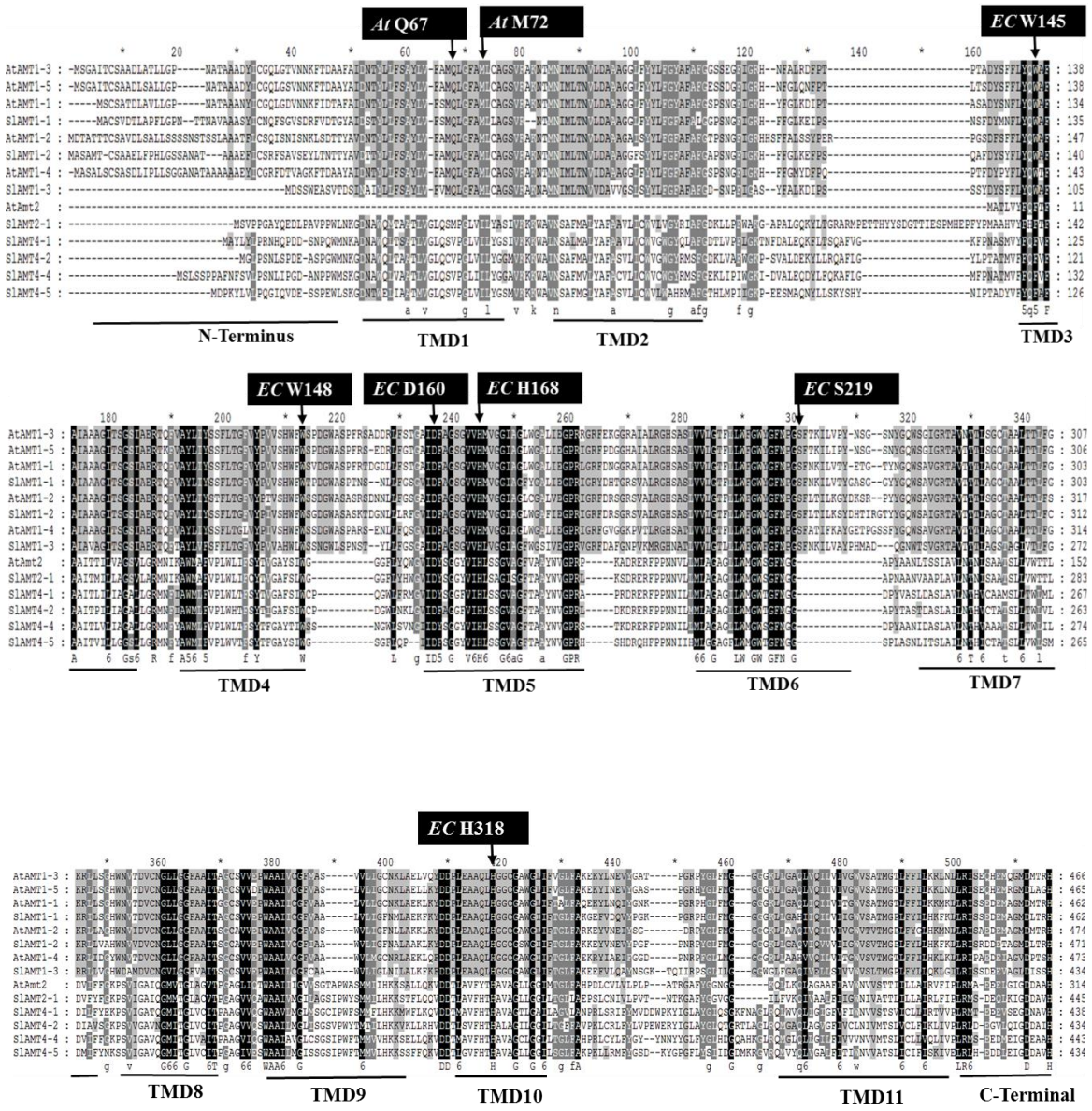
At Q67
At M72
EC W145
N-Terminus
TMD1
TMD2
TMD3
EC W148
EC D160
EC H168
EC S219
TMD4
TMD5
TMD6
TMD7
EC H318
TMD8
TMD9
TMD10
TMD11
C-Terminal

Fig. 4.3. Alignment of AMT proteins from tomato and *Arabidopsis*. All AMT proteins from tomato and *Arabidopsis* were collected from Sol Genomics Network database and GenBank, respectively. Multiple protein sequence alignments of AMTs were conducted using PSI-Praline multiple sequence alignment tool (www.ibi.vu.nl/programs/pralinewww). Functionally important conserved residues (amino acids) identified in *E. coli* (*Ec*) and *Arabidopsis* (*At*) (Pantoja, 2012) are highlighted with black squares at the top of sequences. The underlay lines are the predicted 11 trans-membrane domains (TMD1-TMD11) as well as N-terminal and C-terminal domains (Pantoja, 2012).

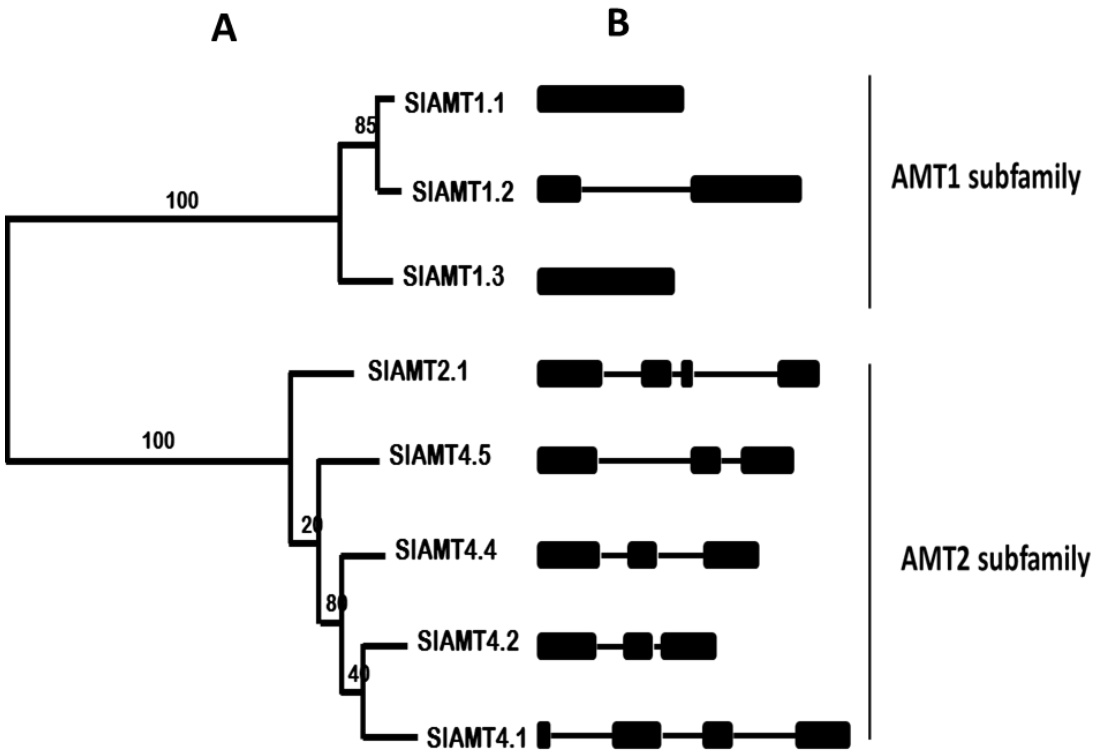

Fig. 4.4. Genetic tree and gene structures of tomato AMTs. (A) Genetic tree for the full-length AMT protein sequences. (B) Gene structure of *AMT*. Black boxes represent coding exons and back lines represent introns.

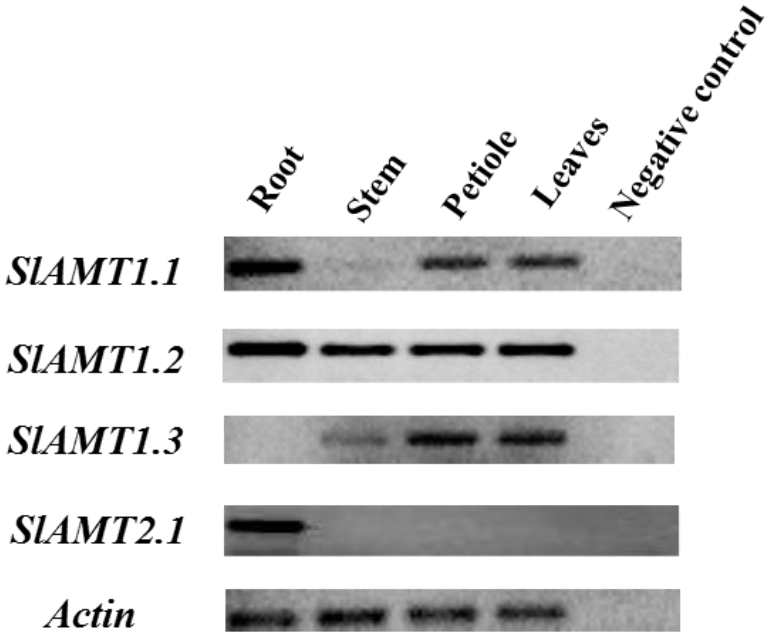

Fig. 4.5. Expression of *SlAMT1.1, SlAMT1.2, SlAMT1.3* and *SlAMT2.1* was analyzed by reverse transcriptase polymerase chain reaction (36 cycles) in different tissues. Aliquots of 1 μg total RNAs were reverse-transcribed into cDNA. The *actin* gene was amplified (26 cycles) and used as an internal control. Experiments were repeated at least three times, with similar results.

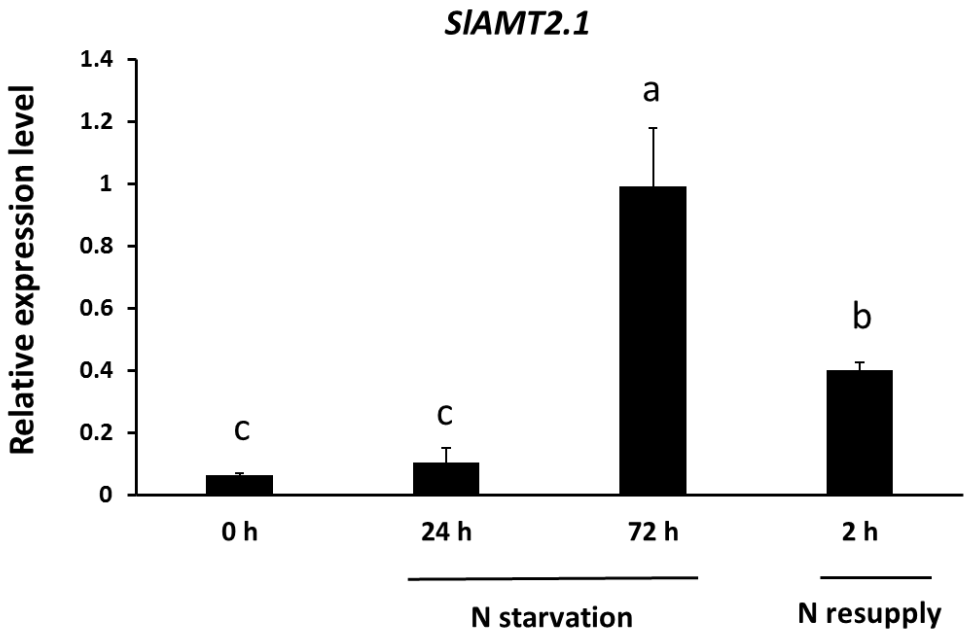

Fig. 4.6. Relative mRNA levels of *AMT2.1* in root tissue of tomato seedlings, grown with different N availability. The ratio of absolute mRNA levels of the *AMT2.1* and the *actin* gene was calculated. Data represent means ± SE, n =4. Different letters indicate significant differences between treatments ($P<0.05$).

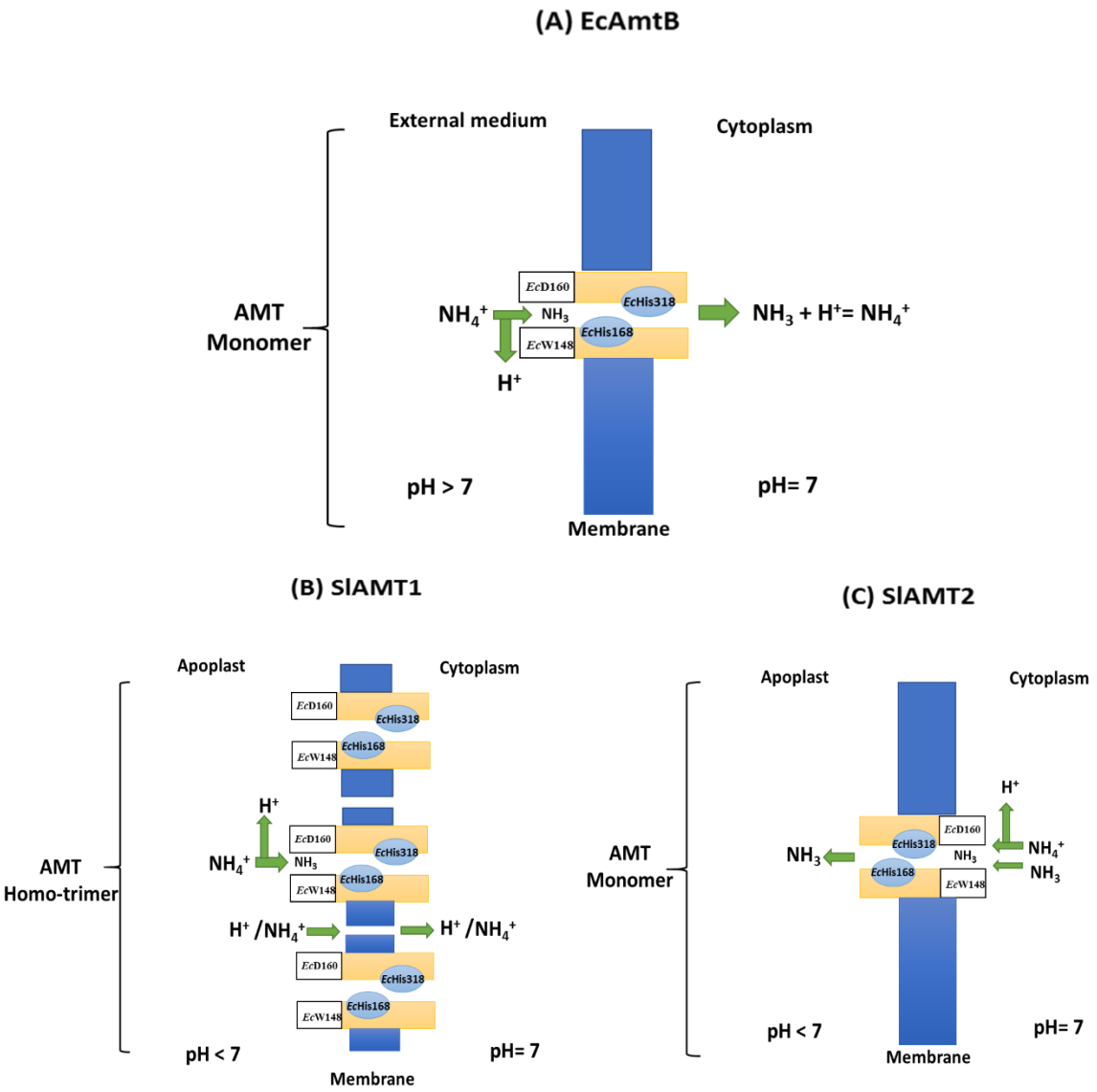

Fig. 4.7. Putative molecular mechanism of SlAMTs (*S. lycopersicum* ammonium transporter) and EcAmtB (*E. coli* ammonium transporter). (A) In EcAmtB, NH_4^+ is recruited by the selective NH_4^+ binding (recruitment) sites formed by specific amino acids such as EcD160 and EcW148. Subsequently, the recruited NH_4^+ is deprotonated in the central section of EcAmtB channel by the deprotonation region, which contains His168 and His318. At the cytoplasmic pore exit, NH_3 is finally re-deprotonated (addition of H^+) in the compartment with neutral pH. This model was designed according to information provided by Khademi et al. (2004) and Zheng et al. (2004). (B) SlAMT1 transports both NH_3 through the hydrophobic channels and NH^+_4 or H^+ through unknown pathway, probably between AMT monomers. (C) SlAMT2 transports NH_3 through the hydrophobic channels driven by the transmembrane NH_3 concentration gradient. The presented models for SlAMT1 and SlAMT2 were designed in this study.

	SlAMT1.1	SlAMT1.2	SlAMT1.3	SlAMT2.1	SlAMT4.1	SlAMT4.2	SlAMT4.4	SlAMT4.5	AtAMT2	EcAmtB
SlAMT1.1	**x**	**73**	**60**	**19**	**17**	**18**	**18**	**18**	**17**	**22**
SlAMT1.2		**x**	**57**	**18**	**17**	**17**	**19**	**18**	**16**	**21**
SlAMT1.3			**x**	**19**	**19**	**20**	**20**	**21**	**16**	**24**
SlAMT2.1				**x**	**56**	**54**	**54**	**54**	**52**	**26**
SlAMT4.1					**x**	**63**	**66**	**58**	**44**	**29**
SlAMT4.2						**x**	**65**	**59**	**45**	**28**
SlAMT4.4							**x**	**59**	**43**	**28**
SlAMT4.5								**x**	**42**	**25**
EcAmtB										**x**

Fig. 4.8. Identity (%) analysis of AMTs from tomato (*S. lycopersicum*) and bacteria *(E. coli*). Alignments of AMT amino acid sequences and sequence identity were performed with ClustalX (Jeanmougin et al., 1998) and GeneDoc (Nicholas and Nicholas, 1997).

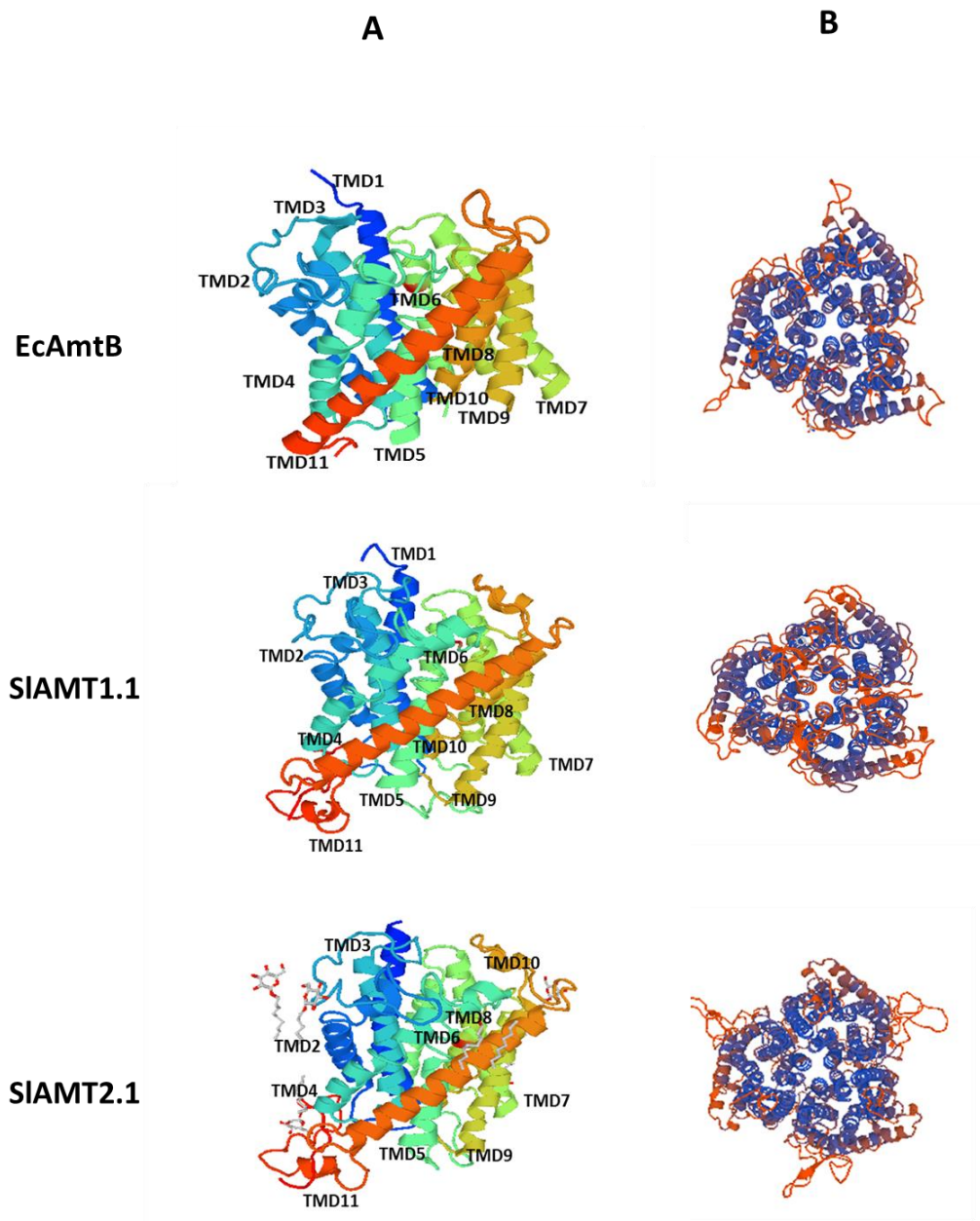

Fig. 4.9. Putative protein structure of SlAMTs (*S. lycopersicum* AMT) and EcAmtB (*E. coli* AMT). (A) Stereo representation of AMTs monomer from tomato and *E. coli*. The predicted 11 trans-membrane domains (TMD1-TMD11) which transport ammonium into the cell are indicated. The protein chain is colored from dark blue at the TMD1 to red at the TMD11 in accordance with Fig. 4.3. (B) The AMT trimer from tomato and *E. coli* seen from the extracellular side. The AMT protein structure models were automatically generated by SWISS-MODEL (https://swissmodel.expasy.org/).

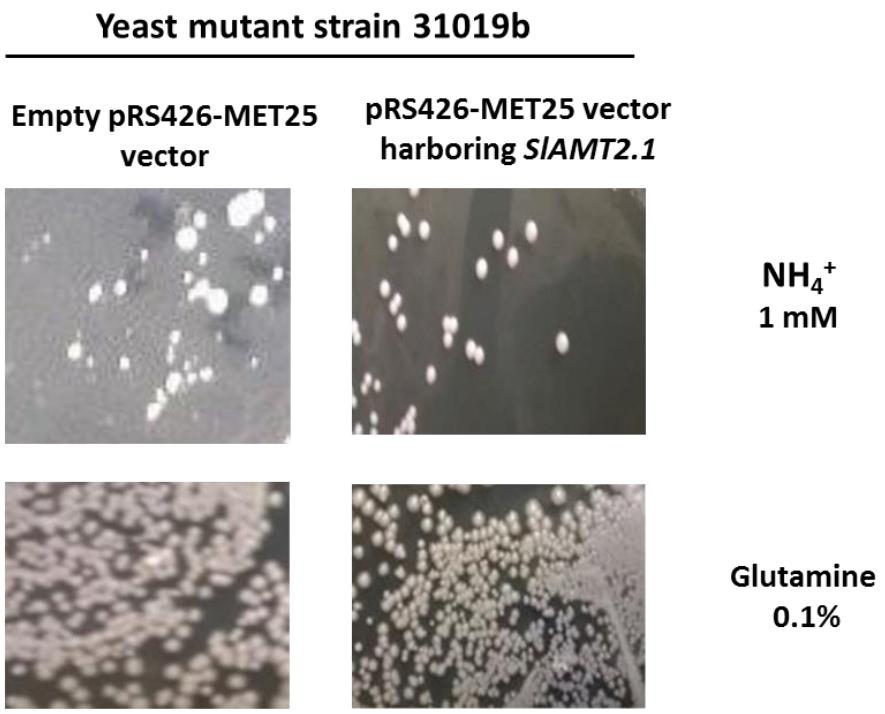

Fig. 4.10. Growth test of yeast (*S.cerevisiae*) mutant strain 31019b on YNB-N minimal media (Amresco, USA) supplemented with 1 mM $(NH_4)_2SO_4$ or 0.1% glutamine (positive growth control) as a sole nitrogen source. Yeast mutant strain 31019b was transformed with either the empty pRS426-MET25 vector (negative control), or, with the same vector harboring the open reading frame of *SlAMT2.1* (positive control).

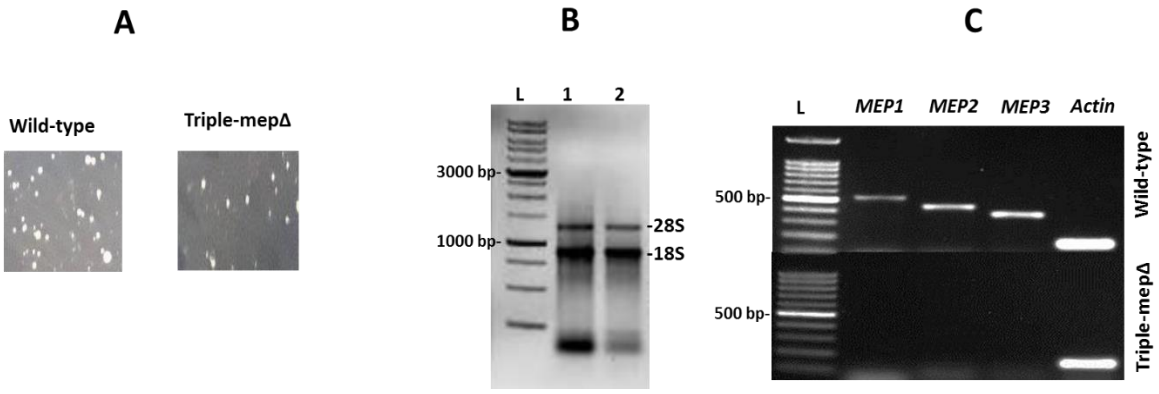

Fig. 4.11. Growth of yeast (*S. cerevisiae*) and agarose gels electrophoresis (A) Growth test of both yeast mutant strain 31019b (Triple-mepΔ) and wild-type of yeast, strain 23344c, (without vector insertion) after 5 days of incubation (37° C) on YNB-N minimal media (Amresco, USA) supplemented with 2% agar (w/v), 3% glucose (w/v) and 1 mM $(NH_4)_2SO_4$ as the sole nitrogen source. (B) Analysis of the integrity of RNA extraction from yeast cells collected from Figure 4.11A. Lane 1 represents the wild-type strain 23344c cells and lane 2 the triple-mepΔ cells (31019b). (C) Expression of *MEP1*, *MEP2* and *MEP3* was analyzed by reverse transcriptase polymerase chain reaction in yeast cells. The endogenous *actin* gene from yeast was used as an internal control. For bands visualization, the PCR products were separated using agarose gel (1%) containing ethidium bromide (Sigma- Aldrich Canada).

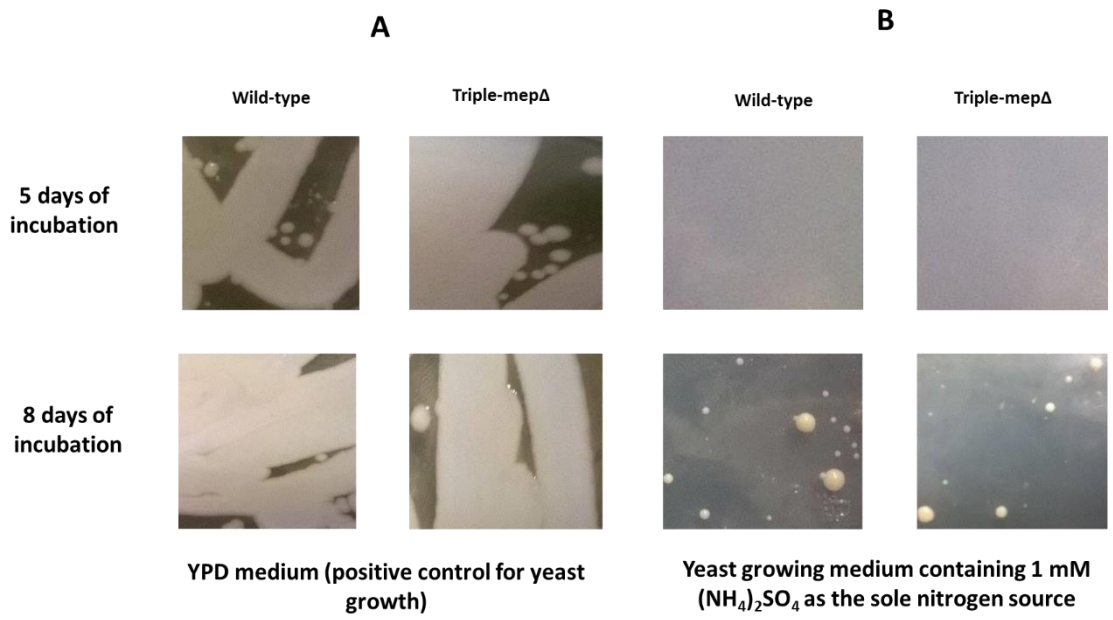

Fig. 4.12. Growth test of yeast (*S. cerevisiae*) on solid media after 5 and 8 days of incubation at 37° C. (A) Growth of both yeast mutant strain 31019b (Triple-mepΔ) and wild-type yeast (strain 23344c) on YPD (Yeast Peptone Dextrose) medium as a positive control for yeast growth. (B) Growth of both yeast mutant strain 31019b (Triple-mepΔ) and wild-type yeast (strain 23344c) on a yeast growing medium containing vitamins, micro and macro elements required for yeast growth (formula by Acumedia, USA) and supplemented with 2% agar (w/v), 3% glucose (w/v) and 1 mM $(NH_4)_2SO_4$ as the sole nitrogen source.

CHAPTER 5. EFFECTS OF SALT STRESS ON THE EXPRESSION OF KEY GENES RELATED TO NITROGEN ASSIMILATION AND TRANSPORT IN THE ROOTS OF THE CULTIVATED TOMATO (*SOLANUM LYCOPERSICON*) AND ITS WILD SALT-TOLERANT RELATIVE (*SOLANUM PENNELLII*)*

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Note: According to comments of the PhD committee, minor changes in wording have been made in this chapter compared to the original paper.

My contribution:

I conducted all experiments, analyzed the data and wrote the manuscript.

5.1. Abstract

Inorganic nitrogen is a key element for plant growth under salt stress. A comparative study including physiological responses, ion content, transcript regulation of ammonium/nitrate transporters (*AMTs*/*NRTs*) as well as key enzymes for nitrogen assimilation was undertaken in wild salt-tolerant tomatoes (*Solanum pennellii*) and cultivated tomatoes (*Solanum lycopersicon*) exposed to 100 mM NaCl for 1 and 7 days. In comparison to *S. lycopersicon*, *S. pennellii* was more salt tolerant as evidenced by its higher survival rate, lower biomass reduction, and less salt injury (reduced electrolyte leakage and proline accumulation). In root tissues of both species, salt exposure (7 days) reduced the mRNA levels of low affinity nitrate transporters (*NRT1.1* and *NRT1.2*). This was associated with a decline in both nitrate content and transcript level of the nitrate reductase gene (*NR*). Salt-stressed root tissues of *S. pennellii* showed relatively higher mRNA level of the high affinity ammonium transporters (*AMT1.1* and *AMT1.2*) compared to *S. lycopersicon.* The root ammonium content was increased only in *S. lycopersicon* going hand in hand with a reduction in mRNA level of cytosolic glutamine synthetase (*GS1*) after 7 days of salt stress, whereas the transcript level of *GS1* was unchanged in *S. pennellii*, suggesting a lower salt-induced inhibition in ammonium assimilation in this species. Our comparative study demonstrated that the salt-tolerant and salt-sensitive tomato species show differential contribution of the nitrogen transporters and key genes associated with nitrogen assimilation under salt stress. While the reduction in the mRNA level of key components of NO_3^- uptake (*NRT1.1, NRT1.2*) and assimilation (*NR* gene) in both species, likely contributed to the reduction in plant growth under salt stress, the observed salt tolerance for *S. pennellii* was associated with relative

higher mRNA level of ammonium uptake and assimilation genes. These results provide crucial knowledge for tomato breeding employing salt-tolerant wild species in salt-induced nitrogen-deficient environments.

Key-words: salt stress; *Solanum lycopersicon*, *Solanum pennellii,* nitrate transporter; ammonium transporter

5.2. Introduction

Salinity is one of the most prevalent stresses that limits plant growth and crop productivity. While elevated salinity is common in arid and semiarid regions, agricultural practices and the limitation of fresh water supplies have contributed to increased saline lands in all climatic regions (Niu and Cabrera, 2010). The adverse effects of salinity on plants result primarily from osmotic and/or ionic stresses caused by the salt-induced decrease in soil water potential and the accumulation of ions (mainly Na^+ and Cl^-) in the plant tissues respectively (Hasegawa et al., 2000). In addition, salt-stressed plants often exhibit nutrient imbalances (Hasegawa et al., 2000); for example, uptake, assimilation and content of nitrogen (N) in plant cells can be reduced by high salt levels in soils in a number of economically important plant species, including tomato (*Solanum lycopersicon*) (Flores et al., 2000; Dluzniewska et al., 2007). This decrease in N content in plants exposed to salt stress has been attributed to the reduction in NO_3^- uptake by roots due to the competition with NO_3^- and Cl^- (Botella et al., 1994; Yao et al., 2008). In addition, salt-induced changes in membrane integrity (Ca^{2+} displacement and oxidative damage) could further affect NO_3^- uptake (Frechilla et al., 2001). Nitrogen fertilization plays a critical role in the alleviation of salinity stress (Flores et al., 2001). Therefore, maintenance of N homeostasis under saline environment seems to be a key element for

salt tolerance (Popova et al., 2003; Ehlting et al., 2007) and N uptake through root transporters may play a key role for maintaining N balance in salt-stressed plants (Senadheera et al., 2009; Zhang et al., 2014).

Nitrate (NO_3^-) and ammonium (NH_4^+) ions, the two main sources of N available in soils for plants (von Wirén et al., 2000), are absorbed by roots using a variety of transporters. Nitrate transporters (NRTs) mediate NO_3^- uptake across the plasma membrane of the plant root cells by a mechanism of proton-coupled symport (Ullrich and Novacky, 1990) using two kinetically distinct nitrate transporters depending on the nitrate concentration in the soil (Forde, 2000; Hildebrandt et al., 2002; Gojon et al., 2011). The high affinity transport system (HATS) is encoded by the *NRT2* gene family and is used with optimal environmental NO_3^- concentrations below 100 µM. The low affinity transport system (LATS) is encoded by *NRT1* and operates at optimal NO_3^- concentrations higher than 1 mM. In *S. lycopersicon*, two families of nitrate transporter genes, *NRT1* and *NRT2*, have been identified as contributors to the NO_3^- uptake system. Ammonium transporters (AMTs) function as NH_4^+ uniporters and mediate NH_4^+ uptake through high-affinity ammonium uptake system (Ludewig et al., 2007). The proteins responsible for low-affinity system for ammonium have not been identified yet, but it has been suggested that the low-affinity system might be mediated by aquaporins and cation channels (Tsay and Hsu, 2011). In *S. lycopersicon* roots, two ammonium transporters, *AMT1.1* and *AMT1.2*, are involved in root ammonium uptake, while the expression of *AMT1.3* was detected in leaves, suggesting for this transporter a different role in N metabolism (von Wirén et al., 2000).

Plant roots are key organs for N uptake but also represent the first defense barrier against salt stress as they are in direct contact with soil solutions (Senadheera et al., 2009). In salt-stressed roots of *Oryza sativa* (rice) and *Populus simonii* (Chinese poplar) changes in mRNA levels of NH_4^+ and NO_3^- transporters have been suggested to be involved in salt tolerance through the regulation of the uptake and transport of NH_4^+ and NO_3^- (Senadheera et al., 2009; Zhang et al., 2014). In NO_3^- fed *O. sativa*, NH_4^+ and NO_3^- transporter genes have been reported to be differentially expressed in roots of salt-tolerant *O. sativa* and salt-sensitive plants (Senadheera et al., 2009). These differences in gene expression in salt-stressed root tissues could be beneficial to withstand salt-induced N deficiency in the salt-tolerant cultivar (Senadheera et al., 2009). In their study, the regulation of nitrogen transporters during salt stress was investigated in the presence of only one source of N. Since NO_3^- and NH_4^+ are the two major nitrogen sources for non-legume plants (Tsay and Hsu, 2011), studying the regulation of different N transporters in plants fed with both NH_4^+ and NO_3^- could be key to understand nitrogen uptake mechanisms during salt stress. A recent study in salt-stressed *Populus simonii*, a moderately salt-tolerant species, showed that the mRNA expression of NH_4^+ transporters were up-regulated in salt-stressed roots, whereas the expression of most of the NO_3^- transporters were down-regulated. This suggests a role of NH_4^+ transporters in NH_4^+ flux during acclimation to salinity stress (Zhang et al., 2014). Despite the efforts made to understand the effect of salt stress on nitrogen transporters and their involvement in salt tolerance, most of the research has focussed on glycophytes; while halophytes that have the ability to cope with salinity, have been rather neglected.

The use of comparative studies of closely related species that differ in their salt tolerance is a powerful strategy to understand the mechanisms of tolerance to this particular stress (Sun et al., 2010). *Solanum lycopersicon*, one of most important vegetable crops in the world, is moderately sensitive to salinity (Sun et al., 2010). The availability of wild halophytic *Solanum* species makes this species an ideal model crop for studying salinity tolerance. Thus, the objective of our study was to compare salinity responses of a well-characterized salt-sensitive species, *S. lycopersicon* cultivar "Manitoba", and the halophyte species, *S. pennellii* (Mittova et al., 2004; Shalata and Tal 1998), to identify the contribution of different N transporters and N assimilation genes of roots in salt tolerance. Therefore, the physiological responses and the mRNA levels of selected key genes encoding N transporters and N assimilation enzymes in salt-stressed roots of both species with $NH_4\,NO_3$ supplies have been investigated. We hypothesized that under salt stress, the expression pattern of ammonium and nitrate transporter genes will be different between salt tolerant and salt sensitive tomato species and that these differences could be related to salt tolerance. Investigating the effect of salinity-stress on root mRNA levels of N-transporter genes and genes important in N-assimilation will make a valuable contribution to breed tomato cultivars that can better withstand salt stress.

5.3. Materials and methods

5.3.1 Plant growth and salt treatment

Seeds of the cultivated tomato, *Solanum lycopersicon* (cv. Manitoba, obtained from T&T seeds, Winnipeg, Canada), and its wild salt-tolerant relative, *S. pennellii* (obtained from Tomato Genetics Resource Center, Davis, CA, USA - accession

LA0716), were selected for this study. In general, tomato plants are more sensitive to salinity stress at the seedling stage (Sun et al., 2010); thus, our study was carried out at this stage. The seeds were sown into seedling trays containing a mixture of peat moss: perlite (2: 1, v: v). When the first true leaf emerged (two to three weeks), the seedlings were transferred for one week to an aerated hydroponic solution (half strength modified Hoagland solution containing 2 mM NH_4NO_3; 1 mM KH2PO4; 1.5 mM $CaCl_2$; 0.5 mM KCl_2; 1 mM $MgSO_4$; 23 µM H_3BO_3; 5 µM $MnCl_2$; 0.4 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.07 µM H_2MoO_4 in addition to 0.007 µM Fe-EDTA) (Sabra et al., 2012) for acclimation (six plants per 10-L containers). The nutrient solution was renewed every 3 days to prevent nutrient deficiency. Plants were grown under the following greenhouse conditions: 24/19 °C day/night temperatures, 18h/6h photoperiod with natural light supplemented by sodium lamps (P.L. light systems, Beamsville, ON, Canada). Seedlings from both species were exposed to salinity stress at the stage of three true leaves by adding 100 mM NaCl to the nutrient solution. Three plants from each of the two species were placed in 10-L container (replicate), which were distributed in a completely randomized block design of six replicates.

After 1 day and 7 days of treatment, root tissues were collected and stored in RNAlater® (Ambion) at -80°C for subsequent mRNA analysis. To confirm the difference in salt tolerance of the two species, several key physiological and biochemical parameters (Sun et al., 2010; Sabra et al., 2012) were measured after 7 days of salt treatments, including relative water content (LRWC), electrolyte leakage (EL) and proline content. The remaining plants were harvested and washed three times with distilled water; the area of fully-expanded leaves was measured with a leaf meter (model 3000, LI-COR Inc.

Nebraska, USA) and the tissues were lyophilized for determining dry weight, and ion analysis.

As all seedlings survived after one week of 100 mmol l^{-1} NaCl, in a second experiment, we exposed *S. lycopersicon* and *S. pennellii* plants to 200 and 300 mmol l^{-1} NaCl for 7 days in parallel experiments (similar set up as the one described above) with four replicates. Survival rates were recorded to further determine the difference in salt tolerance of the two tomato species.

5.3.2. Leaf relative water content (LRWC)

Leaf relative water content was estimated after 7 days of salt treatment in fully-expanded detached leaflets. After recording the fresh weight (FW), leaf samples were placed in distilled water in a closed Petri dish in the dark at 4°C for 24 h to determine the turgid weight (TW). At the end of the imbibition period, leaf samples were freeze dried (Labconco Co., USA) for 3 days, in order to obtain dry weight (DW). Leaf relative water content (LRWC) was measured using the following formula LRWC (%) = [(FW - DW)/ (TW - DW)] * 100 (Goal et al., 2011).

5.3.3. Electrolyte leakage (EL)

Electrolyte leakage, an indicator of membrane damage, was calculated using one leaf disc (1 cm diameter) collected from the upper most fully-expanded leaf tissues. The leaf discs were then rinsed with deionized water and incubated in 20 ml of deionized water on a shaker for 6 h. The electrical conductivity of this solution (EC1) was measured using a conductivity meter (Fisher Scientific, ON, Canada). To measure the total electrolytes, discs were boiled for 15 min and incubated at room temperature; the

conductivity was measured (EC2) after 5 h. The results were presented as percentage of electrolyte leakage (EL %) = (EC1/EC2) x 100 (Sullivan and Ross, 1979).

5.3.4. Free proline content

The free proline content was determined in fully-expanded leaves (stored at -80°C) according to Bates et al. (1973). One gram fresh tissue was homogenized with 10 ml sulphosalicylic acid (3%), and centrifuged at 4900 x g for 5 min. One ml extract was mixed with 1 ml of glacial acetic acid and 1 ml ninhydrin reagent (3% (w/v) ninhydrin in 60% (v/v) 6 M phosphoric acid) in a 5 ml glass tube. After 1 h incubation at 100°C, the tubes were cooled for 10 min using an ice bath to stop the reaction. Following addition of 3 ml of toluene, the absorbance of the upper phase was determined at 520 nm. Proline concentration was determined using a calibration curve (0- 0.250 µmol/ml proline) and expressed as µmol proline g^{-1} FW.

5.3.4. Sodium and chloride analysis

To determine Na^+ and Cl^- content in both species after salt treatment, lyophilized leaves and roots were ground separately using a coffee grinder (Black & Decker, Brockville, ON Canada). The Na^+ content in plant tissues was analyzed using a Direct Current Plasma (DCP) spectrophotometer. The Na^+ analyses were performed by Stratford Agri-Analysis (Stratford, ON, Canada). For Cl^- determination, 50 mg of ground tissues were extracted with 0.5 M HNO_3 for 30 min (Rieger and Litvin, 1998). Chloride was determined using a chloride selective electrode (Accumet, Fisher Scientific, Canada) (Sabra et al., 2012).

5.3.5. Determination of NO_3^- and NH_4^+

Nitrate determination in roots was performed as described by Meng et al. (2016). A total of 100 mg of dry tissues was mixed with 2 ml of deionized water and incubated in a water bath for 1h at 45 °C. The mixture was centrifuged at 5000 x g for 15 min. The supernatant (0.2 ml) was mixed with 0.8 ml of 5% (w/v) salicylic acid in concentrated H_2SO_4 and incubated for 20 min at room temperature. To raise the pH to above 12, a total of 19 ml of 2 M NaOH was added and the absorbance of the sample was read at 410 nm. Nitrate concentration was determined using a calibration curve with KNO_3.

The NH_4^+ content in the roots was measured spectrophotometrically as previously described by Weatherburn (1967). Ammonium was extracted from 100 mg of plant material with 3 ml of 0.3 mM sulphuric acid (pH 3.5). The mixture was centrifuged at 39000 x g for 10 min. One-hundred µl of the supernatant were diluted to a final volume of 4 ml using 0.3 mM sulphuric acid. To get the colour reaction, 0.5 ml of solution A (25 mg sodium nitroprusside and 5 g phenol dissolved in 100 ml deionized water) and 0.5 ml of solution B (2.5 g NaOH and 40 ml 5% sodium hypochlorite were mixed, and deionized water was added to a final volume of 100 ml) were added. The absorbance was measured at 625 nm after incubation in a water bath at 37 °C for 20 min, and NH_4^+ contents were estimated using ammonium sulfate standard.

5.3.6. RNA extraction and analysis

Total RNA was extracted from root tissues stored in RNAlater® using CTAB (Cetyltrimethyl ammonium bromide) extraction buffer as described in Lelièvre et al. (1997) under RNase-free conditions. RNA samples were treated with DNase (DNase 1, Invitrogen, Carlsbad, CA, USA), prior to the synthesis of cDNA to remove any traces of

genomic DNA. Following DNase treatment, RNA was tested for DNA contamination by polymerase chain reaction employing the primer pair actinF/actinR (Table 5.1) targeting the *actin* gene. First-strand cDNA was synthesized from 1 µg of total DNased RNA using iScript cDNA synthesis kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions. The quality of cDNAs generated from all samples was tested by a PCR assay employing the primers actinF/actinR, where each reaction should produce a single, equally strong band after gel-separation and UV visualization. All primers to be employed in quantitative real-time PCR targeting ammonium transporters (*AMT1.1* and *AMT1.2*), nitrate transporters (*NRT1.1, NRT1.2, NRT2.1* and *NRT2.3*), glutamine synthetase 1 (*GS1*) and nitrate reductase (*NR*), were tested for the appropriate annealing temperature (50 °C- 60° C). PCR products for each employed primer pair produced single amplicon of the predicted size using the imager Biorad Versadoc 4000 Mp and Image Lab^{TM} 3.0 software. Primer pairs, the respective employed annealing temperature as well as the product size are given in Table 5.1. To verify correctness, PCR products were sequenced (Robarts Research Institute, London, Ontario, Canada). In order to assess absolute quantity of mRNA, a standard curve was performed, consisting of a dilution series of known quantities (100 fg- 0.001 fg cDNA) of the respective gel purified PCR product (QlAquick Gel Extraction Kit, Qiagen). For the standard curve, a minimum R^2 value of > 0.98 was demanded. Quantitative real-time PCR (Bio-Rad, Mississauga, ON, Canada) analysis was carried out in 15 µl of the reaction mixture containing cDNA transcribed from 50 ng total DNased RNA, 1 µmol l^{-1} of each primer and SSO FastEvaGreen Supermix (Biorad). Melting curve analysis of the amplification products was performed to confirm that the primers amplified a single product. For normalizing

the qRT-PCR reaction, the endogenous *actin* gene was used as a constitutive internal control (Ghanem et al., 2011).

5.3.7. Data analysis

All data were subjected to a two-way analysis of variance (NaCl treatments and species), and Tukey's HSD test was used to determine the significant differences between means at a probability level of ≤0.05. As gene expression data were not normally distributed, they were log-transformed prior to conduct statistical analysis to approach normal distribution. For absolute mRNA level, data were subjected to a one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$).

5.4. Results

5.4.1. Physiological responses to salt stress in S. pennellii and S. lycopersicon

The survival rate (100%) of both species was not affected by 100 and 200 mM NaCl after one week of treatment; while under 300 mM NaCl, the survival rate of *S. lycopersicon* was reduced significantly by 75% with no significant effect observed in S. *pennellii* (Fig. 5.1). Plant growth was reduced after one week of exposure to 100 mM NaCl in both species, with a more pronounced effect in *S. lycopersicon* (Table 5.2). *Solanum lycopersicon* showed a reduction in shoot and root biomass (ca. 35% and 22%, respectively) under salt treatment, while in *S. pennellii*, the shoot biomass showed less reduction (ca. 21%) and no significant change in root biomass (Table 5.2). A similar trend was observed for leaf area, where salt treatment reduced the leaf area of both species, with a more pronounced decrease (ca. 39%) in *S. lycopersicon* (Table 5.2). The physiological responses varied depending on the salt tolerance of the two species. In *S. lycopersicon*, relative water content showed a decrease of 25% after one week of salt

stress, whereas the salt-stressed *S. pennellii* was able to maintain a leaf relative water content comparable to control plants (Table 5.2). Salt stress increased electrolyte leakage in the leaves of both species (Fig. 5.2A); however the increase was more severe in the salt sensitive species, *S. lycopersicon* (ca. 48%) than in *S. pennellii* (ca. 36%). The level of proline in leaf tissues following exposure to NaCl increased by 4.71 fold in *S. lycopersicon* while it was only increased by 2.84 fold in *S. pennellii* (Fig. 5.2B).

5.4.2. Elemental analysis

Sodium and Cl^- content increased in both leaf and root tissues after salt treatment in the salt- tolerant and salt-sensitive species (Table 5.3). In leaves, the accumulation of Na^+ was significantly higher in *S. pennellii* (26.53 fold) than in *S. lycopersicon* (22.4 fold) (Table 5.3). However, in roots, the accumulation of Na^+ was higher in *S. lycopersicon* (31.2 fold) than in *S. pennellii* (21.07 fold) (Table 5.3). For both leaves and roots, the concentration of Cl^- was higher in *S. pennellii* (fold 22 for leaves and 17 fold for the roots) than in *S. lycopersicon* (14 fold for leaves and 12.9 fold for the roots). In control conditions, NO_3^- content of roots was higher in *S. lycopersicon* than in *S. pennellii* (Table 5.4). After salt treatment, NO_3^- content was decreased significantly in roots of both species, without significant differences between species. However, NH_4^+ content was increased in the roots of *S. lycopersicon* after salt stress and *S. pennellii* showed no change compared to control plants (Table 5.4).

5.4.3. Expression of AMTs and NRTs in roots

Transcripts of all the selected *AMTs* and *NRTs* were detected in root tissues of both species. However, the transcript abundance (absolute mRNA level) of the tested transporters in roots showed remarkable differences in both species (Tables 5.5 and 5.6).

Among the six N transporters genes, *AMT1.2* and *NRT1.2* exhibited the highest transcript abundance in *S. lycopersicon,* while in S. *pennellii*, only *AMT1.2* displayed the highest transcript abundance. *NRT2.3* exhibited the lowest transcript abundance in both species (Tables 5.5 and 6). Salt stress increased the mRNA level of the high affinity *AMT1.1* in *S. pennellii* after 1 and 7 days compared to control conditions (Fig. 5.3), while in *S. lycopersicon* a similar increase occurred only after 7 days of salt treatment (Fig. 5.3). The mRNA level of *AMT1.2* was increased (ca. 80 %) after 1 day of salt stress and reduced (ca. 85 %) with the prolonged salt exposure (7 days) in *S. lycopersicon*, whereas *AMT1.2* was not affected by salt stress in *S. pennellii* after the same salt-exposure period (Fig. 5.3). After 1 and 7 days of salt stress, the mRNA level of low affinity nitrate transporters (*NRT1.1* and *NRT1.2*) decreased remarkably in both species with the exception of 1 day of salt treatment for *NRT1.2* in *S. lycopersicon* (Fig. 5.4). For the *NRT1.1* gene, the decrease was significantly more pronounced in *S. pennellii* (ca. 81 % and 78% reduction after 1 day and 7 days, respectively) than in *S. lycopersicon* particularly after 7 days of treatments (ca. 70% and 34% reduction after 1 day and 7 days, respectively). The mRNA level of high affinity nitrate transporter *NRT2.1* appeared unchanged after 1 day and 7 days of salt treatment in *S. lycopersicon*, whereas in *S. pennellii* a significant decrease was recorded after 7 days of salinity stress conditions (Fig. 5.4). *NRT2.3* was down-regulated in salt-stressed *S. lycopersicon* after 7 days only (Fig. 5.4); while this gene showed no significant change after 1 and 7 days in *S. pennellii* (Fig. 5.4).

5.4.4. Expression of GS1 and NR genes in roots

The results showed that salt stress induced a down-regulation of *GS1* in *S. lycopersicon* (ca. 30% reduction) only after 7 days of NaCl treatment (Fig. 5.5), while no change occurred in the salt tolerant species, *S. pennellii* (Fig. 5.5). After 1 day of salt stress, mRNA level of *NR* was reduced significantly in *S. lycopersicon* by 31% and in *S. pennellii* by 30%, while after 7 days, the reduction in the mRNA level of *NR* was 68 % in *S. pennellii* and 51% in *S. lycopersicon* (Fig. 5.5).

5.5. Discussion

5.5.1. Salt tolerance was related to genotypic variation

Our results confirmed findings from earlier studies (Bolarin et al., 1995; Santa-Cruz et al., 1999) showing that the glycophyte *S. lycopersicon* was more affected by salinity than the halophyte *S. pennellii*. Although no plant death occurred at NaCl concentrations of 100 and 200 mM, at 300 mM NaCl the survival rate of *S. pennellii* was higher than that of *S. lycopersicon*. Under salt stress, *S. pennellii* maintained a higher biomass than *S. lycopersicon* as indicted by shoot DW, root DW and leaf area. The ability of *S. pennellii* leaves to maintain a relative water content similar to control plants during salinity stress suggests a typical halophytic mechanism involving maintenance of relatively high solute concentration for osmoregulatory purposes (Santa-Cruz et al., 1999). There is evidence that osmotic adjustment in *Solanum* species is achieved by both organic and inorganic solute accumulation such as Na^+ and Cl^- (Torrecillas et al., 1994). In our study, Na^+ and Cl^- accumulation in leaf tissues were lower in the salt-sensitive species, *S. lycopersicon*, than in the salt tolerant species, *S. pennellii*, after salt exposure;

a result which is in agreement with those obtained previously by Bolarín et al., (1995). Furthermore, Torrecillas et al., (1994) indicated that uptake of inorganic solutes by *S. pennellii* prevailed over the synthesis of organic solutes, confirming the ability of halophytes to use ions, such as Na^+ and Cl^-, for osmoregulation, an energy saving mechanism for this species. Although salt stress increased proline accumulation in many plant species including *Solanum* species (Popova et al., 2003; Sun et al., 2010), the role of proline in osmotic adjustment is still a matter of debate (Blum et al., 1996; Ehlting et al., 2007). The lower accumulation of proline in the wild salt-tolerant tomato species compared to the salt-sensitive cultivated species reported in this paper had previously been described in other studies (Bolarín et al., 1995, Sun et al., 2010). Therefore, the higher level of proline in *S. lycopersicon* may confirm that this species was more affected by salinity.

Excessive Na^+ can induce the accumulation of ROS that triggers the oxidative damage of lipid membranes, thereby resulting in the disruption of the membrane integrity and inducing the leakage of solutes (Parida and Das, 2005). Electrolyte leakage was reported as an appropriate index of the membrane stability during salt stress (Li et al., 2010). In the current study, although *S. pennellii* accumulated higher levels of Na^+ in leaf tissues than *S. lycopersicon*, the membrane integrity (as indicated by electrolyte leakage) was less impaired by NaCl in the salt-tolerant species *S. pennellii* than in the salt-sensitive species *S. lycopersicon*. These findings suggested that *S. pennellii* cells may be protected from the oxidative damage induced by Na^+ accumulation through a sufficient sequestration mechanism of Na^+ into the vacuole (Yamaguchi and Blumwald, 2005) and/or the activation of multiple antioxidant enzymes such as superoxide dismutase

(SOD) and ascorbate peroxidase (APX) (Shalata and Tal, 1998). Overall, in our study, variation of the physiological parameters among both species indicated that *S. pennellii* is more adapted to salt stress than *S. lycopersicon.*

5.5.2. Levels of mRNA for N transporter genes were modified by salt stress

Our results showed that salt stress had different effects on *AMT* mRNA levels in *S. lycopersicon* and *S. pennellii,* strongly suggesting that each species might have different NH_4^+ uptake mechanisms under salt stress. In *S. pennellii,* the earlier up-regulation of *AMT1.1* and the maintenance of the mRNA level of *AMT1.2* (the predominant gene for this species) similar to the control values after one week of salt stress might help to facilitate N uptake.

During salinity stress, NH_4^+ uptake over NO_3^- has been suggested for several plant species (Flores et al., 2001; Zhang et al., 2014). In *Populus simonii,* net NH_4^+ flux was higher than NO_3^- flux in fine roots after acclimation to salt stress (Zhang et al., 2014). This preference of NH_4^+ uptake during salt stress was associated with up-regulation of mRNA levels of 13 *AMT* genes in the root tissues of this species. NH_4^+ uptake by the roots have been associated with energy saving for N assimilation (Bloom et al., 1992). When NH_4^+ is assimilated in the root tissue, the overall energy cost for growth is lowered by 8-12% as compared to NO_3^- (Raven, 1985; Salsac et al., 1987). In a previous study on *S. lycopersicon,* the limitation of the deleterious effect of salinity stress on biomass production due to changes in the NO_3^-: NH_4^+ ratio from 14:0 to 12:2 or 10:4 supports the idea that NH_4^+ uptake during salt stress reduces the energy requirement for growth (Flores et al., 2001). Such a saving of energy that goes along with NH_4^+ uptake

might lead to a better distribution of energy required for salt tolerance mechanisms and to promote plant growth during salt stress (Kant et al., 2007).

In the present study, the mRNA levels of low affinity nitrate transporters *NRT1.1* and *NRT1.2* were reduced in the roots of both species after salt treatment. Yao et al., (2008) found that NaCl reduces the mRNA levels of *NRT1.1* and *NRT1.2* in young *S. lycopersicon* roots, suggesting that the *NRT1* subfamily is responsible for the reduction in nitrate uptake under salt stress. However, Popova et al., (2003) reported that the mRNA level of the halophyte *M. crystallinum McNRT1* was stimulated in the roots in response to salinity stress. The different expression pattern of *NRT1* in response to salt stress might vary due to different experimental conditions. In addition to their roles in NO_3^- uptake, members of both NRT1 and NRT2 families in *Arabidopsis* were proposed to act also as NO_3^- sensors or signal transducers to mediate the development of the root system (Little et al., 2005). Our results showed that a reduction in mRNA level of *NRT2.1* occurred only in the salt-tolerant species, *S. pennellii* during salt treatment. *NRT2.1* was previously reported as a repressor of lateral root initiation (Little et al., 2005). Therefore, down-regulation of this gene during salt treatment may be associated with better root growth in *S. pennellii* than in *S. lycopersicon*. In a previous comparative transcriptomic study, the mRNA level of *NRT2.1* was repressed strongly in the wild halophytic tomato species, *Solanum pimpinellifolium* 'PI365967' while it remained unchanged in *S. lycopersicon* 'Moneymaker' (salt-sensitive) in response to salt stress, suggesting a contribution of *NRT2.1* to better growth of salt-stressed root in this species (Sun et al., 2010). However, further reverse genetic studies are needed to understand how *NRT2.1* is related to salt tolerance and root development during salt stress. Among 6 members of the *AMT* and

NRT gene family that are expressed in roots of both tomato species at different abundance, *NRT2.3* was expressed at the lowest level, and is thus presumably the least important transporter in roots.

5.5.3. Salinity affected NO_3^-, NH_4^+ and N assimilation genes

Nitrate reductase (NR) and glutamine synthetase (GS) are key enzymes responsible for N assimilation (Masclaux-Daubresse et al., 2010). Nitrate is reduced to nitrite by NR and then to NH_4^+ by nitrite reductase (NiR). Then NH_4^+ is incorporated into organic amino acid by GS and glutamate synthase (Fd-GOGAT and NADH-GOGAT) or in an alternative pathway, by glutamate dehydrogenase (GDH) (Shi et al., 2010). Many studies have reported that salt stress can inhibit the uptake of NO_3^- due to transport competition between Cl^- and NO_3^-, with resulting decreases in cell NO_3^- content and NR activity (Flores et al., 2000; Debouba et al. 2006). In our study, the salt-stressed roots of both species showed a down-regulation of *NR* and a reduction in NO_3^- content, which seems to be associated with lower mRNA level of *NRT1.1* and *NRT1.2.* These results indicate that *NRT1.1* and *NRT1.2* may have contributed to the reduction of NO_3^- content in roots observed in both species during salt stress. In control conditions, the observed lower NO_3^- content in the wild species *S. pennellii* roots compared to the cultivated species *S. lycopersicon* may be explained by the adaptation of both genotypes to their native environment. The proportion of NO_3^- and NH_4^+ absorbed by tomato species adapted to different native soils is known to vary (Smart and Bloom 1988).

The cytosolic isoform of GS (GS1) is involved in the re-assimilation of NH_4^+ released from increased amino acid catabolism during stresses (Bernard and Habash,

2009). In our study, the salt-sensitive species, *S. lycopersicon*, accumulated higher amount of NH_4^+ in roots after 7 days of salt treatment, in parallel to a slight but significant reduction in *GS1* transcript level. Such a difference between salt-tolerant and salt-sensitive species has previously been obtained in a study in mulberry leaves (*Morus alba*) where the salt-sensitive species showed higher NH_4^+ content coupled with a lower activity of GS (the first enzyme of NH_4^+ assimilation pathway) compared to the salt-tolerant species under salt stress (Surabhi et al., 2008). Therefore, our results suggest that variation in NH_4^+ content could also be related to the differences in the stress tolerance between salt-tolerant and salt-sensitive species. In addition, the elevated level of NH_4^+ in the salt-stressed tissues of salt-sensitive species (considered as toxic symptoms of salt stress) have previously been reported in different plant species and largely resulted from the inhibition in NH_4^+ assimilation enzymes and enhanced proteolysis rather than from an increased NH_4^+ uptake (Feng and Barker, 1992; Debouba et al. 2006; Surabhi et al. 2008).

5.6. Conclusion

The difference in salt-tolerance between wild salt-tolerant tomato (*S. pennellii*) and cultivated tomato (*S. lycopersicon*) has been exploited to elucidate the molecular responses of N transporters in roots upon salt stress. Our comparative study provides evidence that in contrast to *S. lycopersicon*, salt tolerance in *S. pennellii* was coupled with relative higher mRNA levels of ammonium uptake genes (*AMT1.1* and *AMT1.2*) and lower salt-induced inhibition of ammonium assimilation (mRNA level of GS). On the other hand, the decrease in the mRNA level of key transporters responsible for NO_3^- uptake (*NRT1.1* and *NRT1.2*) and N assimilation (*NR* gene) observed in both species, is

likely to have played a role in the salt stress-induced reduction in plant growth. Further research might be required to determine if the salt tolerance of *S. pennellii* could have resulted in the higher level of ammonium transporters and assimilation genes or if, to the opposite, the relatively higher level of ammonium transporters and assimilation genes could have partially contributed to the salt tolerance in *S. pennellii.* Nevertheless, findings from this study may pave the way to develop strategies (e.g. genetically modified crops and marker-assisted breeding) to increase nitrogen uptake in saline soil and improve salt tolerance in tomato cultivars. Wild salt-tolerant species, possessing multiple physiological traits for salt tolerance, can serve as valuable source of material for identifying salt-tolerance genes for breeding purposes.

Table 5.1. Primer sequences employed in qPCR. F: forward primer, R: reverse primer.

Gene	GenBank accession no.	Nucleotide sequence (5′ ➔ 3′)	Annealing T (°C)	Product size (bp)
AMT1.1	X92854.1	*AMT1.1* F: CGTGGACATAGCGCGTC *AMT1.1* R: CAGCGCTCCATTGACCA	55	139
AMT1.2	NM_001247324	*AMT1.2* F: TTCTGGTCCGGTGATGGTTG *AMT1.2* R: CCGATTGGTGGTCCTTCGAT	58	155
NRT1.1	X92853.1	*NRT1.1* F: ATCCAGCGTGTCCGGGTTC *NRT1.1* R: CCATAACCCCATTCTCTTCCC	60	174
NRT1.2	X92852.1	*NRT1.2* F: GGTGGTTGGGCAAGTGGT *NRT1.2* R: CCTTCCAAGGAAAGTGTCAGC	60	210
NRT2.1	NM_001247205.1	*NRT2.1* F: TCATGTTGTCAGCGCCAACTG *NRT2.1* R: TATTACCCCATCCAGCAGCCG	60	186
NRT2.3	NM_001247198.1	*NRT2.3* F: GGAGGGTACGTTGCTGTGAGG *NRT2.3* R: TGCCCCTGCTCTACGTATT	60	201
NR	X14060.1	*NR* F: CGGTTCGTGGTTGCAACTTC *NR* R: CCAATTATCAGCGGTACCTTC	60	213
GS1	U14754.1	*GS1* F: GGCTATTGAGAAGCTTGGCT *GS1* R: TATCCCTTGCCTGCCTTCTCT	58	168
Actin	AB695290	*Actin* F:GAAATAGCATAAGATGGCAGACG *Actin* R: ATACCCACCATCACACCAGTA	55	160

Table 5.2. Shoot and root dry weight (DW), leaf relative water content (LRWC) and leaf area of *S. lycopersicon* and *S. pennellii* plants exposed to 0 or 100 mM NaCl for 7 days. Values are the means of 6 replicates ± S.E. Different letters show significant differences between means in the same row at $p \leq 0.05$.

	S. lycopersicon			*S. pennellii*		
	0 mM NaCl	100 mM NaCl	Change (%)	0 mM NaCl	100 mM NaCl	Change (%)
Shoot DW (g)	2.49±0.06 [a]	1.61±0.06 [b]	-35.53	0.97±0.02 [c]	0.76±0.03 [d]	-21.37
Root DW (g)	0.24±0.01 [a]	0.19±0.01 [b]	-22.39	0.21±0.01 [ab]	0.20±0.01 [b]	-5.06
Leaf area (cm^2)	412 ±12 [a]	251±7[b]	-39.20	160±6 [c]	129±4 [d]	-19.29
LRWC (%)	80.99±0.99 [a]	60.75±0.83 [b]	-24.99	78.23±1.66 [a]	76.69±20 [a]	-1.97

Table 5.3. Na and Cl content (%) in leaf and root tissues of *S. lycopersicon* and *S. pennellii* plants, grown with either 0 or 100 mM NaCl for 7 days. Values are the mean of 4 replicates ± S.E. Different letters show significant differences between means in the same row at $p \leq 0.05$.

	S. lycopersicon		*S. pennellii*	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Leaf Na	0.11±0.03 ^{c}	2.47±0.01 ^{b}	0.15±0.005 ^{c}	3.98±0.01 ^{a}
Root Na	0.10±0.01 ^{c}	3.12±0.02 ^{a}	0.14±0.03 ^{c}	2.95±0.03 ^{b}
Leaf Cl	0.24±0.04 ^{c}	3.39±0.26 ^{b}	0.24±0.030 ^{c}	5.31±0.25 ^{a}
Root Cl	0.32±0.02 ^{c}	4.24±0.26 ^{b}	0.31±0.027 ^{c}	5.40±0.45 ^{a}

Table 5.4. NO_3^- and NH_4^+ ($mg.g^{-1}$ DW) content in root tissues of *S. lycopersicon* and *S. pennellii* plants, grown with either 0 or 100 mM NaCl for 7 days. Values are the mean of 4 replicates ± S.E. Different letters show significant differences between means in the same row at $p \leq 0.05$, within one species.

	S. lycopersicon		*S. pennellii*	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
NO_3^-	1.86±0.28 ^{a}	0.48±0.07 ^{b}	0.21±0.06 ^{c}	0.036±0.008 ^{d}
NH_4^+	0.20±0.01 ^{b}	0.29±0.02 ^{a}	0.13±0.01 ^{c}	0.15±0.012 ^{c}

Table 5.5. Absolute mRNA levels *(*fg cDNA /50 ng total RNA) of ammonium transporters (*AMT1.1* and *AMT1.2)* and nitrate transporters (*NTR1.1, NRT1.2, NRT2.1* and *NRT2.3*) in root tissues of *S. lycopersicon* plants, grown with 0 mM NaCl, at 1 and 7 days (n=4- 5). Different letters show significant differences between different genes at each time point.

Genes	1 day	7 days
AMT1.1	2.07±0.38 [d]	1.14±0.26 [c]
AMT1.2	26.77±3.30 [a]	29.48±2.71 [a]
NRT1.1	7.75±0.58 [b]	4.40±0.45 [b]
NRT1.2	21.93±2.01 [a]	29.19±3.39 [a]
NRT2.1	4.54±0.46 [c]	1.63±0.17 [c]
NRT2.3	0.14±0.02 [e]	0.02±0.01 [d]

Table 5.6. Absolute mRNA levels *(*fg cDNA /50 ng total RNA) of ammonium transporters (*AMT1.1* and *AMT1.2)* and nitrate transporters (*NTR1.1, NRT1.2, NRT2.1* and *NRT2.3*) in root tissues of *S. Pennellii* plants, grown with 0 mM NaCl, at 1 and 7 days (n=3- 5). Different letters show significant differences between different genes in each time point.

Genes	1 day	7 days
AMT1.1	0.90±0.07 [c]	1.16±0.12 [c]
AMT1.2	25.70±3.00 [a]	11.90±1.00 [a]
NRT1.1	5.14±0.50 [b]	1.33±0.12 [c]
NRT1.2	5.54±0.60 [b]	6.90±0.60 [b]
NRT2.1	7.25±0.79 [b]	6.00±0.54 [b]
NRT2.3	0.10±0.02[d]	0.05±0.01 [d]

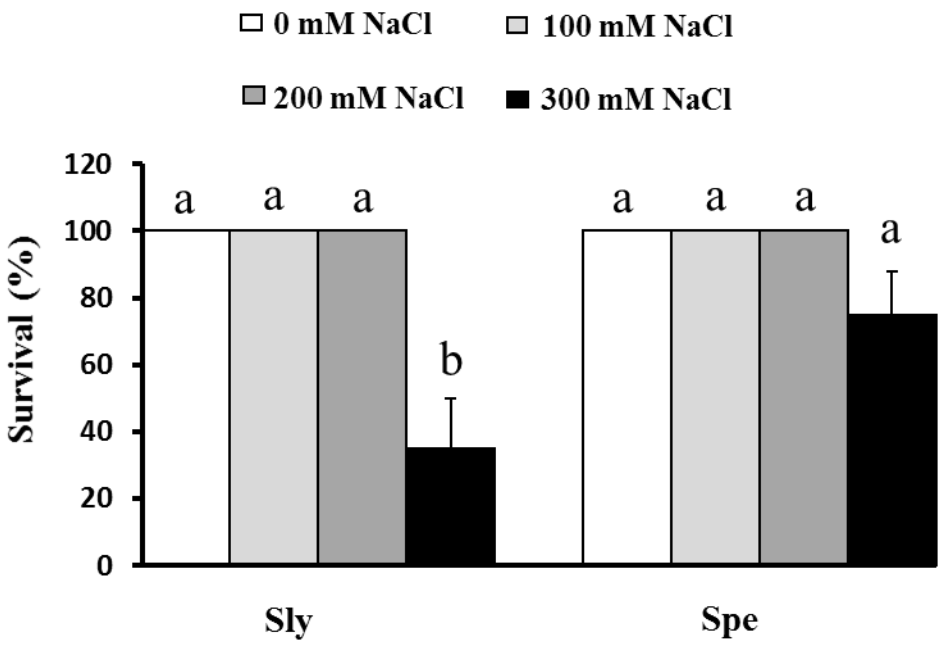

Fig. 5.1. Survival rate (%) of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants treated with 0, 100, 200 and 300 mM NaCl for 7 days. Bars are the mean ± SE of four replicates. Means are considered different at $p \leq 0.05$ when followed by different letters.

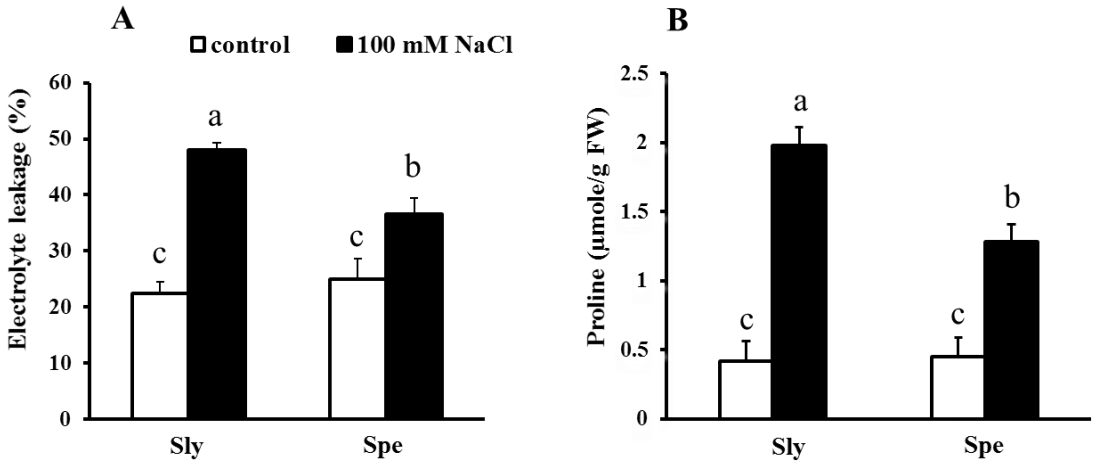

Fig. 5.2. Electrolyte leakage (A) and proline accumulation (B) of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants treated with 0 or 100 mM NaCl for 7 days. Values are the means of 6 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same parameter.

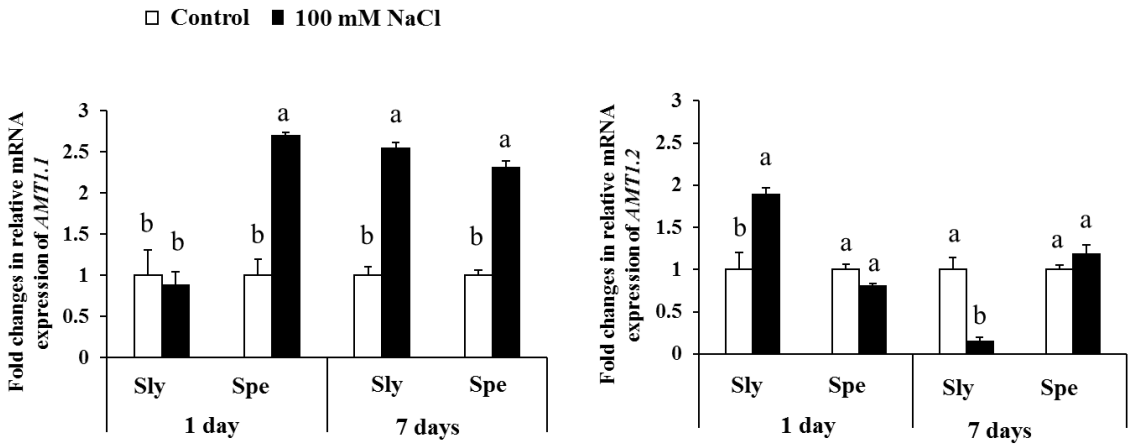

Fig. 5.3. Fold changes in relative mRNA levels of ammonium transporters (*AMT1.1* and *AMT1.2)* in root tissues of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants, grown with either 0 or 100 mM NaCl for 1 or 7 days. The ratio of absolute mRNA levels of the target gene and the *actin* gene was calculated and relative mRNA levels of roots tissues from control plants were set as 1. Data represent means ± SE, n =4- 5. Different letters represent significant differences between means for each date at $p \leq 0.05$. Absolute mRNA expression levels for both species under control conditions are presented in Tables 5.5 and 5.6.

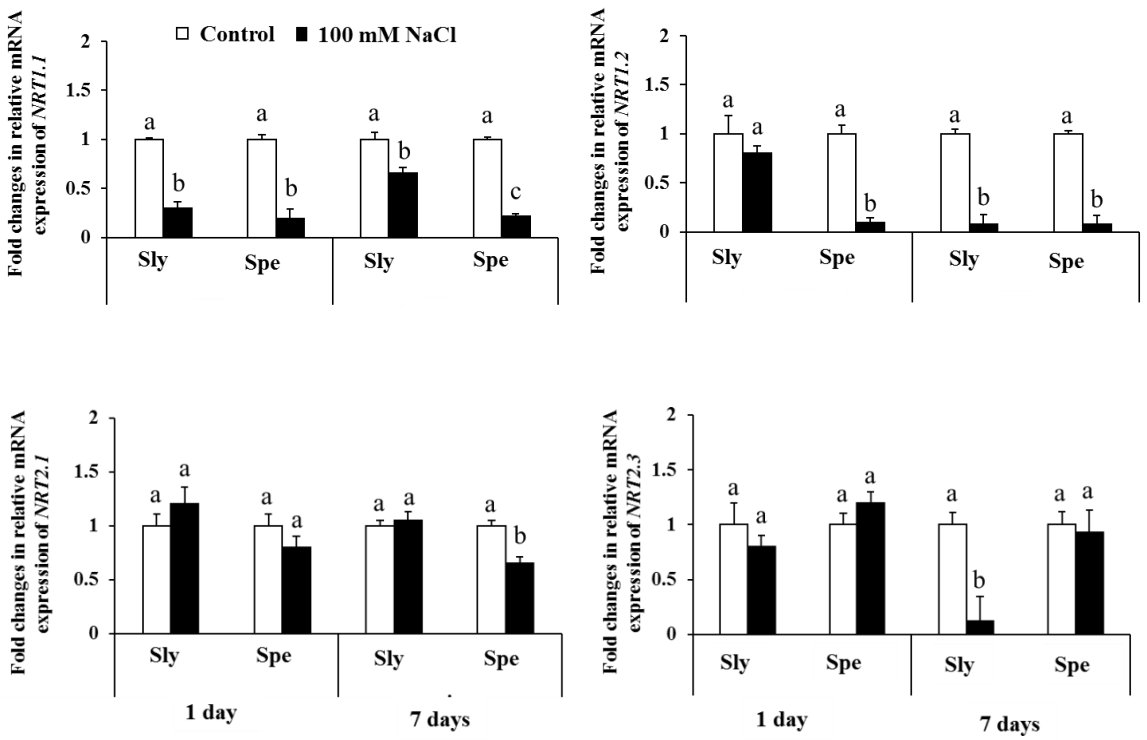

Fig. 5.4. Fold changes in relative mRNA levels of nitrate transporters (*NRT1.1, NRT1.2, NRT2.1* and *NRT2.3*) in root tissues of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants, grown with either 0 or 100 mM NaCl for 1 or 7 days. The ratio of absolute mRNA levels of the target gene and the *actin* gene was calculated and relative mRNA levels of root tissues from control plants were set as 1. Data represent means ± SE, n =4- 5. Different letters represent significant differences between means for each date at $p \leq 0.05$. Absolute mRNA levels for both species under control conditions are presented in Tables 5.5 and 5.6.

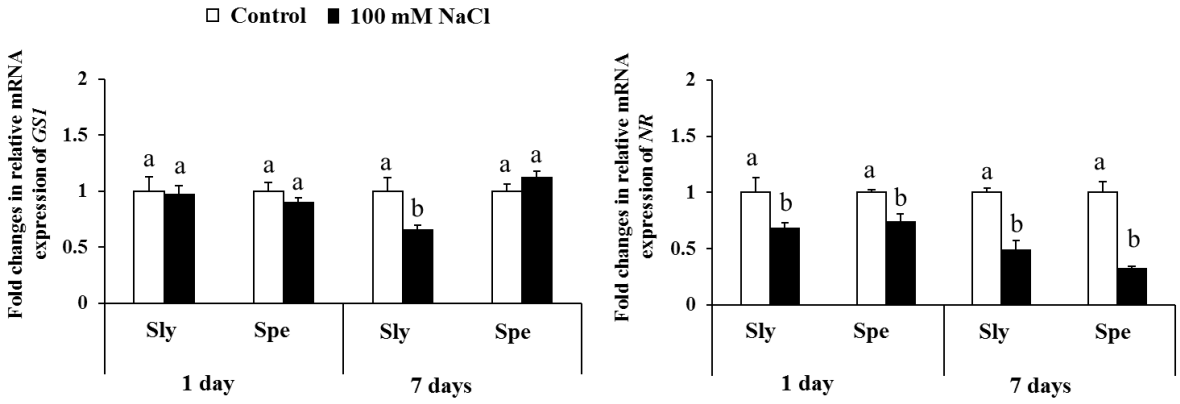

Fig. 5.5. Fold changes in relative mRNA levels of N-assimilation enzymes including glutamine synthetase (*GS1*) and nitrate reductase (*NR*) in root tissues of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants, grown with either 0 or 100 mM NaCl, for 1 and 7 days. The ratio of absolute mRNA levels of the target gene and the *actin* gene was calculated and relative mRNA expression levels of root tissues from control plants were set as 1. Data represent means ± SE, n = 4- 5. Different letters represent significant differences between means for each date at $p \leq 0.05$.

CHAPTER 6. SUMMARY AND CONCLUSIONS

Saline soil is a major abiotic stress that affects the growth and productivity of field crops worldwide (Munns and Tester, 2008). In the current thesis, the decision to use tomato to study salt tolerance was motivated by the agricultural value of this crop. In addition, the current advance on the tomato genome sequencing project enables tomato research to be advanced. Moreover, the availability of tomato plants bearing mutations in genes involved in phytohormone metabolism and wild species (halophytes) made this species very attractive to conduct stress physiology research. The knowledge from studies conducted on tomato which belongs to the *Solanaceae* family, could then be applied to the closely related commercially important crops such tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*) and peppers (*Capsicum annuum*). Overall, tomato can serve as a model plant for salt tolerance studies.

Salt tolerance in plants is achieved through both molecular and physiological changes (also known as salt tolerance mechanisms) that occur after the onset of stress. The first step in switching on such salt tolerance mechanisms is to perceive the salt stress through a signal transduction pathway (Munns and Tester, 2008). Salt stress signaling activates salt tolerance mechanisms via changes in the expression of many genes that encode osmolytes, antioxidants and different membrane transporters (Sairam and Tyagi, 2004; Parida and Das, 2005; Munns and Tester, 2008). Salt stress signaling mediated by JA and the roles of plasma membrane transporters such as AMTs in salt tolerance were two key areas of salinity stress that remained to be investigated to improve plant growth in saline soil.

Although the role of JA in salt stress signaling has been recognized (Walia et al., 2007; kang et al., 2005; Waternack, 2015), hardly any investigations have focused on the underlying mechanisms that trigger salt tolerance. In the first study (chapter 3), I studied the responses of wild-type (WT) tomato cv Castlemart and its JA-deficient mutant *defenseless-1* (*def-1*) under salt stress to determine the role of JA in salt tolerance. In a first step, the impairment of JA production in *def-1* was confirmed by the observed lower mRNA level of JA responsive genes compared to WT, under non-stressful conditions (*JAZ1*, *JAZ3*, *JAR1* and *PDEF1*) and following wounding stress (*PIN II*) (Appendix C, Fig. 8.1).

One of the most important findings in this study (chapter 3) were that *def-1* mutant showed higher levels of both H_2O_2 and MDA (biochemical marker for oxidative damage of cell plasma membranes), a slight decrease of photosynthetic pigments (Chl a and carotenoids) as well as a lower activity of both enzymatic antioxidants (glutathione reductase, glutathione-s-transferase and superoxide dismutase) and non-enzymatic antioxidants (indicated by the lower scavenging activity for DPPH) compared to WT plants when exposed to salt stress. These results support my hypothesis that the *def-1* mutant, clearly displayed oxidative stress as characterized by the ROS-associated injury phenotype during salt stress. In addition, *def-1* plants exhibited a more pronounced decrease of N content in both leaves and roots and a slightly higher level of Na in roots compared to WT plants. In the current study, I provide evidence for the role of endogenous JA in protecting the plants from salt-induced oxidative stress through the activation of both enzymatic antioxidants and non-enzymatic antioxidants. In fact, many studies have shown that JA has a positive role in the pathogen resistance (Waternack,

2007; Waternack, 2015). Therefore, JA seems to act as a signaling nexus for plant tolerance/defense responses. This knowledge of the positive contribution for JA to both biotic and salt stress could be useful for breeding of crops (e.g. overexpression of the JA biosynthetic genes) that maintain high productivity under diverse stressful conditions.

In the second study (chapter 4), prior to investigate the contribution of the root AMTs in salt tolerance, I identified the entire set of AMTs in the tomato genome and determine their tissue expression patters. The results showed that roots expressed multiple *AMT* members including members of the *AMT1* subfamily (*SlAMT1.1* and *SlAMT1.2*) and one member of the *AMT2* subfamily (*SlAMT2.1*). In different plant species (*Arabidopsis thaliana*, *Medicago truncatula*, *Marchantia polymorpha* and *Lotus japonicus*), AMT1 and AMT2 subfamilies are consistently found to transport the substrates, NH_4^+ and NH_3, respectively (Neuhäuser et al., 2009; Guether et al., 2009; Straub et al., 2014; McDonald and Ward 2016). This implies that the primary role of the AMT1 members could be to mediate NH_4^+ uptake in the outer root cells, where cation uptake is driven by the transmembrane voltage gradient. On the other hand, electroneutral transporters (AMT2.1) will transport NH_3 based on the transmembrane NH_3 concentration gradient. However, electrophysiological experiments on tomato AMTs are needed to confirm which chemical species (NH_4^+ or NH_3) are transported by those proteins.

In previous studies, the expression of the *AMT2.1* homologue from *Medicago truncatula* and *Arabidopsis* has been localized in the root outer cell layers (epidermis and cortex) and the root vascular tissues, respectively (Straub et al., 2014; Sohlenkamp et al., 2002). Therefore, it is likely that AMT2.1 mediates NH_3 efflux from rhizodermal and

cortical root cells and/or from root cells surrounding the xylem for loading into xylem vessels (long-distance root-to-shoot transport of NH_3). In fact, ions moving in the symplast stream need to move across the plasma membrane to enter the xylem for root-shoot transport. Multiple types of nutrient membrane transporters for nitrate, copper and boron have been found in the pericycle cells to mediate the loading of those ions in the root xylem (Takano et al., 2002; Lin et al., 2008; Deng et al., 2013). It is the first time such a function for plant AMT2.1 has been suggested. In order to confirm this hypothesis, knockdown or knockout mutants could be used to study the function of *SlAMT2.1* by measuring the NH_3 concentration in the xylem sap. In addition, fluorescence based in the situ hybridization (FISH) technique (Popova et al., 2003) could be used to visualize this transcript within root cells. Overall, the results presented in chapter 4, provides basic genomic and transcriptomic information for tomato AMTs. This knowledge is also needed for understanding N transport pathways under salt stress.

The difference in salt tolerance between the wild (naturally inhabiting saline environments) and domesticated tomato species was used to further investigate the contribution of *AMTs* to salt tolerance. In the third study (chapter 5), I compared salinity responses of the well-characterized salt-sensitive species, *S. lycopersicon* cultivar "Manitoba", and the halophyte wild species, *S. pennellii*, exposed to 100 mM NaCl for 1 and 7 days. The physiological responses and the mRNA expression levels of *AMTs* (*AMT1.1, AMT1.2* and *AMT2.1*), *NRTs* (*NRT1.1, NRT1.2, NRT2.1* and *NRT2.3*) and key genes encoding N assimilation enzymes (*GS1* and *NR*) in salt-stressed roots of both species have been investigated. This study confirmed that in comparison to *S. lycopersicon*, *S. pennellii* was more salt tolerant as evidenced by its higher germination

rate (Appendix D, Fig. 8.2), higher survival rate, lower biomass reduction, lower reduction in leaf relative water content and limited salt injury (determined by electrolyte leakage and proline accumulation) under salt stress conditions. In addition, *S. pennellii* accumulated higher levels of Na and Cl in leaf tissues than *S. lycopersicon*. This suggests that *S. pennellii*, as a halophyte plant, was able to use ions as inorganic solutes for osmoregulation, an energy saving mechanism for this species (Torrecillas et al., 1994). Hence, *S. pennellii* can serve as a model to study mechanisms of salt tolerance. In fact, in tomato there are 13 recognized wild species that show a large variety of phenotypes (e. g. abiotic stress tolerance and morphological structure) (kimura and Sinha, 2008). These wild tomatoes are potentially important not only for salt tolerance studies but also for plant breeding projects (as sources of desirable traits, by crossing with cultivated tomato) and plant evolutionary studies.

Although, there were no significant differences in total N reduction between both genotypes after one week of exposure to 100 mM NaCl (Appendix E, Table 8.2). My comparative study (chapter 4) showed that salt tolerance of *S. pennellii* was coupled with relative higher mRNA level of NH_4^+ uptake genes (*AMT1.1* and *AMT1.2*), which may facilitate NH_4^+ uptake in root cells of this species. In order to further advance the knowledge on the roles of AMTs in response to salinity stress, it would be interesting to analyze not only the mRNA levels, but also the ammonium uptake in both species using radiolabeled ^{15}N-ammonium ions. This will allow to validate our results on the role of AMTs in salt tolerance.

In the salt-stressed root for both species, the observed up-regulation of mRNA level for *AMT2.1* (Appendix F, Fig. 8.3) indicates that the transport of NH_3 was activated

under such conditions. In roots, according the two hypotheses I have proposed (chapter 4), that NH_3 might be loaded into the root xylem for the later assimilation in leaves or be effluxed from the root cells to avoid toxicity (chapter 4). Salt stressed roots of *S. lycopersicon* showed higher level of NH_4^+ content compared to control conditions. In such conditions, we could expect an activation of NH_3 efflux by root cells, mainly through AMT2.1. However, *S. pennellii* was able to maintain a NH_4^+ content in salt-stressed root similar to control conditions in spite of an increased level of *AMT2.1* in the presence of salt; making it difficult to draw any conclusions on the involvement of *AMT2.1* in ion efflux from root cells. Alternatively, *SlAM2.1* may contribute to NH_3 loading into xylem elements. In different plant species including tomato, the increase in NH_4^+ to NO_3^- ratio in the nutrient solution enhances the accumulation of nitrogen content in leaves under salt stress (Ben-Oliel et al., 2006; Kant et al., 2007). This suggests that the root-to-shoot transport of NH_4^+/NH_3 may be stimulated under salinity. On the other hand, the expression of key components of NO_3^- uptake (*NRT1.1* and *NRT1.2*) and assimilation (*NR* gene) were reduced in both species. Overall, the study provide information at the molecular level about the importance of ammonium, as a source of N, for tomato salt tolerance. Therefore, under saline soils, the N source may need to be supplemented as ammonium fertilizer to improve tomato growth. In fact, the ratio between ammonium and nitrate is of a great significance for salt tolerance. The most appropriate ratio to be applied varies with the plant growth stage and the pH of soil (Bloom et al., 1992; Flores et al., 2001). Future studies testing the involvement of AMT in salt tolerance with different ammonium and nitrate ratios under a range of pH and at

various plant growth stages would be required to fully understand the role of AMT in salt tolerance.

As described above, I have shown that JA signaling and *AMTs* are involved in tomato salt tolerance. In addition, others salt stress signaling molecules (e.g. Ca^{2+}, NO and ABA) and transporter proteins (e.g. NHX1, SOS1 and HKTs) were previously reported to regulate plant salt tolerance. Thus, it can be concluded that plants fine-tune salinity tolerance in a complex manner. Studying JA-signaling in isolation is valuable for our understanding of plant salt tolerance, but it is likely part of larger signaling networks. In fact, cross-talk can also occur between several salt-stress pathways. To date, the elements sharing JA-signaling during salt stress are largely unknown. To demonstrate those events, powerful tool such as RNA sequencing or microarrays could be used to generate transcriptome information on the responses of JA mutants such as *def-1* to salt stress. This will lead to a greater understanding of other potentially connected pathways, such as MAP kinase cascades and calcium-regulated portions. In addition, analyzing the levels of other plant hormones such Abscisic acid, salicylic acid and ethylene by HPLC (high performance liquid chromatography) in *def-1* would be also required to draw an overall picture of the role of JA in salt tolerance. For example, several lines of evidence demonstrate the interaction between ABA and JA at both biosynthesis and signaling levels during stomatal closure (Hossain et al., 2011). Stomatal closure diminish the water loss from the leaves during salt stress (Hassine and Lutts, 2010). Hence, the physiological role of JA in salinity stress-induced stomatal closure could be also an interesting point for future research. It has been shown that jasmonic acid acts as a master switch for a wide range of genes including nitrogen transporters (*AMTs* and *NRTs*) (Cho

et al., 2007). Interestingly, the results of chapter 3 provide evidence that JA may have a role in N homeostasis during salt stress. Therefore, future studies will also be required to investigate the involvement of nitrogen transporters in N homeostasis of salt-stressed JA mutants. This could be performed by monitoring the mRNA level of different N transporters (e.g. *AMTs* and *NRTs*) in roots of salt-stressed JA mutants and their corresponding WT plants. Although plant roots are equipped with different types of N transporters (e.g. *AMTs* and *NRTs*), my comparative study between *S. lycopersicon* and halophyte tomato *S. pennellii* provided information about the important role of *AMTs* in salt tolerance. More importantly, the function of *AMTs* in plant salt tolerance could be further confirmed by knockdown mutants and/or transgenic plants over-expressing those genes.

Overall, the results of the present thesis contribute substantially to our knowledge of signaling components and key genes that help with plant salt tolerance. This will provide the potential to develop for new strategies using the different means of molecular breeding (e.g. genetically modified crops and marker-assisted breeding) to improve the salt tolerance of crop plants.

CHAPTER 7. LITERATURE CITED

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8. APPENDICES

Appendix A: DNA sequences of tomato *AMT2*-type genes identified in chapter 4.

SlAMT4.1 **(Solyc09g065740)**

ATGGCATACTTGTACTTGCCTAGAAATCATCAACCAGATGATTCAAATCCCCAATGGATGAAC
AAAGCTGACAATGCTTGGCAATTGACTTCAGCCACCCTCGTTGGCCTTCAAAGTGTTCCAGGT
CTTGTTATCCTTTATGGTAGCATTGTGAAGAAGAAATGGGCTCTCAACTCGGCGTTGATGGCC
CTTTACGCCTTTGCAGCAGTCCTTGTTTGTTGGGTTGGCTGGGGATACCAATTAGCCTTTGGTG
ACACATTAGTTCCATTTCTTGGAAAAACAAATTTTGATGCATTGGAACAAAAGTTCCTAACGA
GTCAAGCATTTGTTGGCAAGTTTCCAAATGCCTCAATGGTCTATTTCCAGTTTGTATTCGCGGC
GATCACTTTGATTTTAATAGCTGGTGCCCTGCTCGGGAGGATGAATTTCATCGCGTGGATGTTG
TTTGTTCCTCTTTGGCTTACTTTTTCATATACAGTAGGTGCATTTAGTATCTGGTGCCCTCAAGG
TTGGCTGTTCAGGATGGGAGTGATTGACTATTCGGGAGGTTTTGTTATTCACCTCTCTTCTGGT
GTTGCTGGTTTCACTGCTGCATATTGGGTTGGACCAAGGGCACCTAGAGACAGGGAGAGGTTT
CCACCAAATAACATATTGTTAATGTTGGCTGGTGCTGGCATTCTATGGATGGGATGGACAGGA
TTCAACGGCGGTGATCCATATGTAGCTAGCCTTGATGCATCCTTAGCCGTCCTTAACACGCAT
GTTTGTGCTGCAATGAGCTTGCTAACTTGGCTAATGTTAGACATTCTCTTCTATGAAAAGCCTT
CCGTCATCGGTGCTACCCAAGGGATGATCACTGGCTTAGTTTGCATTACGCCTGCAGCAGGGG
TGGTGCAAGGTTGGGCAGCAGTACTAATGGGGCTAATGTCCGGATGCATTCCATGGTTCAGTA
TGATGTTCCTTCATAAAAAAATGTGGTTCCTTAAACAAGTAGACGACACCATGGCTGTGTTCC
ACACACACGCAGTAGCAGGAACCTTAGGCGCGATTCTAGCAGGTGTATTGGCAAATCCAAGA
CTGAGTCGCATCTTCTACATGGTTGATGATTGGCCAAAGTATATAGGCCTTGCTTATGGCATTC
AAAGTGGAAAGTTCAATGCAGGGCTCAGGCAATTATGGGTACAATTGATAGGTATCGGATTT
GTTTTCATTTGGAATGTCGTTAGCACTAGTGTGATTTGCTTATTAATTAGGACAGTGGTTCCTT
TGAGGATGACAGAGGAAGAGGTCAGTGAGGGAGATAACGCGGTACATGGAGAAGAGGCGTA
TGCATTGTGGGGTGATGGGGAGAAATTCGACAACTCAAAACTCCAGTTTGTTCATGAAATTGA
AGAACATCAGTTGTCTAAAAGTGGCTATGGACTTTAA

SlAMT4.2 **(Solyc01g097370)**

ATGGGACTTCCCTCAAATCTATCACCAGATGAGGCAAGTCCGGGATGGATGAACAAAGGTGA
CAACGCGTGGCAGCTCACAGCTGCCACCCTAGTCGGGCTCCAGTGTGTGCCCGGCTTAGTCAT
TCTCTATGGTGGCATGGTGAAGAAGAAATGGGCAATTAATTCCGCGTTCATGGCACTCTATGC
CTTTGCCTCCGTTCTCATATGTTGGGTTGGATGGGGCTACCGGATGTCATTCGGTGACAAGCTC
GTGGCCTTCTGGGGAAAACCATCCGTCGCCTTGGACGAGAAGTACCTTCTTAGACAAGCATTT
CTGGGATACCTCCCCACAGCAACAATGGTGTTTTTTCAGTTTGTTTTCGCAGCCATTACGCCAA
TCTTGATAGCTGGGGCGCTTCTGGGGAGGATGAATTTTATAGCTTGGATGTTGTTCGTACCTTT
GTGGCATACTTTTTCGTATACCATTGGTGCATTTAGCATTTGGTGCCCAGATGGATGGCTGAAC
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SlAMT4.4 **(Solyc08g067080)**

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Appendix B: Composition of yeast nitrogen medium used in chapter 4.

Table 8.1. Composition of yeast nitrogen medium without amino acid (formula by Acumedia, USA).

The medium components	Quantity per liter
Vitamins	
Biotin	2 µg
Calcium Pantothenate	400 µg
Folic Acid	2 µg
Inositol	200 µg
Nicotinic Acid	400 µg
Pyridoxine HCl	400 µg
Riboflavin	200 µg
Thiamine HCl	400 µg
Citric Acid	0.1 g
Trace Elements	
Boric Acid	500 µg
Copper Sulfate	40 µg
Potassium Iodide	100 µg
Manganese Sulfate	400 µg
Sodium Molybdate	200 µg
Zinc Sulfate	400 µg
Salts	
Potassium Phosphate. Monobasic	1.0 g
Magnesium Sulfate	0.5 g
Sodium Chloride	0.1 g
Calcium Chloride	0.1 g

Appendix C: Expression of Proteinase Inhibitor II (*PIN II*) in tomato leaves after wounding stress.

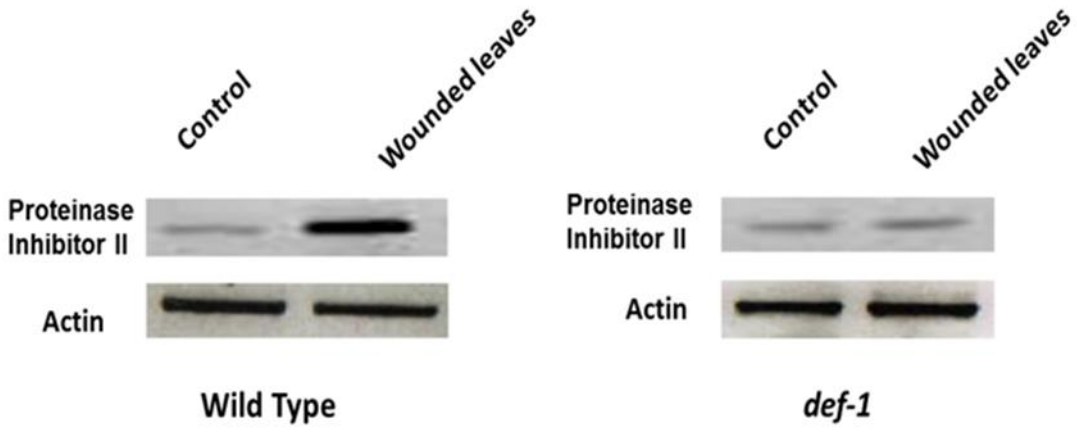

Fig. 8.1. Expression of Proteinase Inhibitor II (*PIN II*) in leaves of WT and *def-1* after 7 hours of wounding treatment. The seeds of both genetypes were sown into pots containing a mixture of peat moss: perlite (2: 1, v: v). Plants were grown under the following growth chamber conditions: 24/19 °C day/night temperatures, 18h/6h photoperiod. When the seedligs reached one month old (4-5 true leaves), the third leaf from the plant bottom was wounded using a hemostat (Howe et al., 1996). Seven hours later, the wounded and un-wounded (control) leaf tissues were collected in liquid nitrogen and stored at -80°C for subsequent mRNA analysis (Howe et al., 1996). Aliquots of 1 µg total RNAs form leaf tissues were reverse-transcribed into cDNA. The experssion of *PIN II* were analyzed by reverse transcriptase polymerase chain reaction (36 cycles) using specific primers (F, 5'- CGTTCAGAAGGAAGTCCGCT-3' and R, 5'-AGCAACCCTTGTACCCTGTG -3'). The endogenous *actin* gene (amplified at 26 cycles) was used as internal control. The PCR products were separated using agarose gel electrophoresis containing ethidium bromide (Sigma- Aldrich Canada) for band visualization. Images of the gels were observed using the imager Biorad Versadoc 4000 Mp and Image Lab^{TM} 3.0 software. Experiments were repeated at least three times, with similar results.

Appendix D: Germination and root growth of *S. pennellii* and *S. lycopersicon* under salt stress.

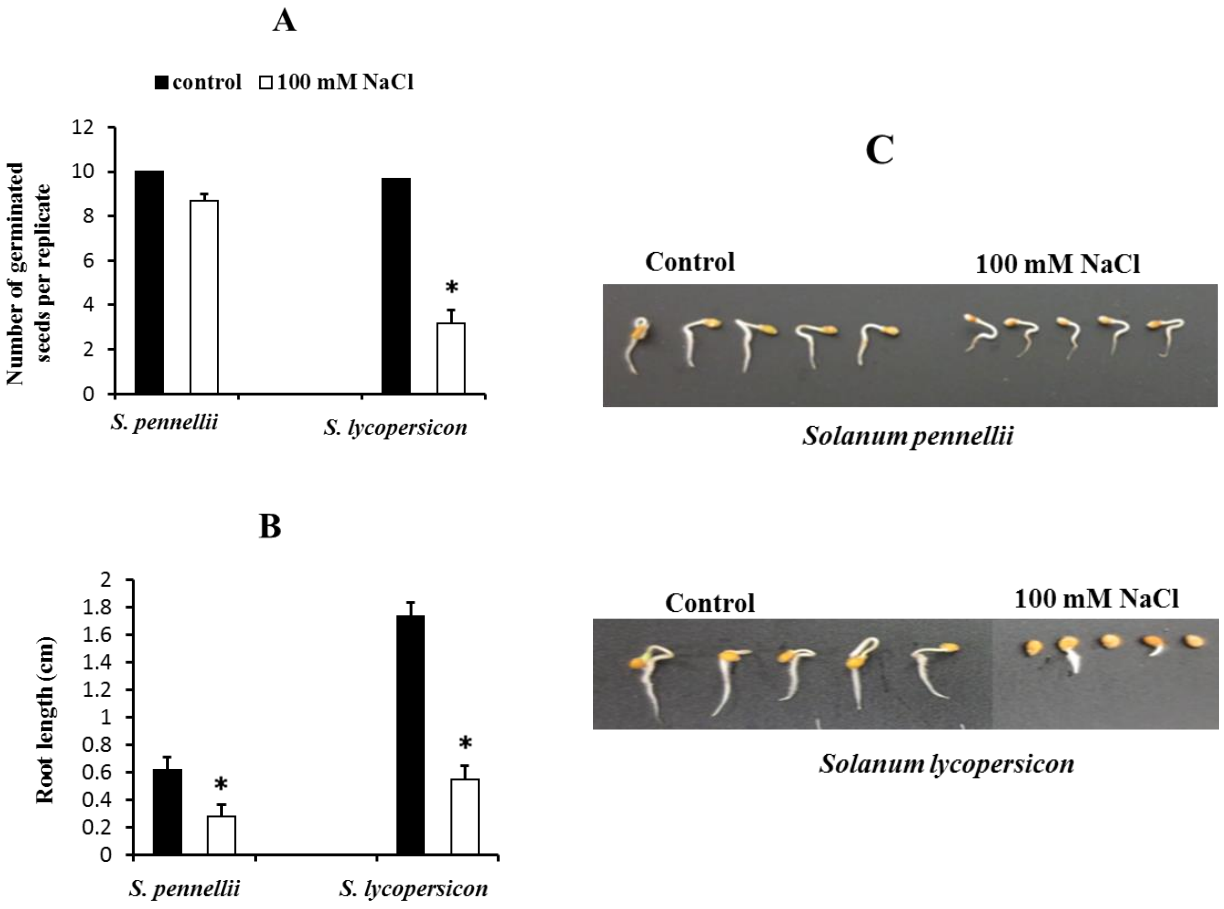

Fig. 8.2. Effect of salinity on germination and root growth of *S. pennellii* (salt- tolerant genotype) and *S. lycopersicon* (salt- sensitive genotype) after 0 or 100 mM NaCl for 4 days. Tomato seeds were surface sterilized for 10 min with 5% sodium hypochlorite. The seeds were then rinsed several times with distilled water and germinated in 9-cm Petri dishes containing two filter papers (Whatman No.1). The dishes were moistened with 5 ml of half strength Hoagland solution supplemented with 0 or 100 mM NaCl. The Petri dishes were incubated at 24 °C for 4 days in the dark. (A) Number of germinated seeds of *S. pennellii* and *S. lycopersicon.* (B) Root length (cm) of *S. pennellii* and *S. lycopersicon.* (C) Germination and root growth comparison between both genotypes. Root length (cm) (right) of *L. esculentum* (salt-sensitive genotype) and *L. pennellii* (salt-tolerant genotype) seeds treated with 100 mM NaCl for 4 days. Five replicates were used in each treatment (10 seeds per replicate or Petri dish). * denotes significant differences between control and salt treatment (t-test with $p \leq 0.05$).

Appendix E:

Table 8.2: Nitrogen contents (%) in shoot and root tissues of *S. lycopersicon* and *S. pennellii* plants, grown with either 0 or 100 mM NaCl for 7 days. Values are the mean of 4 replicates ± S.E.Different letters show significant differences between both genotypes in the same parameter at $p \leq 0.05$.*

	S. lycopersicon		*S. pennellii*	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Shoot N	5.07±0.08 [a]	4.41±0.0 [b]	4.86±0.01 [a]	4.09±0.02 [b]
Root N	4.79±0.09 [a]	3.42±0.03 [b]	4.68±0.08 [a]	3.54±0.06 [b]

* Lyophilized shoots and roots were ground separately using a coffee grinder (Black & Decker, Brockville, ON Canada). The CHNOS elemental analyser 'vario Micro' (Elementar, Hanau, Germany) was used For N analysis. The N analyses were performed by Stratford Agri. Analysis (Stratford, ON, Canada).

Appendix F: Expression levels of *AMT2.1* in roots tissue of *S. lycopersicon* and *S. pennellii*, under salt stress.

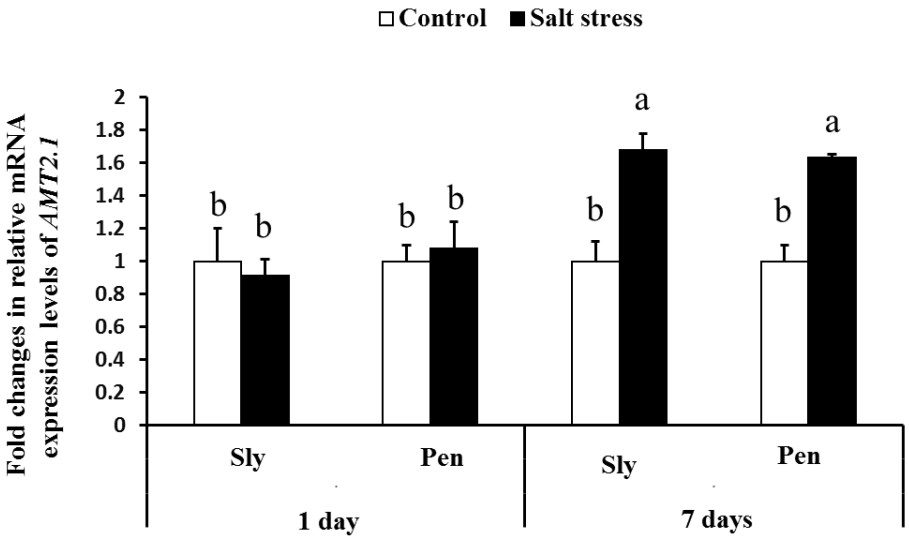

Fig. 8.3. Fold changes in relative mRNA levels of *AMT2.1* in roots tissue of *S. lycopersicon* (Sly) and *S. pennellii* (Spe) plants, grown with either 0 or 100 mM NaCl for 1 or 7 days. The ratio of absolute mRNA levels of the target gene and the *actin* gene was calculated and relative mRNA levels of roots tissue from control plants were set as 1. Data represent means ± SE, n =4. Different letters represent significant differences between means for each date at $p \leq 0.05$.

Appendix G: Tomato plants growing in hydroponic culture

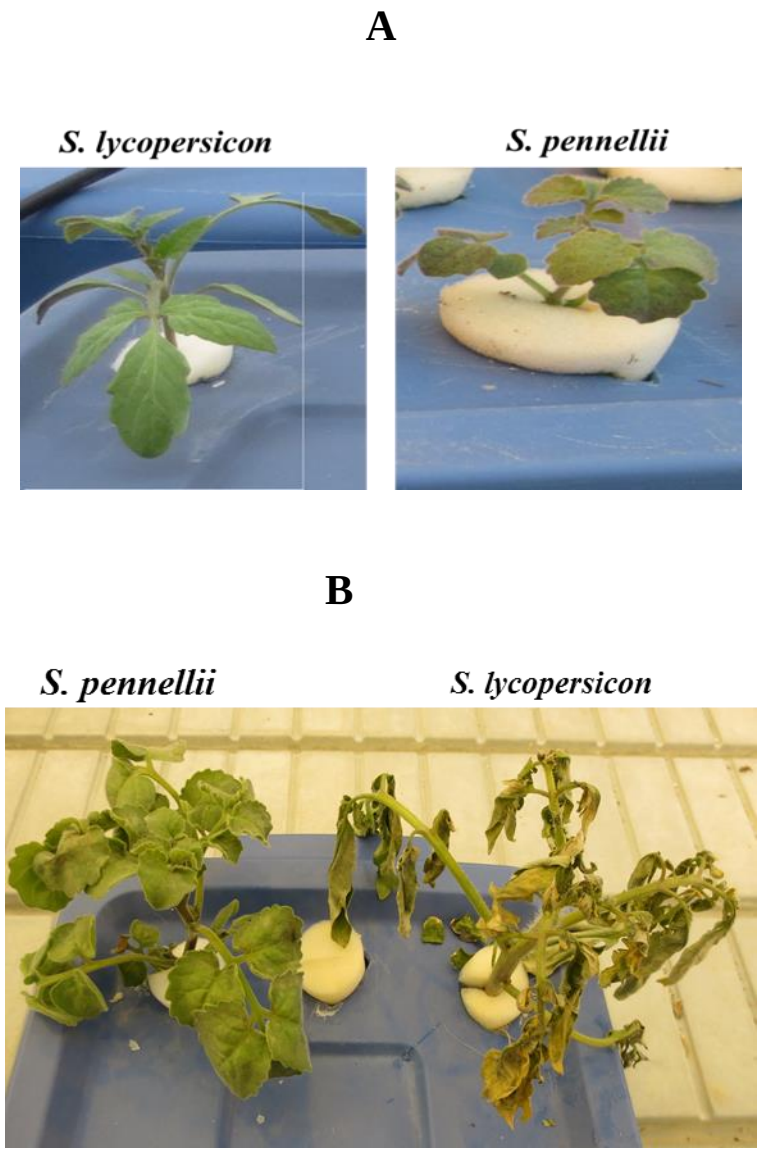

Fig. 8.4. Tomato plants growing in hydroponic culture. (A) Tomato seedlings at the stage of three true leaves in aerated hydroponic solution. (B) Tomato plants exposed to 300 mM NaCl for one week.