DEVELOPMENT OF SUBNILS FOR FINE MAPPING OF SALT TOLERANCE IN TOMATO (*SOLANUM LYCOPERSICUM*)

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ABSTRACT

DEVELOPMENT OF SUBNILS FOR FINE MAPPING OF SALT TOLERANCE IN TOMATO (*SOLANUM LYCOPERSICUM*)

Salt tolerance is a complex trait that is not easily bred into plants. Salt tolerance can be manifested in several ways such as the ability to increase shoot and/or root mass despite salt stress, a greater than normal accumulation of antioxidants like phenolic compounds and flavonoids, and an improved ability to exclude sodium ions. In previous work, *S. lycopersicum* M82, *S. pennellii* LA716 and a *S. pennellii* IL population were evaluated for growth and their levels of antioxidant activity and content under both control and salt stress conditions (150 mM NaCl) (Frary *et al.*, 2010). These data were used to identify QTLs responsible for controlling antioxidant parameters under both control and stress conditions. Salt tolerance characteristics were observed in IL11-1, IL6-1 and IL7-4-1. The aim of this project was the development of subNILs for fine mapping of salt tolerance related traits within these introgression regions. To this end, each *Solanum pennellii* IL was crossed with *Solanum lycopersicum* M82 to produce F2 populations of approximately 1600 individuals for IL6-1, 1600 individuals for IL7-4-1 and 3000 individuals for IL11-1. These individuals were screened with molecular markers that delimit the 30-40 cM introgressions contained in each line. Because of low number of recombinants in IL6-1 and IL7-4-1 populations, they were not selfed to produce subNILs for future analysis. Recombinant F2 plants in IL11-1 population were self-pollinated to generate F3 recombinant families. Each F3 recombinant plant was characterized with several codominant molecular markers in the introgression region. 228 homozygous recombinant F3 plants and 620 heterozygous recombinant plants were identified. At the end of this work, fine mapping populations were developed and in the future they will be grown hydroponically under both control and salt conditions and will be screened for physiological, mineral and biochemical parameters. By statistical comparison between control and salt-treated plants, it will then be possible to identify which recombinants carry regions with significant effects on the various salt tolerance responses. Thus, each gene will be narrowed down to a particular chromosome region.

ÖZET

DOMATES'TE (*SOLANUM LYCOPERSICUM*) TUZA TOLERANSIN YÜKSEK ÇÖZÜNÜRLÜKTE HARiTALANMASI İÇİN SUBNILS HATLARIN GELİŞTİRİLMESİ

Tuza tolerans kolaylıkla bitkilere ıslah edilemeyen kompleks bir özelliktir. Tuz toleransı stres koşullarında bitkinin kök ve gövde kütlesinin artması, fenolik ve flavanoid gibi antioksidanların normalden fazla birikimi ve sodyum iyonlarının dışarı atım kabiliyetinin geliştirilmesi gibi çeşitli şekillerle kendini gösterir. Önceki çalışmada, S. lycopersicum M82, S. pennellii LA716 ve S. pennellii introgresyon hatlarının control ve tuz stresi koşullarındaki büyümeleri, antioksidan aktiviteleri ve miktarları belirlenmiştir (150 mM tuz stresi). Bu veriler kontrol ve stres koşulları altında antioksidan parametrelerini kontrol eden kantitatif karakter lokusların belirlemenmesinde kullanılmıştır. Tuza tolerans özellikleri introgresyon hatları 11-1, 6-1 ve 7-4-1'de gözlemlenmiştir. Bu projenin amacı tuza toleransla ilişkili özelliklerin yüksek çözünürlükte haritalanması için subNIL hatlarının geliştirilmesidir. Bu amaçla her bir Solanum pennellii introgresyon hattı F2 populasyonlarının oluşturulması amacıyla, yaklaşık 1600 birey introgresyon hattı 6-1'den yaklaşık 1600 birey introgresyon hattı 7-4-1'den ve yaklaşık 3000 birey introgresyon hattı 11-1'den, Solanum lycopersicum M82 ile melezlenmiştir. Bu bireyler 30-40 cM'lık introgresyon segmentleri sınırlayan moleküler markörlerle taranmıştır. 6-1 ve 7-4-1 populasyonlarında gözlemlenen düşük sayıdaki rekombinant bireyler nedeniyle sonraki analizlerde kullanılmamıştır. 11-1 introgresyon hattından elde edilen rekombinant F2 bitkileri kendilenerek F3 populasyonu oluşturulmuştur. Her bir F3 bitkisi introgresyon segmentinde bulunan birkaç kodominant moleküler markörle karakterize edilmştir. 228 homozigot rekombinant birey, 620 heterozigot rekombinant bitki belirlenmiştir. Bu çalışmanın sonunda yülksek çözünürlükte haritalama populasyonu geliştirilmiş olup geleceteki çalışmalarda bu bitkilerin kontol ve tuz stresi koşulları altında fizyolojik, mineral ve biyokimyasal parametreleri ölçülecektir. Tuz uygulanmış bitkiler ile kontrol şartlar altına yetiştirilen bitkilerin istatistiki olarak kıyaslanması ile, değişik tuz toleransı faktörleri üzerine önemli etkileri olan bölgeleri taşıyan rekombinantları belirlemek mümkün olacaktır. Sonuç olarak, her bir etki (veya gen) çok özel bir kromozom bölgesine daraltılacaktır.

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CHAPTER 1

INTRODUCTION

1.1. Tomato (*Solanum sp.*)

Tomato, *Solanum lycopersicum* (synonym: *Lycopersicon esculentum*) is one of the economically most important members of the Solanaceae family which also includes potato, pepper, eggplant and tobacco. Tomato is in second place after potato as the most consumed vegetable in the family. Tomato is composed of high amounts of minerals, vitamins, and antioxidants and is an important part of the human diet in most countries (Grierson and Kader, 1986).

Tomato is believed to have originated in western South America: Chile, Bolivia, and Ecuador, and the coastal region of Peru. Tomato was classified initially under genus Lycopersicon in the 18th century, however, molecular and phylogenetic studies by Peralta *et al.* (2005) classified tomato in genus Solanum with potato and eggplant. After this change in phylogeny, the genus Lycopersicon became a section of the genus Solanum with 13 species including *S. pimpinellifolium, S. pennellii, S. habrochaites, S. peruvianum, S. chmielewskii* and *S. chilense* (Peralta *et al.* 2006). *S. lycopersicum cerasiforme* is considered as the ancestor of cultivated tomato because it is widely present in Central America and it has a short style length in the flower (Cong *et al.*, 2002). But later, genetic investigation showed that "cerasiforme" plants are just a mixture of wild and cultivated tomatoes (Nesbitt and Tanksley, 2002). The genus Solanum is divided into two groups: "esculentum complex" and "peruvianum complex" according to fruit color and hybridization barriers (Peralta and Spooner 2000). Domestication of tomato caused changes in morphological and physiological traits and these traits are called the domestication syndrome (Frary and Doganlar, 2003). Compact growth habit, increased earliness, reduction/loss of seed dispersal and dormancy, fruit set, fruit size, fruit shape, fruit color, flavor, yield, heterosis, and disease and stress resistances are examples of domestication syndrome characteristics. During the domestication of tomato, only phenotypic properties were consciously selected by farmers. Since domestication, further improvement of tomato has occurred through

plant breeding. Often the best source for improvement of a crop plant is its wild species (Tanksley and McCouch, 1997). Wild species retain allelic (trait) diversity which has been lost during domestication and breeding, therefore, wild tomato species are sources of both desired and undesired traits (for example, Top *et al.*, 2014?).

Tomato is currently grown in almost every country of the world. Worldwide tomato production reached 162 million tonnes in 2012 (FAOSTAT 2012). Asia ranks first with 50.3% of total production, Europe ranks second with 17.8% of production, followed by the Americas with 19.4%, Africa with 12.1% and Oceania with 0.4%. Turkey is in the top five tomato-producing countries and placed after China, India, and United States of America with production of more than 11 million tonnes (FAOSTAT 2012).

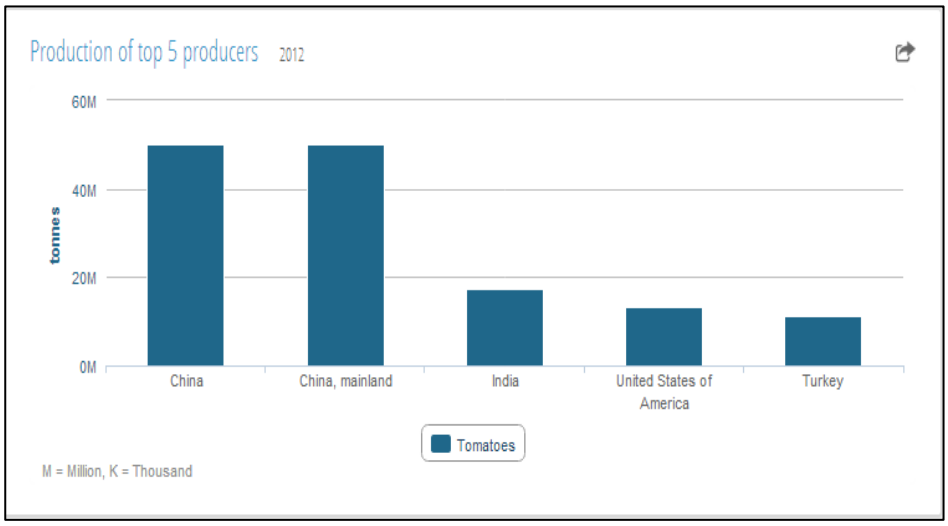


Figure 1.1. Production of top five tomato producers in world
(Source: FAOSTAT 2012)

Tomato is not only important for the economy but is also essential in the human diet. High levels of antioxidants like lycopene, phenolics, vitamins E and C, and β-carotene are important constituents of tomato (Adalid *et al.*, 2004). It has been shown that, although lycopene is not an essential ingredient for humans, it has many benefits for human health such as inhibition of human cancer cell growth, prevention or reduction in DNA mutations and prevention of liver fibrosis (Heber and Lu, 2002; Zhou *et al.*, 2008; Kitade *et al.*, 2002). The precursor of vitamin A, β-carotene, is an antioxidant that plays a role in the prevention of cellular or tissue damage, cardiovascular disease and major cancers (Mantzouridou *et al.*, 2001; Zhang and

Omaye, 2001). In addition to these constituents, molybdenum, iron, phosphorus, magnesium, niacin and potassium are present in tomato and their beneficial roles for human health have been reported (Agarwal and Rao, 2000).

In addition to all of these benefits, tomato also is an important model system for genetic studies in plants. It is a widely used plant in such studies and is diploid (2n=24). Tanksley *et al.,* (1992) constructed the first plant species high-density DNA-based molecular map in tomato. Paterson *et al.,* (1988) did whole genome quantitative trait locus (QTL) mapping in a single segregating population for the first time. The first plant resistance gene and first plant QTL that were cloned in plants were in tomato (Martin *et al.,* 1993; Frary *et al.,* 2000). The tomato (inbred tomato cultivar Heinz 1706) genome was sequenced and assembled using Sanger and next generation technologies and published in Nature on May 31, 2012 (Tomato Genome Consortium, 2012).

1.2. Definition of Salinity

Salinity is one of the major factors that limit plant growth and productivity all over the world. Some plants are able to tolerate high levels of salinity while others are able to tolerate little or no salinity. Salt tolerance is defined as the relative growth of the plant in the presence of salinity and is indicated based on the stage of plant growth over electrical conductivity (EC) levels. Electrical conductivity is the ability of a solution to transmit electric current and is determined using electrodes and a soil extract solution. The units are defined in deciSiemens per metre (Ds/m).

Table 1.1. Soil and water salinity criteria based on plant salt tolerance groupings (Source: Foolad, 2004)

Plant Salt Tolerance Grouping	Water or Soil Salinity Rating	Average Root Zone Salinity EC (Ds/m)
Sensitive Crops	Very Low	< 0.95
Moderately Sensitive Crops	Low	0.95 – 1.9
Moderately Tolerant Crops	Medium	1.9 – 4.5
Tolerant Crops	High	4.5 – 7.7
Very Tolerant Crops	Very High	7.7 – 12.2
Generally Too Saline	Extreme	> 12.2

One billion ha of the total 14 billion ha of land available on earth is saline soil and it is reported that 20% of cultivated land and 33% of irrigated agricultural land worldwide are affected by high salinity. These areas are increasing because of low precipitation, high surface evaporation, and irrigation with saline water (Ghassemi *et al.*, 1995).

Two main approaches are proposed to prevent the harmful effects of salinity in agriculture (Epstein *et al.*, 1980). The first approach is the use of technology-based applications such as reclamation, drainage and irrigation with high quality water. This approach is successful in some areas but it is very expensive and is just a temporary solution. The second approach is the use of biological strategies mainly concentrated on the development of plants that are able tolerate high levels of salinity. If these two approaches are applied simultaneously, sustainable crop production can be achieved in saline conditions (Epstein *et al.*, 1980).

1.3. Effects of Salt Stress on Plants

Plants are affected by salinity in different ways such as osmotic effects, specific-ion toxicity and/or nutritional disorders (Lauchli and Epstein, 1990). These effects can change based on many factors including species, genotype, plant age, plant organ and salinity level. Plants undergo characteristic changes from the time they face salinity until they reach maturity (Munns, 2002a). After salinization, high levels of salt cause cells to dehydrate and shrink. Although they re-gain their original size within hours, cell elongation and cell division are reduced. These reductions in cell elongation and division cause alterations in leaf appearance and size. Under high saline conditions, visual injury symptoms can be observed on the leaves. These changes also affect lateral shoot development and, eventually, overall growth (Munns, 2002a).

Munns (2002a, 2005) explained the effect of salinity on plants according to a "two-phase growth response to salinity" (Figure 2). The first phase is the quick response of plants to salinity due to osmotic effects which reduce the ability of the plant to absorb water. After the quick response, there is a limited recovery period to reach a steady-state dependent upon the salt concentration outside of the plant (Munns, 2002a). The second phase is not as quick as the first one because it can take days, weeks or even months, depending on the accumulation of salt in leaves. This accumulation is very toxic to

leaves and therefore, results in leaf injury and death (Munns and Termaat, 1986; Munns 2002a; 2005; Munns et al, 2006). The rate at which leaves die, in other words, the rate at which total photosynthetic leaf area is reduced is the key parameter that determines the survival of the plant. If new leaves are produced at a greater rate than the rate at which old leaves die, the plant can tolerate salinity and can flower and produce seeds. If old leaves die faster than the production of new leaves, the plant cannot survive.

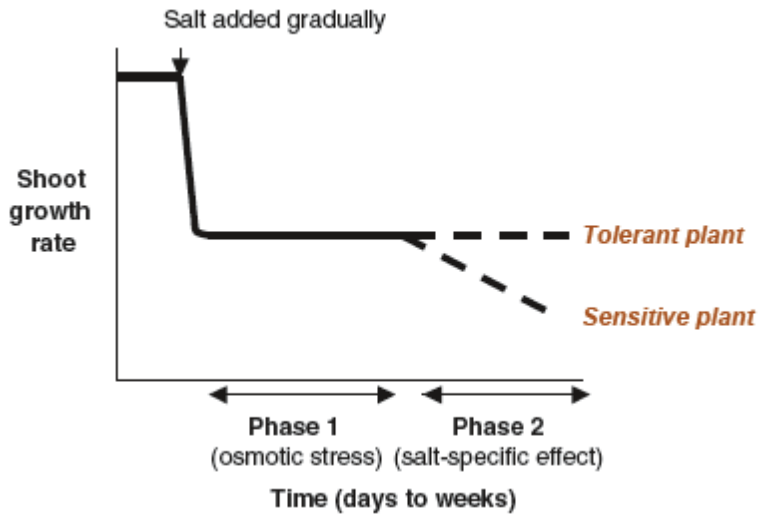


Figure 1.2. Schematic illustration of the two-phase growth response to salinity for genotypes that differ in the rate at which salt reaches toxic levels in leaves (Source: Munns, 2005).

Salinity causes four detrimental effects on plants. The first one is osmotic stress due to high salinity which causes plants to lose their turgor pressure (Xiong and Zhu, 2002). The second effect is nutrient deficiency. Due to decreased water uptake, the levels of essential minerals like phosphorus, potassium, nitrate, and calcium are lowered (Xiong and Zhu, 2002). Ion cytotoxicity is the third detrimental effect of salinity and is based on excessive levels of Na^+, Cl^-, and SO_4^{2-}. Oxidative stress is a secondary effect of salinity and caused by excessive amounts of reactive oxygen species (ROS). When plants are affected by salinity stress, ROS cannot be effectively neutralized, therefore they accumulate in the cells and cause oxidative stress (Xiong and Zhu, 2002).

1.4. Salt Tolerance Mechanisms in Plants

Plants are categorized as either halophytes or glycophytes. It is believed that halophytes may be evolved from glycophytes that survived under salinity stress (Zhu, 2000). Therefore the most important difference between halophytes and glycophytes is the ability of halophytes to grow and survive best where salt concentration is 200 mM or more (Figure 3) (Braun *et al.*, 1986; Casas *et al.*, 1991; Hassidim *et al.*, 1990).

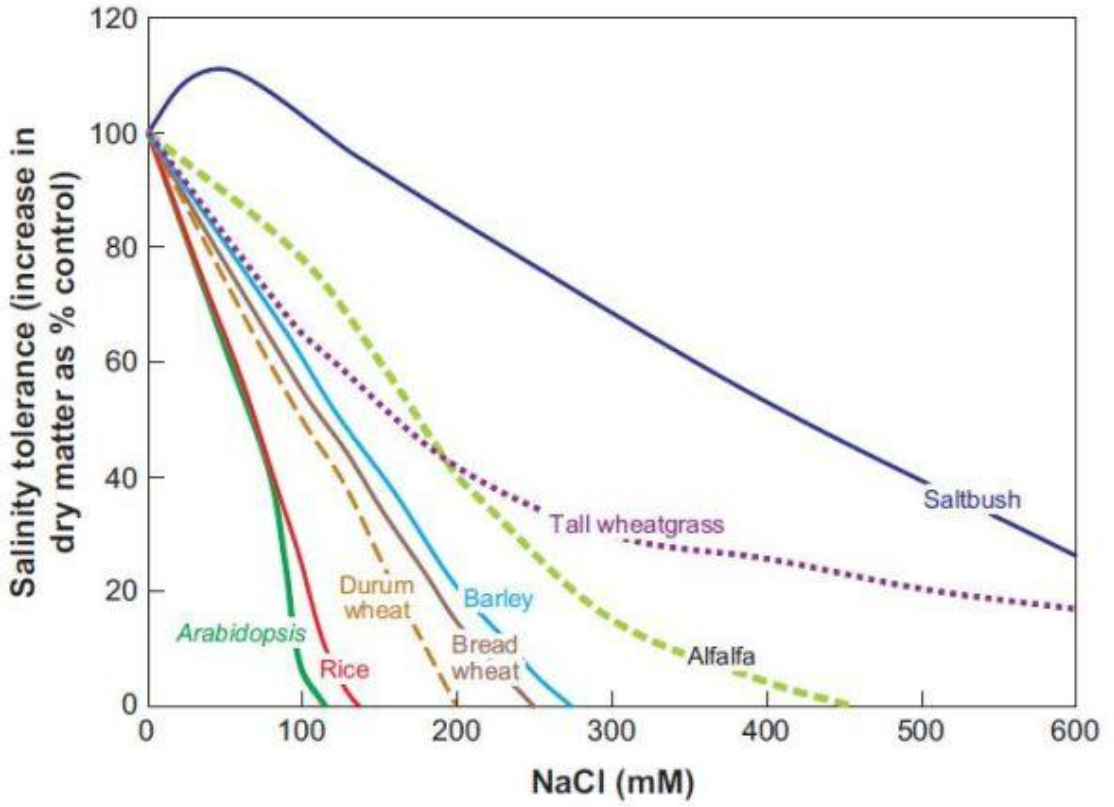


Figure 1.3. Response of glycophytes and halophytes to varying concentrations of NaCl after 3 weeks of treatment after Munns & Tester, 2008 (Source: Javid *et al.*, 2011)

Salinity tolerance can be achieved by several mechanisms in both halophytes and glycophytes and their differences are shown in Figure 4. There are differences in tissue tolerance such as salt compartmentation, synthesis of compatible solutes, and potassium by sodium replacement and avoidance.There are 3 main adaptive strategies: i) avoidance through ion exclusion; ii) tolerance through inclusion and compartmentalization of ions; iii) tolerance to osmotic stress (Blumwald *et al.*, 2004; Munns, 2005; Munns and Tester, 2008). In other words, plants tolerate salt stress through: avoidance or alleviation of salt injury, re-establishment of homeostatic

conditions, and survival and growth under stress (Figure 5; Zhu, 2001). Each of these strategies will be discussed in the following sections.

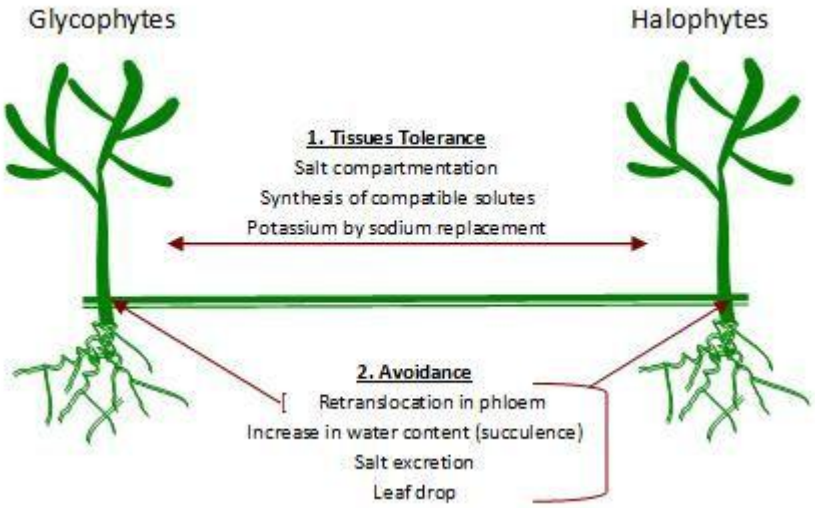


Figure 1.4. Adaptive strategies for salt tolerance in plants
(Source: Javid *et al.*, 2011)

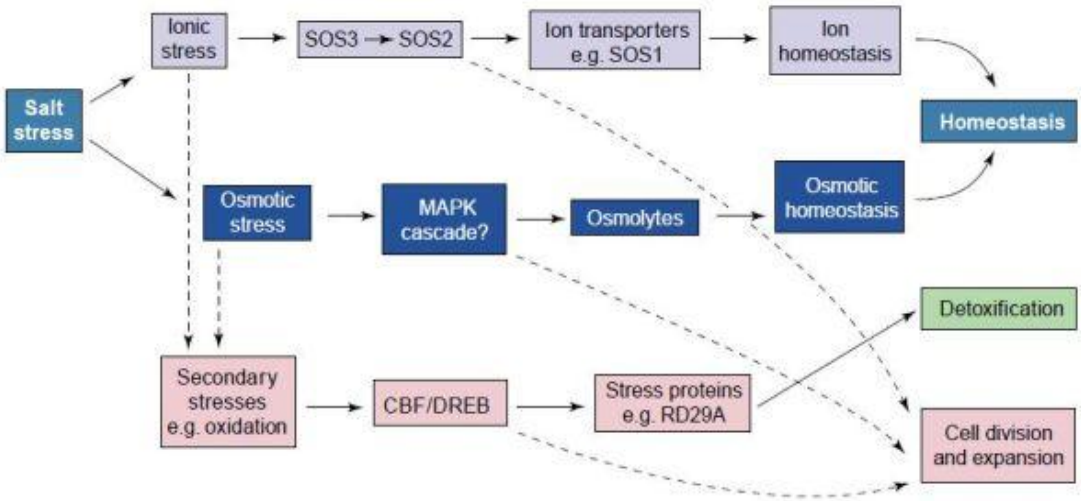


Figure 1.5. Three main paths of salt tolerance in plants
(Source: Zhu, 2001 and Javid *et al.*, 2011).

1.4.1. Ion Exclusion

It has been reported that Na^+ avoidance through exclusion is the most crucial salt tolerance mechanism in glycophytes such as wheat (Munns, 2005; Munns and Tester, 2008), Arabidopsis (Moller *et al.*, 2009; Moller and Tester, 2007), *B. napus* and *B. juncea* (Ashraf and McNeilly, 2004; Ashraf *et al.* 2001). As can be understood from its

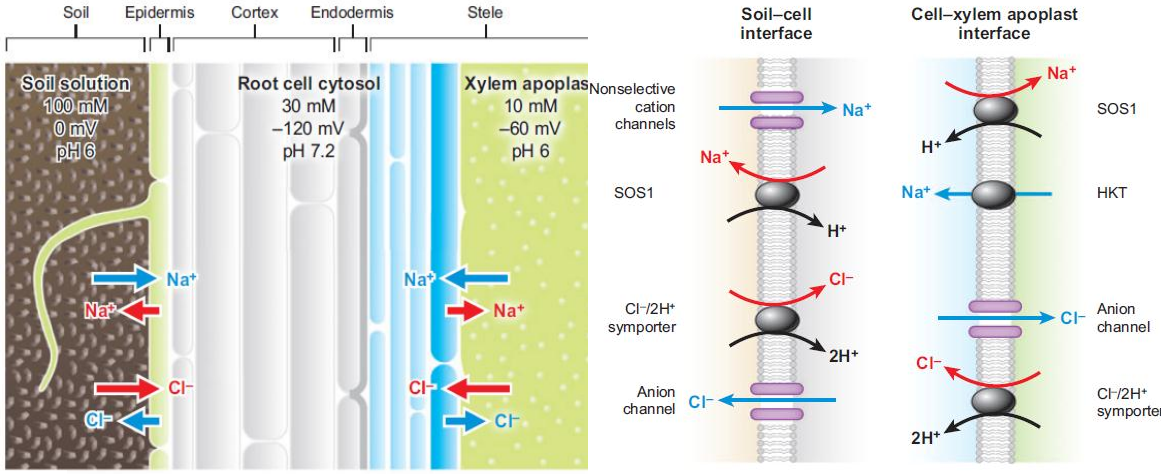
name, ion exclusion is a mechanism by which plants discard most of the Na^+ and Cl^- dissolved in the soil solution, thereby preventing the accumulation of toxic levels of salt in shoots. Plants are able to transpire approximately 50 times more water than they absorb in their leaves which means that 98% of the salt in the soil solution is excluded. Thus, Na^+ concentration is kept stable and its accumulation up to toxic levels is prevented (Munns, 2005). In bread wheat, more than 98% of Na^+ was excluded and Na^+ concentration in the leaves was measured as less than 50 mM (Husain *et al.*, 2004). In contrast, salinity tolerance in *B. juncea* is reported as achieved through partial exclusion (Ashraf and McNeilly, 2004; Ashraf *et al.*, 2001). In addition, Moller and Tester (2007) reported that rather than exclusion of Na^+ ions from the shoots, salinity tolerance could be achieved by tissue tolerance to Na^+ levels in some members of Brassicaceae including Arabidopsis. Interestingly, Huang and Redman (1995) examined two *B. napus* genotypes and found that the genotype with higher Na^+ accumulation in shoots was more salinity tolerant and that accumulation of Na^+ was accompanied by increased levels of proline and K^+. After this study, it could be concluded that tissue tolerance might be more important than Na^+ exclusion in the shoot zone in Brassicaceeae.

1.4.1.1. Thermodynamics of Sodium Transport

It has been elucidated that the concentration of Na^+ ions in the cytosol is 30 mM and that the electrical potential created by the presence of these ions in the cytosol is -120 mV. In figure 6.A., the concentration of Na^+ ions and electrical potential due to Na^+ ions can be seen. Based on concentration and electrical potential differences, Na^+ ions are passively or actively transported across the regions (Munns and Tester, 2008).

As can be seen from figure 6.B., Na^+ ions can enter into roots in different ways: passively with the aid of nonselective cation channels, via Na^+ transporters and by HKT family transporters. In addition Na^+ ions can be transported with the aid of some members of the HKT transporter family but this influx is blocked in the presence of high salt concentrations. The players involved in the nonselective transport of Na^+ ions are not known but recent studies found evidence for cyclic nucleotide-gated channels (CNGC) and ionotropic glutamate receptor-like channels (Kronzucker and Britto, 2011). In Arabidopsis, it has been found that CNGC3 is responsible for Na^+ uptake in the roots and Gobert et al. (2006) reported that this transporter is one of the salinity

tolerance mechanisms in Arabidopsis. In 2008 Guo et al. reported that AtCNGC10 is responsible for sodium uptake. Moreover, in 2006, transcriptomics studies were done by the same group using CNGC isoforms in Arabidopsis and it was shown that their regulation was affected by salinity stress (Guo et al. 2006).



a) b)

Figure 1.6.a.Thermodynamics of Na^+ and Cl^- transport. Figure 1.6.b. Proposed mechanisms of Na^+ and Cl^- transport (Source: Munns and Tester, 2008)

The entry sites of Na^+ ions into roots are uncertain but it is thought that as water enters the root cortex and then moves to the stele (Figure 7), Na^+ ions are sequestered in cell vacuoles (Munns and Tester, 2008).

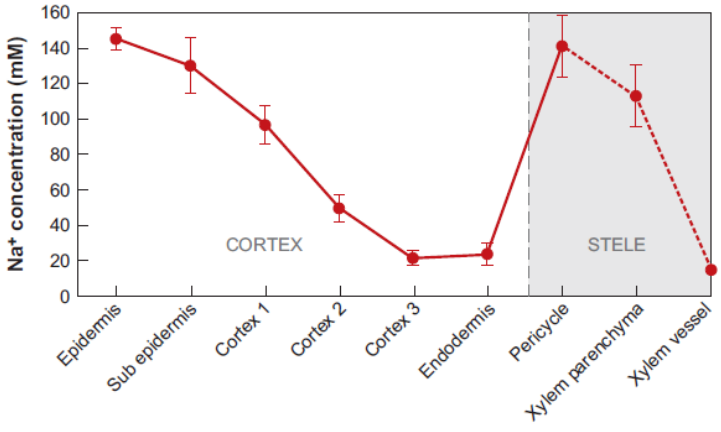


Figure 1.7. The concentration of Na^+ ions in different parts of the plant (Source: Munns and Tester, 2008)

Na^+ efflux proteins have roles in pumping out Na^+ ions that previously entered into root cells. An example is plasma membrane Na^+/H^+ antiporters. AtSOS1, a plasma membrane Na^+/H^+ antiporter in Arabidopsis, is responsible for exchange of H^+ with Na^+

or K^+ ions (Kronzucker and Britto, 2011). It is known that SOS1 is present in root tips and absent in most other root tissues, however, its expression level in the outer part of the root is not known. Because of these facts, scientists concluded that SOS1 is not responsible for most of the Na^+ ion efflux into the apoplast and there should be other transporters that play roles in Na^+ efflux in epidermal and cortical cells (Kronzucker and Britto, 2011).

When Na+ ions are present in the roots, there are two main ways to remove these ions. The first is compartmentation in vacuoles and the second is transport of the ions to shoots. Tonoplast NHX family exchangers (such as Na^+/H^+ antiporters) play a role in the compartmentation of Na^+ ions but, because of the presence of tonoplast nonselective cation channels, there is leakage. Therefore, sequestered Na^+ ions can return to the cytoplasm from the tonoplast. To reduce the leakage of Na^+ ions, there has to be constant sequestration of ions into the tonoplast (Munns and Tester, 2008). For example, Arabidopsis vacuolar H^+ translocating pyrophosphatase (AVP1) is responsible for transport of Na^+ ions into the vacuole and, because it decreases the cytosolic Na^+ concentration and allows sequestration of Na^+ ions into vacuole, it is responsible for salt tolerance in Arabidopsis (Kronzucker and Britto, 2011). Gaxiola et al. (2001), found that the vacuolar proton gradient, solute accumulation and water retention were increased in transgenic plants that overexpressed AVP1 and that these plants survived in high salinity conditions as a result of sequestration of Na^+ into the vacuole, therefore, reducing the cytosolic concentration of ions and eliminating the toxic effects of these ions (Gaxiola et al., 2001). Moreover, it can be seen from the literature that the overexpression of AVP1 in Arabidopsis, cotton, tomato and rice causes sequestration of Na^+ ions and sugars into the vacuole and decreases the water potential such that these plants show increased tolerance to salt stress when compared to wild type plants (Parida and Das, 2005).

High affinity potassium transporters are carrier-type proteins that function as Na^+:K^+ symporters or Na^+ uniporters. In 2002, Laurie et al. showed that HKT1 is responsible for Na^+ uptake from the soil solution. Antisense expression of the wheat HKT1 showed that transgenic plants were able to accumulate less Na^+ from the soil and these plants survived under high salt stress. There are other examples for members of the HKT gene family (Parida and Das, 2005). In rice, OsHKT2;1 is mainly responsible for Na^+ uptake from the soil and, at low concentrations of salt in the soil, OsHKT2;1 has a high affinity to Na^+ ions. However, when the concentration of salt in the soil

increases, studies showed that OsHKT2;1 is downregulated and, therefore, blocks the uptake of Na^+ ions from the soil and prevents the accumulation of toxic levels of Na^+ ions (Parida and Das, 2005). In durum wheat, TmHKT1;4-A2 is able to exclude Na^+ ions. In addition to exclusion of Na^+ ions, it is responsible for the high K^+/Na^+ concentration ratio in leaves.

All of the studies done on the HKT gene family show that this family is divided into two groups with the distinction being the properties of the pore loop region that determine cation selectivity. Group one has serine groups in this pore loop region and, therefore, it favors Na^+. Group two has glycine instead of serine residues and, therefore, favors both K+ transport and high Na^+ influx (Munns and Tester, 2008). To sum up, based on the latest findings, it is suggested that although HKT1 is responsible for Na^+ uptake into cells, its overall role is reduction of movement of Na^+ ions into the shoot. Moreover, the LCT1 (low affinity cation transporter 1) of wheat is responsible for cation transport such as K^+, Rb^+, Na^+, and Ca^{2+} as a nonselective cation carrier in yeast (Munns and Tester, 2008). In 2001, Amtmann et al. showed that, expression of LCT1 in yeast caused accumulation of Na^+ in the cells but the exact roles of LCT1 in plants has not yet been understood.

1.4.2. Ion Compartmentation

Ion compartmentation is an important salt tolerance mechanisms in many glycophytes like Arabidopsis (Moller *et al.*, 2009; Moller and Tester, 2007), wheat, barley (Munns, 2005; Munns *et al.*, 1995; Munns and Tester, 2008), and *B. juncea* (Ashraf and McNeilly, 2004; Kumar *et al.*, 2009). Basically, salinity causes accumulation of higher levels of Na^+ in leaves. This accumulation is because of the entry of Na^+ ions due to the similarities between Na^+ and K^+ ions and the fact that transporters cannot differentiate them (Blumwald *et al.*, 2000). Therefore, to prevent toxicity, ions must be compartmentalized in vacuoles and this compartmentalization keeps K^+, Ca^{2+} and Na^+ at optimum cellular levels (Munns and Tester, 2008). Several reports concluded that an optimal K^+/Na^+ ratio in the cytosol is the key parameter of plant salt tolerance (Singla-Pareek *et al.*, 2003; Singla-Pareek *et al.*, 2008; Tester and Davenport, 2003). A higher K^+/Na^+ ratio means that the plant excludes Na^+ ions and maintains K^+ ions in optimal concentration and prevents any injury due to salinity.

Because of this mechanism, key elements in ion homeostasis such as the salt overly sensitive (SOS) pathway, Na^+/H^+ antiporters and K^+ transmembrane transporters can be targeted for the development of increased salt tolerance in plants (Benke *et al.*, 2010; Blumwald *et al.*, 2004). Antiporters, ion channels, ABC-type transporters, Na^+ and K^+ transporters, plasma membrane and vacuolar ATPases are capable of Na^+ exclusion, ion homeostasis, and compartmentalization of solutes and amino acids under stress conditions (Apse *et al.*, 2003; Takahashi *et al.*, 2009). Indeed, oxidative stress in vacuoles was eliminated by over-expression of vacuolar Na^+/H^+ antiporter in *B. napus* (Ruiz and Blumwald, 2002; Zhang *et al.*, 2001). In addition, overexpression of the vacuolar alkali cation transporter AtNHX1 of *Arabidopsis thaliana* provided elevated levels of salt tolerance in transgenic plants (Apse *et al.*, 1999; Hernandez *et al.*, 2009; Venema *et al.*, 2002).

1.4.3. Osmotic Adjustment

Plants have evolved to tolerate various stress conditions and they survive in salinity and drought conditions by tolerating the low soil water potential caused by the stress. Osmotic stress tolerance is a key feature of most glycophytes and halophytes (Munns and Tester, 2008). Osmotic adjustment keeps turgor pressure at a steady state allowing plants to survive under saline conditions (Ashraf and McNeilly, 2004). Munns and Tester (2008) concluded that genetic variation within species may exist for the osmotic response under saline stress. It has been reported that saline conditions cause low water potential and lead to cell membrane damage, cell toxicity and cell injury (Chen and Murata, 2002; Sreenivasulu *et al.*, 2000). Smaller leaves, in other words leaf area reduction, are the main results in many plants. As the water used by the plant decreases, leaf development and root growth are reduced, soil moisture is preserved and salt concentration levels in the soil are not increased (Munns and Tester, 2008). When subjected to salinity, plants are induced to produce various molecules like soluble sugars, free amino acids, and free proline (Ashraf and Akram, 2009; Ashraf and McNeilly, 2004). Although there are some reports about osmotic adjustment, the mechanisms underlying osmotic adjustment are unknown because of difficulties in determination of this parameter. Therefore the closely linked parameter, tissue tolerance of Na^+ ions, is used in studies. Because it is speculated that plants that can tolerance

high levels of Na^+ ions by storing them in the vacuoles, tolerant plants are capable of tolerating dramatic changes in the osmotic stress (Munns and Tester, 2008).

1.4.4. Other Mechanisms of Salinity Tolerance

The other mechanisms of salinity tolerance can be classified as K^+ accumulation and Cl^- tolerance. Although these mechanisms may seem to be completely independent of Na^+, they are actually related to Na^+ concentration.

1.4.4.1. K^+ Accumulation in the Cytoplasm

Recent studies showed that K^+ ions and especially high cytosolic K^+/Na^+, are important factors that affect salinity tolerance (Shabala and Cuin, 2008). Like Na+ transport, the transport of K^+ is complex and different types of transporters are involved. An overview of this transport is shown in Figure 8 (Shabala and Cuin, 2008). To keep the cytosolic K^+/Na^+ ratio high, the plant restricts the accumulation of Na^+ ions or prevents the loss of K^+ ions from the cell. At least seven major families of cation transporters play roles in the transport of potassium across the plasma membrane. These families are divided into two groups: potassium permeable channels (three families) and potassium transporters (three families). Potassium permeable channels can be categorized as Shaker-type potassium channels, two-pore potassium channels and NSCC (cyclic nucleotide gated channels and glutamate receptors). Potassium transporters can be categorized as KUP/HAK/KT transporters, HKT transporters and K^+/H^+ antiporters (Shabala and Cuin, 2008). The basic features of these transporters will be explained.

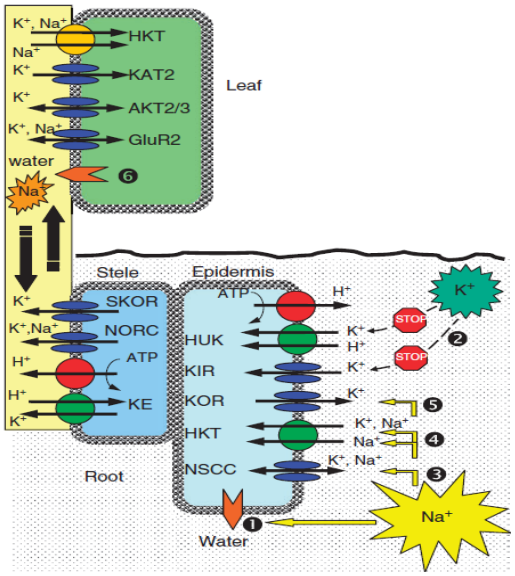


Figure 1.8. Overview of K^+ transport in different parts of the plant (Source: Shabala and Cuin, 2008).

Shaker-type potassium channels function as K^+ selective voltage-gated channels and are present on the plasma membrane of various cell types. In 2004, Qi and Spalding showed that sodium has adverse effects on these shaker-type potassium channels. The direct effect of sodium on these channels is a reduction in the probability of these channels opening. The indirect effect of sodium on these channels is a reduction in the number of expressed channels. Although sodium has negative effects on these channels, it is known that they are mainly responsible for K^+ homeostasis during salt stress (Shabala and Cuin, 2008).

Two-pore potassium channels are another class of potassium channels that are mostly found in the tonoplast. They are $Ca2^+$ dependent channels and are responsible for the removal of K^+ ions from the tonoplast. Although their main role is not known, it is thought that during salt stress, these channels are responsible for the maintenance of cytoplasmic K^+ concentrations and possibly responsible for the exchange of vacuolar K^+ for Na^+ (Shabala and Cuin, 2008).

Non-selective cation channels are present in the plasma membrane and endomembranes. In 2007, Demidchik and Maathuis showed that these channels are highly selective for cations instead of anions, their selectivity ratio for K^+/Na^+ changes from 0.3 to 3.0 and the main function of these channels is low-affinity uptake. They may be gated either by gates or ligands. For example, there are depolarization-activated, hyperpolarization activated, calcium-dependent, cyclic nucleotide and glutamate-gated

channels. In addition, several reports indicated that these channels are a primary player for Na^+ influx into plant roots; and, in 2006, Maathuis concluded that the expression levels of these channels are affected by salt stress. Moreover, with their function, these channels are important for the reduction of the osmotic potential of tissue and, therefore, important for prevention of water stress (Shabala and Cuin, 2008).

KUP/HAK/KT transporters are another group of transporters that are responsible for both uptake of high and low affinity K^+. They are present in both plasma membrane and tonoplast. Reports indicated that during salt stress conditions, these transporters are responsible for Na^+ influx. Santa-Maria et al. (1997) expressed genes responsible for HvHAK1 in barley and saw that these transporters are responsible for both low affinity Na^+ transport and high affinity K^+ uptake (Shabala and Cuin, 2008).

The main function of HKT transporters is K^+/Na^+ symport. In addition, Laurie et al. (2002) showed that HKT transporters may play a role in the transport of Na^+ into roots when there is a low ratio of K^+/Na^+. Moreover the studies by Apse and Blumwald in 2007, concluded that these transporters are responsible for uptake of Na^+ and recirculation of sodium during salt stress circumstances.

The main function of K^+/H^+ antiporters has not been elucidated but, according to speculation, they play important roles in K^+ homeostatis and it was also shown that during salt stress, the transcript levels of these antiporters increase (Shabala and Cuin, 2008).

1.4.4.2. Cl^- Tolerance

Like K^+ transport, there is not much known about Cl^- transport. In 2006, Li et al. showed that Cl^- transport is complicated and, similar to K^+ transport, it is mostly dependent on cation transport, especially Na^+ transport. Therefore, they concluded that both cation and anion transport have to be studied at the same time.

Studies also showed that Cl^- tolerance and salinity tolerance of plants change from species to species and even within a species. Moreover studies were done with Cl^- and Na^+ ions to test which ion is more toxic to plants, but no convincing result was found (Teakle and Tyerman, 2010). The mechanism of Cl^- transport varies according to location. For example, the loading of Cl^- into xylem is mostly actualized by passive mechanisms through anion channels. Gilliham and Tester showed that these channels

are subject to downregulation by ABA and this downregulation causes blockage of Cl^- transfer to shoot during salt stress. In addition, it is known that the transfer of Cl^- into root xylem is correlated with accumulation of Cl^- in the shoots. A lower accumulation of Cl^- into shoots causes a lower amount of Cl^- to be transferred into root xylem (Teakle and Tyerman, 2010).

The transport of Cl^- to shoots can be controlled by reduced transfer of Cl^- by anion channels and increased recovery of Cl^- from the xylem. Although there is some information about transport of Cl^- in the xylem and shoots, the transport in tonoplasts is unknown. Cl- transport circuits are schematically represented in Figure 9.

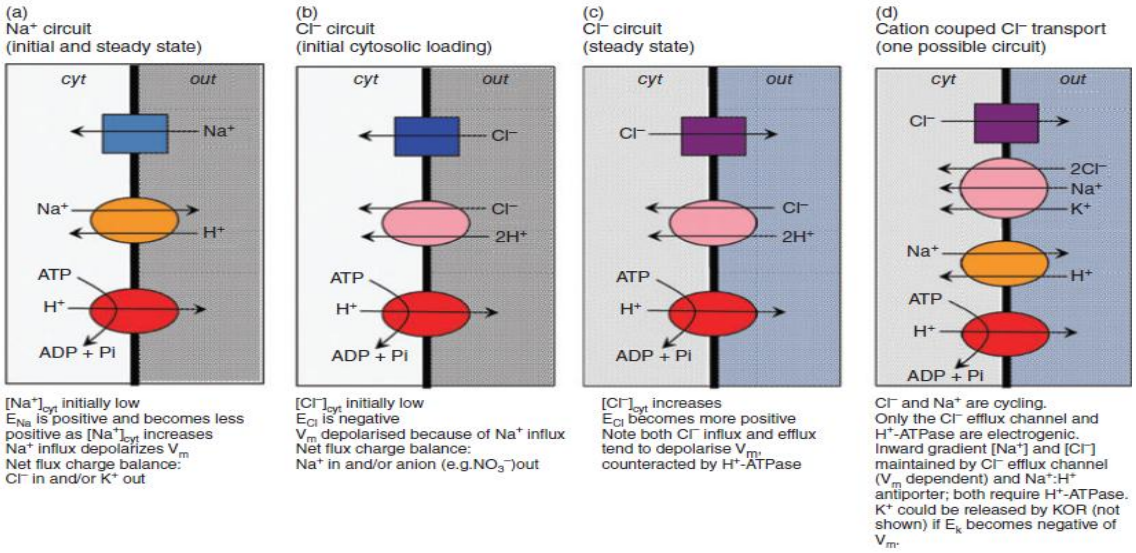


Figure 1.9. Hypothetical transport circuits in the plasma membrane (Source: Teakle and Tyerman, 2010).

1.4.5. Genetic Control of Salt Tolerance

The physiological and biochemical responses produced by plants after being exposed to salinity are controlled by genes that encode salt tolerance mechanisms (Cases *et al.* 1992). Salinity tolerance is a quantitative trait which means that it is controlled by the interaction of many salt responsive genes (Sahi *et al.*, 2006; Winicov, 1998). Ion homeostasis, ion transport proteins, osmotic adjustment, osmolyte production and ROS scavenging enzymes are a few consequences of these salt responsive genes (Benke *et al.*, 2010; Blumwald *et al.*, 2004). Hormones, mediators, transcription factors and regulatory genes are modulators of salinity tolerance (Mishra *et al.*, 2006). Genetic,

transcriptomic and proteomic analysis showed that there are two main groups of abiotic-stress inducible genes: i) genes that directly protect the plant from environmental stress; and ii) genes that indirectly protect the plant by modulating gene expression and signal transduction pathways (Hasegawa and Bressan, 2000; Kawaura *et al.*, 2008; Mishra *et al.*, 2006; Popova *et al.*, 2008; Ueda et *et al.*, 2002). In Table 1.2, major genes/proteins that are activated under salinity are shown. The exact functions of these molecules must be elucidated to understand the molecular mechanisms of stress tolerance in plants.

Table 1.2. Major categories of genes/proteins related to salt-stress responses/tolerances in plants (Source: directly taken from Javid *et al.*, 2011)

Functionality class	Possible role in stress	References
Signalling molecules	Stress signal transduction and gene expression	Cardinale *et al.* 2002; Pardo *et al.* 1998; Saijo *et al.* 2000; Ulm *et al.* 2002
Transcriptional and post-transcriptional machinery	Transcriptional regulation of stress gene expression, transcript stability, turnover, processing	Cooper *et al.* 2003; Lee *et al.* 2001; Park *et al.* 2001; Sanan-Mishra *et al.* 2005
Translational machinery	Stress-regulated protein translation, selective translation, transport, localization	Wood *et al.* 2000; Wood and Oliver 1999
Protein folding	Maintenance of protein structures, protein folding, preventing protein denaturation, protein sorting, targeting	Sun *et al.* 2001
Protein turnover	Regulation of protein metabolism, targeted protein degradation in response to stress	Khedr *et al.* 2003; Moon *et al.* 2004
Osmoprotectants	Osmotic adjustment, protection of cellular structures and macromolecules	Nomura *et al.* 1998; Tarczynski *et al.* 1993
Transport protein	Ion homeostasis during stress, compartmentalization of solutes and amino acids	Apse *et al.* 1999; Gisbert *et al.* 2000; Shi *et al.* 2000; Zhang and Blumwald 2001
ROS scavengers, cell death, senescence and ageing	Detoxification of free oxygen radicals, cell death, hypersensitive response	Reddy and Sopory 1999; Roxas *et al.* 1997

(Cont. on next page)

Table 1.2. (cont.)

Metal-binding proteins	Affecting cellular metabolism, metal ion homeostasis, acting as cofactors for critical reactions, signaling, metal toxicity, secondary stress responses, oxidative stress	Kawasaki *et al.* 2001; Sahi *et al.* 2003
Photosynthesis	Regulation of photosynthesis	Kawasaki *et al.* 2001; Sahi *et al.* 2003
Defense-related proteins	Protection against biotic stress including viral, bacterial and fungal infestation	Cheong *et al.* 2002; Dombrowski 2003; Reymond *et al.* 2000
Hormone-related proteins	Hormonal homeostasis and gene expression	Kalifa *et al.* 2004
General metabolism	Overall cellular function, housekeeping metabolic pathways carbohydrate, fatty acid and protein synthesis and modifications membrane fluidity, nitrogen metabolism, carbon and nitrogen fixation	Hoshida *et al.* 2000; Jeong *et al.* 2002

1.5. Current Techniques for Improving Crop Salinity Tolerance

Several different methods including germplasm selection, marker assisted selection, transcriptional profiling, metabolomics, proteomics and transgenics are employed in crop salinity improvement studies.

Messenger RNAs can be a target in these studies because mRNAs are differentially transcribed in tolerant and sensitive genotypes. The most important consideration is representation of the stress environmental conditions during the experiment because environmental conditions can easily affect stress responses. Also it is known that stress responses differ among plant growth stages and among genotypes (Ashraf and McNeilly, 2004; Munns, 2005; Munns and Tester, 2008). Gene-specific or genome wide expression patterns and functional genomics are more recent techniques involved in these studies (Kuhn, 2001).

In the last decade, transcriptional technologies have been improved and now they can be used for the analysis of mRNA from samples to generate multi-dimensional measurements of differentially expressed genes. These techniques are mainly divided

into two classes: open and closed systems. Amplified Fragment Length Polymorphism (AFLP), Serial Analysis of Gene Expression (SAGE), Massively Parallel Signature Sequencing (MPSS) and Real-time RT-PCR are some examples of open systems. They can be used in the discovery of novel genes but they do not guarantee whole genome coverage (Cheng *et al.*, 2008; Drea *et al.*, 2009; Nakano *et al.*, 2006; Sreenivasulu *et al.*, 2010). On the other hand, closed systems like microarrays are developed based on annotated information, therefore, they can be used to study hundreds and thousands of genes from a single experiment (Lee *et al.*, 2005; Seki *et al.*, 2002). Because of these facts, microarrays have become the major technique to determine differential gene expression in salinity, drought and cold tolerance studies (Dai *et al.*, 2007; Nakashima *et al.*, 2009; Seki *et al.*, 2002).

1.6. Developing Salt-Tolerant Crop Plants

Salinity tolerance is a quantitative trait. Poehlman (1987) reported the features of multiple gene inheritance: i) the phenotype is affected by a number of genes at different loci, ii) each gene can have a small effect on the phenotype, iii) it shows continuous variation because of the additive effect of multigenes, iv) phenotype is the result of the interaction between genotype and environment, v) transgressive segregation can be observed.

Genetic transformation, molecular markers and quantitative trait locus (QTL) analysis are used for better understanding of plant salt tolerance. The identification of salt responsive genes is crucial for the development of salt tolerant plants (Shen *et al.*, 1997; Winicov, 1998; Apse *et al.*, 1999; Grover *et al.*, 1999). Molecular markers are tools that can be used in the mapping, identification, characterization and comparison of QTLs with significant effects on plant salt tolerance at different developmental stages (Ellis *et al.*, 1997; Foolad and Lin, 1998; Foolad and Lin, 2001). Fine mapping of QTLs with the aid of molecular marker technology can facilitate identification of causal genes and these genes can be used in marker-assisted selection for the development of cultivars with better tolerance.

Many genes/proteins related to salt-stress responses/tolerances in plants have been found but the use of this information in traditional plant breeding or MAS has not yet resulted in the development of cultivars with increased salinity tolerance.

Transgenic approaches have been employed to obtain genetically modified plants that are tolerant to salt stress. Genes for enzymes that are responsible for the production of osmolytes, such as mannitol (Thomas *et al.*, 1995) and glycine betaine (Lilius *et al.*, 1996), are examples of the transgenic approach.

1.7. *S. pennellii* and salinity

Tomato is sensitive to moderate levels of salt stress and is produced in areas that are increasingly affected by salinity. It is well known that wild relatives of tomato are easy to cross with cultivated tomato and these wild relatives contain traits like resistance and tolerance for biotic and abiotic stresses including salinity. *S. pennellii* accession LA716 has been reported as salt tolerant in several studies (Hajjar and Hodgkin, 2007). In 1977, Dehan and Tal suggested that *S. peruvianum* could be used as a potential source of germplasm for salt resistance. They suggested that wild relatives of tomato like *S. pennellii* and *S. peruvianum* have better osmotic adjustments than *S. lycopersicum* and therefore they perform better under salinity. In 1995, Eshed and Zamir developed an introgression line population of *S. pennellii* in the cultivated tomato that enabled the identification and fine mapping of yield-associated QTL (Figure 10). Each of the lines contains a single introgression from *S. pennellii*, while the rest of the genome is from *S. lycopersicum.* Moreover, these researchers showed that the gene pool of *S. pennellii*, the small, green-fruited tomato, can serve as a source of agriculturally important genes (Eshed and Zamir, 1995).

In previous work, *S. lycopersicum* M82, *S. pennellii* LA716 and *S. pennellii* ILs (introgression lines), which each contain a portion of *S. pennellii* chromosome in the cultivated tomato genomic background, were evaluated for growth and levels of antioxidant activity and content under both control and salt stress conditions (150 mM NaCl) (Frary *et al.*, 2010). These data were used to identify QTLs responsible for controlling antioxidant parameters under both control and stress conditions. Several salt tolerance characteristics like plant height, leaf dry mass, root dry mass, antioxidants, flavonoids, and phenolics were observed in IL11-1, IL6-1, and IL7-4-1 (Frary *et al.*, 2010; Frary *et al.*, 2011). These lines are important because they provide the starting material needed to more precisely localize and identify genes involved in salt tolerance.

Thus, the purpose of this study was development of subNILs for the fine mapping of salt tolerance related traits in chromosome 6, 7, and 11 of tomato.

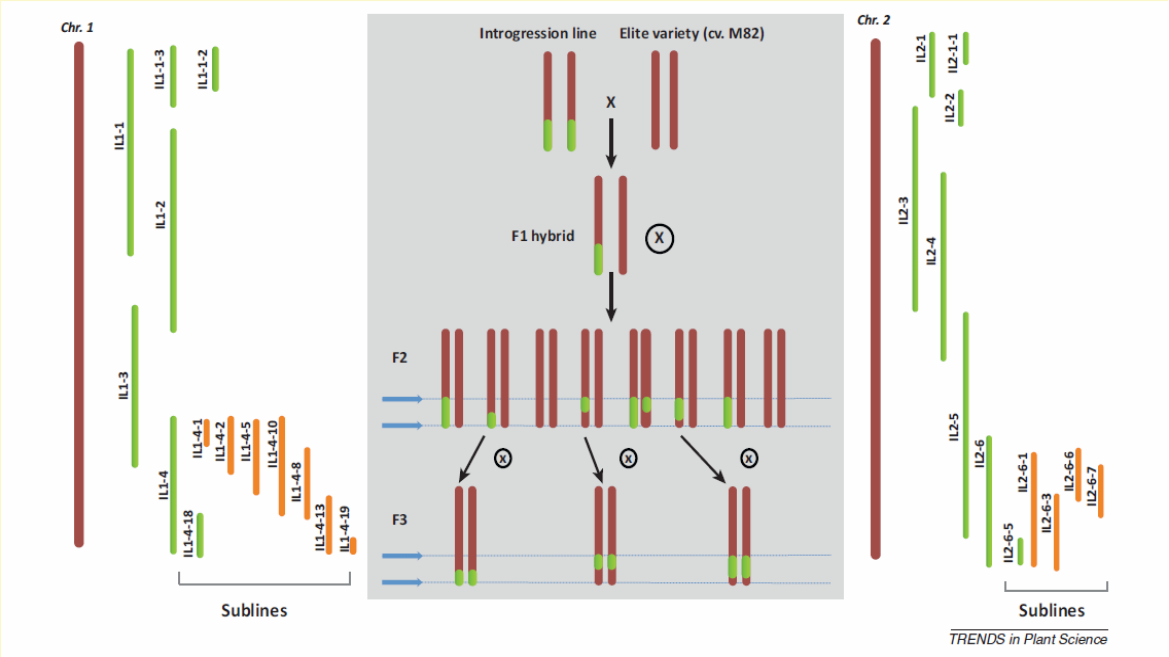


Figure 1.10. Generation of *S. pennellii* sublines
(Source: Alseekh et al, 2013)

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study *S. lycopersicum* M82, a red-fruited cultivated tomato, was used as a salt sensitive line and *S. pennellii* ILs (IL6-1, IL7-4-1, and IL11-1) developed by Eshed and Zamir were used as salt tolerant lines. *S. lycopersicum* M82 was crossed with each *S. pennellii* IL to create F2 populations. These three F2 populations containing 3000 individuals each were screened with molecular markers (Figure 2.1, 2.2, 2.3) to identify recombinant F2 plants. The recombinant individuals in the *S. lycopersicum* M82 X *S. pennellii* IL11-1 F2 population were self-pollinated to generate F3 recombinant classes. In the F3 population, 1600 plants were screened with molecular markers and recombinant individuals were self-pollinated to produce homozygous F4 individuals. All of the crosses were done in Alata Horticultural Research Station in Mersin, Turkey.

2.2. Methods

2.2.1 DNA Extraction

DNA was extracted from the leaves of tomatoes using the CTAB total DNA isolation method described by Doyle and Doyle (1987). All DNA samples were dissolved in distilled water and stored at -20 °C. After isolation, DNA concentration and quality were measured using Thermo Scientific Multiscan Go Spectrophotometer. Each sample of DNA was diluted to ~ 55 ng/µl with distilled water.

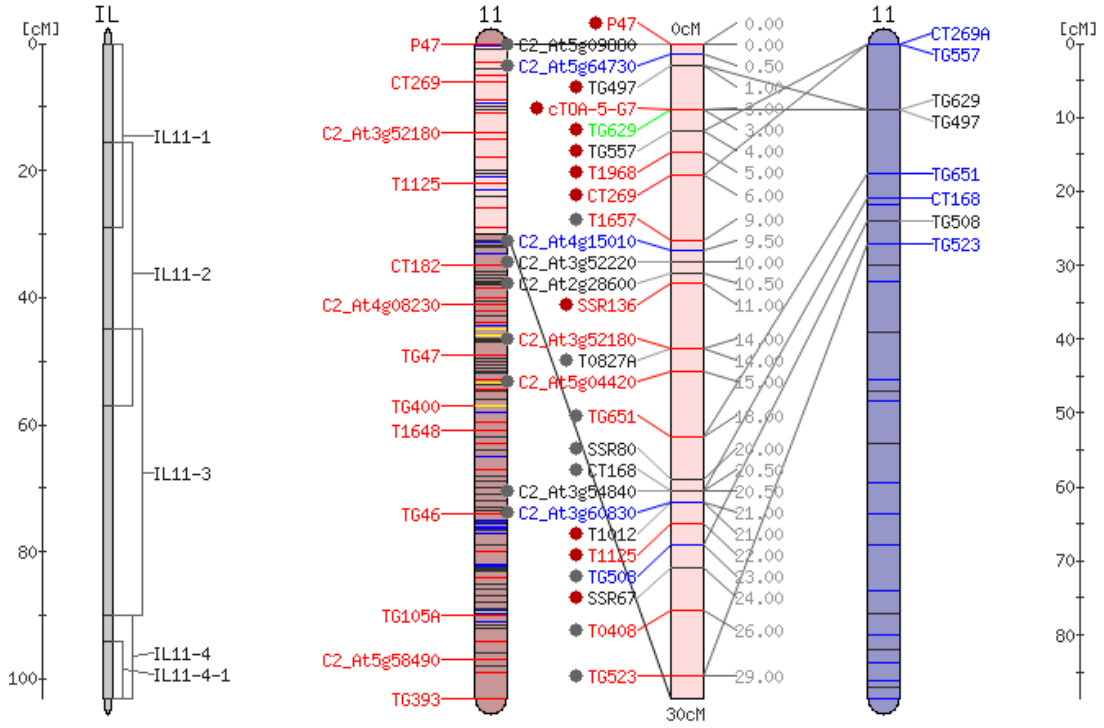


Figure 2.1. *S. pennellii* IL11-1 introgression region which encompasses 29 cM (Source: Fulton *et al*., 2002)

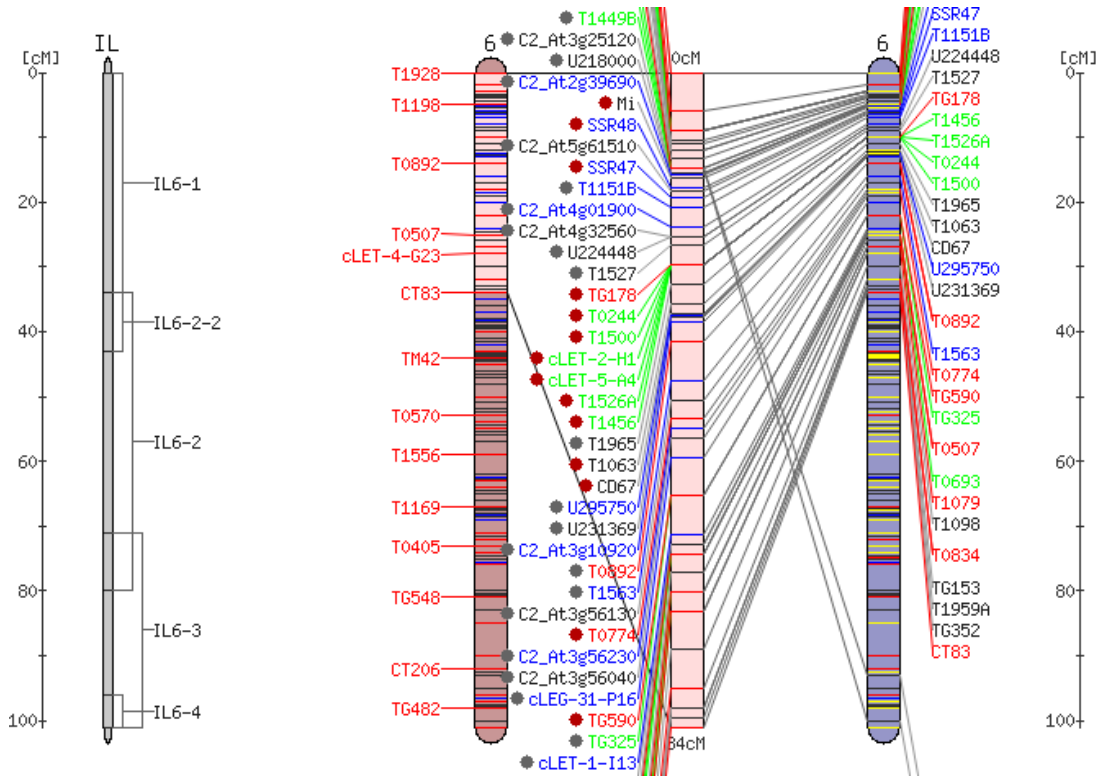


Figure 2.2. *S. pennellii* IL6-1 introgression region which encompasses 34 cM (Source: Fulton *et al*., 2002)

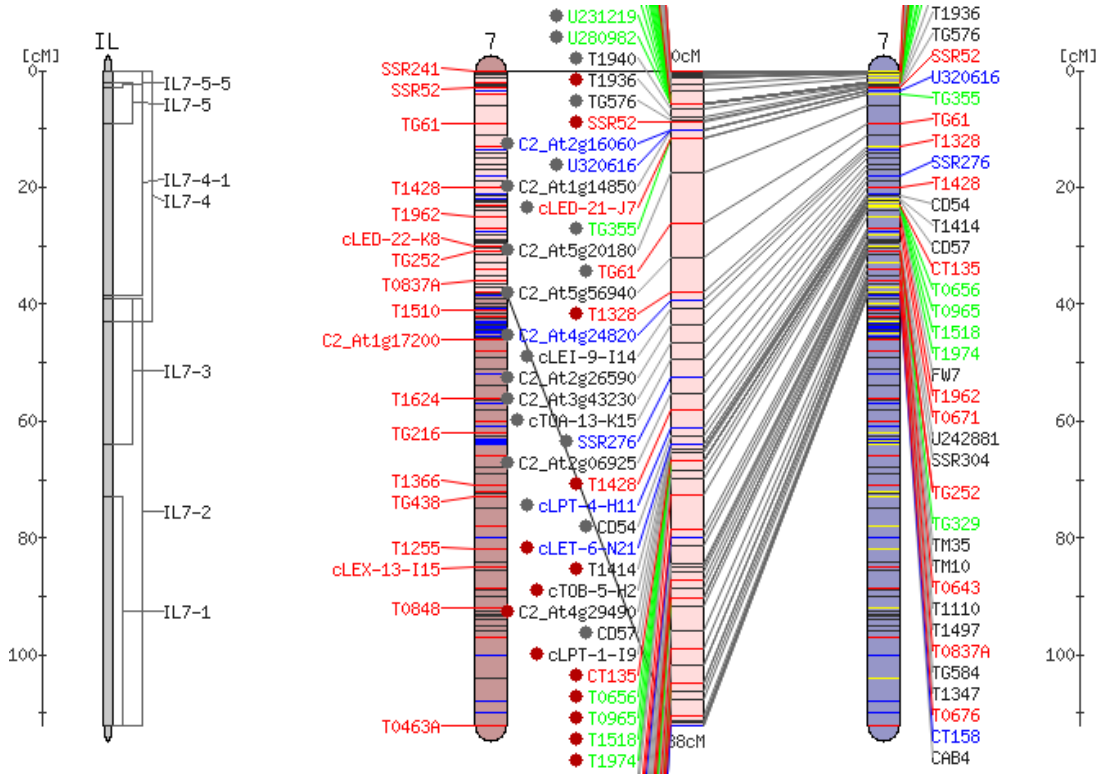


Figure 2.3. *S. pennellii* IL7-4-1 introgression region which encompasses 38 cM (Source: Fulton *et al*., 2002)

2.2.2. Molecular Marker Analyses – Parental Surveys

For genotyping of the samples, Cleaved Amplified Polymorphic Sequence (CAPs) marker analyses were done using COSII molecular markers (Solgenomics). SSR molecular markers were also used (Solgenomics). For the determination of polymorphic markers, parental surveys were first performed. The four parents (M82, IL6-1, IL7-4-1, and IL11-1) were tested with markers present in these chromosome regions according to the map of Fulton *et al.* (2002) which is available in Sol Genomics Network (http://solgenomics.net/). For both COSII markers and SSR markers, 25 µl of PCR mixture was prepared and contained: 2.5 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 0.5 µl dNTP (0.2 mM), 0.5 µl forward and 0.5 µl reverse primers (10 pmol), 0.25 µl Taq polymerase (0.25 U), 18.75 µl sterile distilled water, and 2 µl DNA (~55 ng/µl). Amplified DNA samples were separated on 2 or 3% agarose gels in 1X TAE buffer (0.25 M Tris base, 12.75 M EDTA adjusted to 1 L with distilled water and pH 8.3 with acetic acid). Samples were run at 100 V for 2 hours and visualized under UV light after ethidium bromide staining. For the SSR markers,

Advanced Analytical Fragment Analyzer was used to achieve high precision in polymorphism detection.

2.2.3. Molecular Marker Analyses – Populations

A total of 3000 F2 individuals obtained by crossing *S. lycopersicum* M82, with each introgression line, were used. The F2 individuals were screened with molecular markers that delimit the 30-40 introgressions. C2_At3g25120 and TG590 were used for IL6-1, SSR 241 and C2_At4g29490 were used for IL7-4-1, and C2_At5g09880 and TG523 were used for IL11-1. After screening, individuals that carries recombination events in these introgression lines were identified. Recombinant *S. lycopersicum* X *S. pennellii* IL11-1 F2 plants were self-pollinated to generate F3 recombinant classes. A total of 1600 F3 recombinant plants were characterized with molecular markers in the introgression region listed in Table 2.1. Then recombinant F3 plants were self-pollinated and the F4 individuals, subNILs were produced.

Table 2.1. Molecular markers used in this study.

Marker Name	Location	Sequence (5' to 3')
C2_At3g25120 F	Chr. 6	CCTTCCTCGGATCGAAAACATT
C2_At3g25120 R	Chr. 6	AGCACTTGGATAGGCGACCATTC
TG 590 F	Chr. 6	GTGAACTGGTTCAAACCAAACTTC
TG 590 R	Chr. 6	GGCGTGCTGCTGTTTGATTCTCCT
SSR 241 F	Chr. 7	TCAACAGCATAGTGGAGGAGG
SSR 241 R	Chr. 7	TCCTCGGTAATTGATCCACC
C2_At4g29490 F	Chr. 7	AAGAGCAAACTCGACATTGCACC
C2_At4g29490 R	Chr. 7	ACAAGTAGGCGAAATAGCTCTCCTG
C2_At5g09880 F	Chr. 11	AAAACATGTTTGATCCTGCAACTGAG
C2_At5g09880 R	Chr. 11	CCTTTGAACTTGGCATCATATTCAT
C2_At5g64730 F	Chr. 11	TGAAGTCCGCGATGTCCATGTCAC
C2_At5g64730 R	Chr. 11	ACATGAACAAAGTTTTGAATTGTCC
C2_At3g52220 F	Chr. 11	TGCTCGGGTGGATGGTCTTGG

(Cont. on next page)

Table 2.1. (cont.)

C2_At3g52220 R	Chr. 11	TGATGGTGAACTTGGTTCTTCCC
TG 523 F	Chr. 11	TGGGTCTACAGCTACCACCA
TG 523 R	Chr. 11	GCGAATCACGAAGTGCATAA
SSR 136 F	Chr. 11	GAAACCGCCTCTTTCACTTG
SSR 136 R	Chr. 11	CAGCAATGATTCCAGCGATA
SSR 80 F	Chr. 11	GGCAAATGTCAAAGGATTGG
SSR 80 R	Chr. 11	AGGGTCATGTTCTTGATTGTCA
SSR 67 F	Chr. 11	GCACGAGACCAAGCAGATTA
SSR 67 R	Chr. 11	GGGCCTTTCCTCCAGTAGAC

2.2.4. Bioinformatics

The chromosome 11-1 portion of the *S. lycopersicum* DNA sequence was used to assess the presence/absence of already known abiotic stress tolerance genes in plants. For this purpose, all of the known abiotic stress tolerance genes were used to create a database. Then NCBI-SPIDEY tool (http://www.ncbi.nlm.nih.gov/spidey/) was used to search for similarities between the DNA sequence and database. These results were filtered using a parsing tool.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. DNA Extraction and Quality Control

Genomic DNAs of individuals were isolated and their qualities were determined with the aid of agarose gel electrophoresis. In addition, quantities of samples were analyzed using Thermo Scientific Multiskan Go Spectrophotometer. In this study, genomic DNA of almost 7800 individuals was isolated. Therefore, DNA qualities and quantities of only some individuals are shown (Figure 3.1 and Table 3.1). A260/280 ratio is an indication of purity and pure DNA solutions have an A260/280 ratio greater than or equal to 1.8. Lower values indicate protein contamination. The A260/230 ratio is another measure of DNA purity and can be used to determine chemical contamination. The A260/230 ratio should be ideally between 1.8 and 2.2. All DNA samples were dissolved in TE buffer and stored at -20 °C.

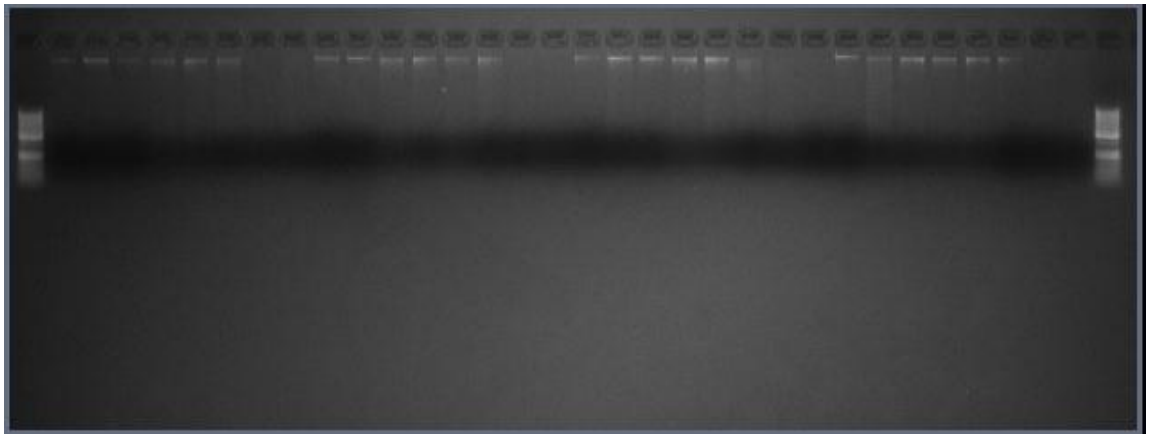


Figure 3.1. DNA quality check from some individuals in agarose gel electrophoresis

Table 3.1. DNA quantity check and absorbance values of individuals shown in Figure 3.1 using Spectrophotometer

ID	ng/ul	A260/280	A260/230	ID	ng/ul	A260/280	A260/230
1	1.52	-2.88	0.01	17	3.40	2.95	0.01
2	4.92	5.64	0.02	18	7.53	2.46	0.01
3	3.33	2.72	0.01	19	7.34	3.03	0.02
4	3.91	2.08	0.02	20	2.29	3.65	0.01
5	5.84	2.66	0.02	21	1.42	0.74	0.00
6	4.56	2.52	0.01	22	9.60	1.93	0.02
7	6.42	1.95	0.02	23	22.59	1.92	0.05
8	6.86	2.03	0.02	24	6.08	2.25	0.01
9	2.47	2.37	0.01	25	7.27	2.06	0.03
10	3.69	1.71	0.02	26	6.40	1.98	0.03
11	5.27	1.83	0.01	27	39.43	1.73	0.09
12	4.95	2.76	0.01	28	2.16	1.72	0.01
13	5.08	2.13	0.02	29	7.25	1.73	0.02
14	5.59	2.09	0.01	30	9.85	2.33	0.02
15	6.19	2.28	0.02	31	7.32	4.05	0.01
16	6.19	2.03	0.02	32	5.97	2.64	0.01

3.2. *S. lycopersicum* X *S. pennellii* IL 6-1 F2 Population

The IL 6-1 F2 population consisted of 1584 individuals which were genotyped with markers C2_At3g25120 and TG590 (Table 3.2). Only four individuals of the 1584 plant F2 population were recombinant and because of this low number, this part of the project was cancelled.

Table 3.2. Genotyping of *S. lycopersicum* X *S. pennellii* IL 6-1 F2 individuals with C2_At3g25120 and TG590. '1' represents *S. lycopersicum* M82 alleles, '3' represents *S. pennellii* IL 6-1 alleles and '2' represents heterozygous individuals.

ID	C2_At3g25120	TG590
S. lycopersicum M82	1	1
S. pennellii IL 6-1	3	3
F2-F42	1	3
F2-E46	3	1
F2-G79	1	3
F2-H21	3	2

3.3. *S. lycopersicum* X *S. pennellii* IL 7-4-1 F2 Population

The IL 7-4-1 F2 population consisted of 1592 individuals which were genotyped with markers SSR 241 and C2_At4g29490 (Table 3.3). Only three individuals of the 1592 individual F2 population were recombinant and because of this low number of recombinants, this part of the project was cancelled.

Table 3.3. Genotyping of *S. lycopersicum* X *S. pennellii* IL 7-4-1 F2 individuals with SSR 241 and C2_At4g29490. '1' represents *S. lycopersicum* M82 alleles, '3' represents *S. pennellii* IL 7-4-1 alleles and '2' represents heterozygous individuals.

ID	SSR 241	C2_At4g29490
S. lycopersicum M82	1	1
S. pennellii IL7-4-1	3	3
F2-A78	1	3
F2-D59	3	1
F2-G11	1	3

3.4. *S. lycopersicum* X *S. pennellii* IL 11-1 F2 Population

The IL 11-1 F2 population consisted of 2976 individuals which were genotyped with the aid of C2_At5g09880 and TG523 (Table 3.4). A total of 30 individuals of 2976 F2 population were recombinant individuals and these individuals were selfed for the creation of the F3 population.

Table 3.4. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F2 individuals with C2_At5g09880 and TG523. '1' represents *S. lycopersicum* M82 alleles, '3' represents *S. pennellii* IL 11-1 alleles and '2' represents heterozygous individuals.

ID	C2_At5g09880	TG523	ID	C2_At5g09880	TG523
S. lycopersicum M82	1	1	13T021	1	2
S. pennellii IL11-1	3	3	13T022	3	2
13T007	1	2	13T023	1	3
13T008	2	3	13T024	1	2
13T009	2	3	13T025	1	2
13T010	1	2	13T026	3	2
13T011	3	2	13T027	3	2
13T012	3	2	13T028	2	3
13T013	1	3	13T029	2	3
13T014	1	2	13T030	1	2
13T015	1	2	13T031	3	2
13T016	3	2	13T032	3	2
13T017	3	2	13T033	1	2
13T018	1	2	13T034	2	3
13T019	2	3	13T035	2	3
13T020	2	3	13T036	1	2

3.5 *S. lycopersicum* X *S. pennellii* IL 11-1 F3 Populations

The F3 population consisted of nearly 1536 individuals which were genotyped with the aid of several markers as described in the Materials and Methods. At the end of genotyping analysis, 228 homozygous and 620 heterozygous recombinant individuals were detected. These individuals belonged to 5 homozygous recombination classes and 20 heterozygous recombinant classes based on the location of the recombination event. Eight recombinants were homozygous for *S. pennellii* alleles from SSR80 to the bottom of the introgression (Table 3.5). Thirty-five recombinants were homozygous for *S. pennellii* alleles from C2_At3g52220 to the bottom of the introgression (Table 3.6). Thirty-six recombinants were homozygous for *S. pennellii* alleles between C2_At3g52220 and SSR136 (Table 3.7). One hundred and eight recombinants were homozygous for *S. pennellii* alleles from the top of the introgression to SSR136 (Table 3.8). Only two recombinants were homozygous for *S. pennellii* alleles except from C2_At5g64730 to SSR80 (Table 3.9).

Table 3.5. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F3 8 homozygous recombinant individuals with C2_At5g09880 (0 cM), C2_At5g64730 (0.50 cM), C2_At3g52220 (10 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM). '1' represents *S. lycopersicum* M82 alleles, and '3' represents *S. pennellii* IL 11-1 alleles. The pattern was 1-1-1-1-3-3-3.

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A78-1	1	1	1	1	3	3	3
A119-3	1	1	1	1	3	3	3
A119-4	1	1	1	1	3	3	3
A120-1	1	1	1	1	3	3	3
A120-120	1	1	1	1	3	3	3
A120-124	1	1	1	1	3	3	3
A120-130	1	1	1	1	3	3	3
A120-135	1	1	1	1	3	3	3

Table 3.6. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F3 35 homozygous recombinant individuals with C2_At5g09880 (0 cM), C2_At5g64730 (0.50 cM), C2_At3g52220 (10 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM). '1' represents *S. lycopersicum* M82 alleles, and '3' represents *S. pennellii* IL 11-1 alleles. The pattern was 1-1-3-3-3-3-3.

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A120-149	1	1	3	3	3	3	3
A120-152	1	1	3	3	3	3	3
A120-156	1	1	3	3	3	3	3
A120-168	1	1	3	3	3	3	3
A120-38	1	1	3	3	3	3	3
A120-46	1	1	3	3	3	3	3
A120-48	1	1	3	3	3	3	3
A120-51	1	1	3	3	3	3	3
A120-63	1	1	3	3	3	3	3
A120-73	1	1	3	3	3	3	3
A120-77	1	1	3	3	3	3	3
A120-89	1	1	3	3	3	3	3
A120-97	1	1	3	3	3	3	3

(Cont. on next page)

Table 3.6. (cont.)

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A120-99	1	1	3	3	3	3	3
A123-11	1	1	3	3	3	3	3
A123-13	1	1	3	3	3	3	3
A123-14	1	1	3	3	3	3	3
A123-17	1	1	3	3	3	3	3
A123-5	1	1	3	3	3	3	3
A126-4	1	1	3	3	3	3	3
A129-15	1	1	3	3	3	3	3
A129-23	1	1	3	3	3	3	3
A129-27	1	1	3	3	3	3	3
A129-40	1	1	3	3	3	3	3
A129-44	1	1	3	3	3	3	3
A129-47	1	1	3	3	3	3	3
A129-48	1	1	3	3	3	3	3
A129-53	1	1	3	3	3	3	3
A129-64	1	1	3	3	3	3	3
A129-66	1	1	3	3	3	3	3
A129-77	1	1	3	3	3	3	3
A129-79	1	1	3	3	3	3	3
A129-8	1	1	3	3	3	3	3
A129-9	1	1	3	3	3	3	3
A140-16	1	1	3	3	3	3	3

Table 3.7. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F3 36 homozygous recombinant individuals with C2_At5g09880 (0 cM), C2_At5g64730 (0.50 cM), C2_At3g52220 (10 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM). '1' represents *S. lycopersicum* M82 alleles, and '3' represents *S. pennellii* IL 11-1 alleles. The pattern was 1-1-3-3-1-1-1.

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A140-21	1	1	3	3	1	1	1
A140-27	1	1	3	3	1	1	1
A178-34	1	1	3	3	1	1	1
A19-10	1	1	3	3	1	1	1
A19-11	1	1	3	3	1	1	1
A19-12	1	1	3	3	1	1	1
A21-1	1	1	3	3	1	1	1
A21-11	1	1	3	3	1	1	1
A21-14	1	1	3	3	1	1	1

(Cont. on next page)

Table 3.7. (cont.)

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A21-15	1	1	3	3	1	1	1
A21-17	1	1	3	3	1	1	1
A21-2	1	1	3	3	1	1	1
A21-22	1	1	3	3	1	1	1
A21-23	1	1	3	3	1	1	1
A21-26	1	1	3	3	1	1	1
A21-3	1	1	3	3	1	1	1
A21-34	1	1	3	3	1	1	1
A21-4	1	1	3	3	1	1	1
A21-42	1	1	3	3	1	1	1
A21-8	1	1	3	3	1	1	1
A44-11	1	1	3	3	1	1	1
A44-13	1	1	3	3	1	1	1
A44-19	1	1	3	3	1	1	1
A44-22	1	1	3	3	1	1	1
A44-23	1	1	3	3	1	1	1
A44-26	1	1	3	3	1	1	1

Table 3.8. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F3 108 homozygous recombinant individuals with C2_At5g09880 (0 cM), C2_At5g64730 (0.50 cM), C2_At3g52220 (10 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM). '1' represents *S. lycopersicum* M82 alleles, and '3' represents *S. pennellii* IL 11-1 alleles. The pattern was 3-3-3-3-1-1-1.

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A46-1	3	3	3	3	1	1	1
A78-1	3	3	3	3	1	1	1
A78-108	3	3	3	3	1	1	1
A78-109	3	3	3	3	1	1	1
A78-111	3	3	3	3	1	1	1
A78-112	3	3	3	3	1	1	1
A78-116	3	3	3	3	1	1	1
A78-135	3	3	3	3	1	1	1
A78-144	3	3	3	3	1	1	1
A78-145	3	3	3	3	1	1	1
A78-149	3	3	3	3	1	1	1
A78-157	3	3	3	3	1	1	1
A78-16	3	3	3	3	1	1	1

(Cont. on next page)

Table 3.8. (cont.)

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A78-157	3	3	3	3	1	1	1
A78-16	3	3	3	3	1	1	1
A78-161	3	3	3	3	1	1	1
A78-176	3	3	3	3	1	1	1
A78-177	3	3	3	3	1	1	1
A78-178	3	3	3	3	1	1	1
A78-18	3	3	3	3	1	1	1
A78-18	3	3	3	3	1	1	1
A78-197	3	3	3	3	1	1	1
A78-199	3	3	3	3	1	1	1
A78-20	3	3	3	3	1	1	1
A78-200	3	3	3	3	1	1	1
A78-207	3	3	3	3	1	1	1
A78-209	3	3	3	3	1	1	1
A78-21	3	3	3	3	1	1	1
A78-212	3	3	3	3	1	1	1
A78-215	3	3	3	3	1	1	1
A78-217	3	3	3	3	1	1	1
A78-226	3	3	3	3	1	1	1
A78-227	3	3	3	3	1	1	1
A78-231	3	3	3	3	1	1	1
A78-235	3	3	3	3	1	1	1
A78-235	3	3	3	3	1	1	1
A78-237	3	3	3	3	1	1	1
A78-24	3	3	3	3	1	1	1
A78-244	3	3	3	3	1	1	1
A78-255	3	3	3	3	1	1	1
A78-258	3	3	3	3	1	1	1
A78-259	3	3	3	3	1	1	1
A78-26	3	3	3	3	1	1	1
A78-262	3	3	3	3	1	1	1
A78-271	3	3	3	3	1	1	1
A78-273	3	3	3	3	1	1	1
A78-281	3	3	3	3	1	1	1
A78-287	3	3	3	3	1	1	1
A78-290	3	3	3	3	1	1	1
A78-298	3	3	3	3	1	1	1

(Cont. on next page)

Table 3.8. (cont.)

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
A78-30	3	3	3	3	1	1	1
A78-290	3	3	3	3	1	1	1
A78-298	3	3	3	3	1	1	1
A78-30	3	3	3	3	1	1	1
A78-300	3	3	3	3	1	1	1
A78-301	3	3	3	3	1	1	1
A78-309	3	3	3	3	1	1	1
A78-31	3	3	3	3	1	1	1
A78-311	3	3	3	3	1	1	1
A78-313	3	3	3	3	1	1	1
A78-39	3	3	3	3	1	1	1
A78-47	3	3	3	3	1	1	1
A78-49	3	3	3	3	1	1	1
A78-52	3	3	3	3	1	1	1
A78-59	3	3	3	3	1	1	1
A78-70	3	3	3	3	1	1	1
A78-8	3	3	3	3	1	1	1
A78-84	3	3	3	3	1	1	1
A78-86	3	3	3	3	1	1	1
A78-9	3	3	3	3	1	1	1
A78-90	3	3	3	3	1	1	1
A78-94	3	3	3	3	1	1	1
A78-96	3	3	3	3	1	1	1
A78-96	3	3	3	3	1	1	1
A78-ERSOY	3	3	3	3	1	1	1
B142-11	3	3	3	3	1	1	1
B142-17	3	3	3	3	1	1	1
B142-41	3	3	3	3	1	1	1
B142-44	3	3	3	3	1	1	1
B142-46	3	3	3	3	1	1	1
B142-49	3	3	3	3	1	1	1
B142-51	3	3	3	3	1	1	1
B142-54	3	3	3	3	1	1	1
B142-56	3	3	3	3	1	1	1
B142-7	3	3	3	3	1	1	1
B151-17	3	3	3	3	1	1	1

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Table 3.8. (cont.)

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
B151-181	3	3	3	3	1	1	1
B151-29	3	3	3	3	1	1	1
B151-33	3	3	3	3	1	1	1
B151-35	3	3	3	3	1	1	1
B151-4	3	3	3	3	1	1	1
B151-46	3	3	3	3	1	1	1
B151-47	3	3	3	3	1	1	1
B151-66	3	3	3	3	1	1	1
B151-7	3	3	3	3	1	1	1
B151-73	3	3	3	3	1	1	1
B151-95	3	3	3	3	1	1	1
B157-121	3	3	3	3	1	1	1
B157-13	3	3	3	3	1	1	1
B157-141	3	3	3	3	1	1	1
B157-15	3	3	3	3	1	1	1
B157-155	3	3	3	3	1	1	1
B157-163	3	3	3	3	1	1	1
B157-167	3	3	3	3	1	1	1
B157-19	3	3	3	3	1	1	1
B157-200	3	3	3	3	1	1	1
B157-218	3	3	3	3	1	1	1
B157-41	3	3	3	3	1	1	1
B157-57	3	3	3	3	1	1	1

Table 3.9. Genotyping of *S. lycopersicum* X *S. pennellii* IL 11-1 F3 2 homozygous recombinant individuals with C2_At5g09880 (0 cM), C2_At5g64730 (0.50 cM), C2_At3g52220 (10 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM). '1' represents *S. lycopersicum* M82 alleles, and '3' represents *S. pennellii* IL 11-1 alleles. The pattern was 3-3-1-1-3-3-3.

ID	C2_At5g09880	C2_At5g64730	C2_At3g52220	SSR136	SSR80	SSR67	TG523
B172-14	3	3	1	1	3	3	3
B172-2	3	3	1	1	3	3	3

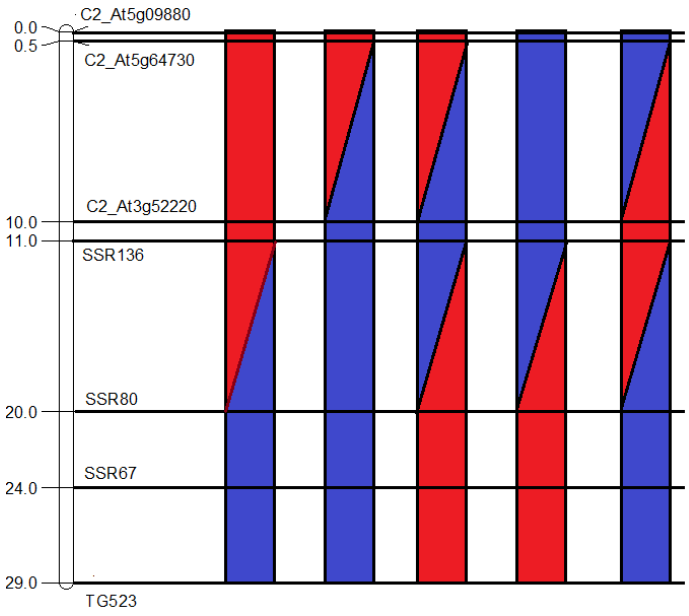

Figure 3.2. Visual representation of recombinant classes in homozygous recombinant individuals (Red color represents *S. lycopersicum* alleles, blue color represents *S. pennellii* alleles and numbers are in 'cM' distance and taken from Fulton *et al*., 2002). The numbers of individuals in each class from left to right are 8, 35, 36, 108, and 2.

Recombination events between C2_At5g64730 – C2_At3g52220 and SSR136 – SSR80 were observed. Recombination events were not observed between C2_At5g09880 – C2_At5g64730, C2_At3g52220 – SSR136, SSR80 – SSR67 and SSR67 – TG523. The lack of recombination events between C2_At5g09880 – C2_At5g64730 and C2_At3g5220 – SSR136 can be explained by the very short distance between these markers. Therefore it might be concluded that these markers are cosegregating in the F3 individuals or that recombination between these markers might be suppressed. In addition, the exact recombinant break points in the chromosome segments between C2_At5g64730 – C2_At3g52220 and SSR136 – SSR80 could not be identified. This might be caused by several reasons: i) Because of being relatively long chromosome segments, there would have been double cross-over that could not been determined with these limited number of markers ii) Because of a low number of codominant locus-specific markers in the region iii) Although these conclusions based on the recombination events in homozygous F3 individuals, in Table 3.10, it is possible to see different recombination events in different segments of the chromosome that could not be seen in these homozygous individuals. It is known that DNA sequence divergence can result in regional suppression of recombination and this might be the

consequence of evolution to preserve the function of genes that have crucial roles in the plant's life cycle.

In addition to homozygous recombinant individuals, 620 heterozygous recombinant individuals were identified and they belonged to 20 different recombination classes. These individuals should be selfed for one or two generations to create homozygous recombinant individuals carrying different types of recombination events.

Table 3.10. Different recombination classes determined by using C2_At5g09880 (0 cM), SSR136 (11 cM), SSR80 (20 cM), SSR67 (24 cM) and TG523 (29 cM) markers. '1' represents *S. lycopersicum* M82 alleles, '3' represents *S. pennellii* IL 11-1 alleles and '2' represents heterozygous ones.

Class	C2_At5g09880	SSR136	SSR80	SSR67	TG523	Number of individuals
1	3	2	1	1	1	108
2	2	2	1	1	1	92
3	2	3	1	1	1	50
4	2	3	3	3	3	49
5	3	3	2	2	2	38
6	1	2	1	1	1	36
7	1	2	3	3	3	35
8	1	2	2	2	2	33
9	2	3	2	2	2	29
10	2	3	2	2	2	29
11	1	2	3	3	3	25
12	1	3	2	2	2	21
13	1	2	1	1	1	21
14	1	2	3	3	3	20
15	2	1	1	1	1	12
16	1	1	2	3	3	8
17	3	2	2	2	2	8
18	1	1	2	2	2	3
19	3	2	3	3	3	2
20	2	1	2	2	2	1

3.6. Bioinformatics

According to a similarity search between *S. lycopersicum* sequence and a database of stress-related genes, an open reading frame (ORF) that is similar to *Coffea arabica* cDNA clone CACATN1-1548TVB were identified. This ORF is similar to Salt-induced AAA-Type ATPase (78.1 % identity). In addition, *Coffea arabica* cDNA clone CACATN1-8E54TV showed similarity with the region of interest in tomato. This cDNA is also similar to Salt-induced AAA-Type ATPase (75% identity). Finally, *Coffea arabica* cDNA clone CACAT36MER_Q4_08_24H03.F similar to DNAJ heat shock protein (81% identity) was found as a candidate stress-related gene in the region (Table 3.11). Lin et al. (2005) concluded that tomato has a nearly perfect gene-for-gene match with tomato and coffee genes share higher similarity to already known tomato genes. This result indicates that there are stress-related genes in the region of our QTLs. Further work should examine these genes more closely in the tomato genome.

Table 3. 11. Candidate genes in chromosome 11-1 segment

mRNA: gi|257023180|gb|GT003946.1|GT003946 TransId-204167 CACATN1 Coffea arabica cDNA clone CACATN1-1548TVB similar to Salt-induced AAA-Type ATPase - Mesembryanthemum crystallinum (Common ice plant), mRNA sequence, 859 bp Strand: minus Number of exons: 6 Number of splice sites: 5 mRNA coverage: 75% overall percent identity: 78.1%
mRNA: gi|257015925|gb|GR994286.1|GR994286 TransId-226870 CACATN1 Coffea arabica cDNA clone CACATN1-8E54TV similar to Salt-induced AAA-Type ATPase - Mesembryanthemum crystallinum (Common ice plant), mRNA sequence, 754 bp Strand: minus Number of exons: 5 Number of splice sites: 4 mRNA coverage: 68% overall percent identity: 75.0%

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Table 3.11. (cont.)

mRNA: gi|257012282|gb|GR989434.1|GR989434 TransId-96860 CACAT36MER Coffea arabica cDNA clone CACAT36MER_Q4_08_24H03.F similar to Symbol: None | DNAJ heat shock protein, putative (J3), identical to AtJ3 (Arabidopsis thaliana) GI:2641638, str>, 774 bp Strand: minus Number of exons: 4 Number of splice sites: 3 mRNA coverage: 81% overall percent identity: 81.2%

CHAPTER 4

CONCLUSION

Tomato is sensitive to moderate levels of salt stress and is produced in areas that are increasingly affected by salinity. Most of the wild relatives of tomato are easy to cross with cultivated tomato and provide a rich source of resistance and tolerance genes for biotic and abiotic stresses including salinity. *S. pennellii* accession LA716 has been reported as salt tolerant in several studies. In previous work (Frary et al., 2010 and 2011), several salt tolerance characteristics were observed in IL11-1, IL6-1 and IL7-4-1, tomato introgression lines which contain portions of chromosome 11, 6 and 7 from *S. pennellii* in the cultivated tomato genomic background. Fine mapping of salt tolerance related traits in these 30-40 cM introgression lines can be achieved by the creation of subNILs, recombinant lines in which the original introgression is broken into smaller genomic fragments.

In this work, F2 populations were obtained by crossing *S. lycopersicum* M82, salt-sensitive cultivated tomato, with each introgression line. The F2 individuals were screened with molecular markers that delimit the 30-40 cM introgressions. After screening, individuals that carry recombination events in these introgression lines were identified. Recombinant F2 plants in the IL11 population were self-pollinated to generate F3 recombinant classes. Each F3 recombinant plant was characterized with several codominant molecular markers in the introgression region. As a result, 228 homozygous recombinant individuals and 620 heterozygous recombinant individuals were identified. These plants will be grown hydroponically under both control and salt (150 mM NaCl) conditions and will be screened for physiological, mineral and biochemical parameters. By statistical comparison between control and salt-treated plants, it will then be possible to identify which recombinants carry regions with significant effects on the various salt tolerance responses. The gene/s in these regions, eventually, will be identified to understand genetic background in tomato.

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