INTRODUCTION
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1.1 Salinity: a major environmental concern

Stress is the environmental factor that limits plants productivity or destroys biomass (Grime 1979). Various type of stresses, affect growth of the plants. Low temperature, drought and high salinity are common stress conditions that adversely affect plant growth and crop production (Xiong et al., 2002). Stresses can be biotic (living) or abiotic (nonliving). Biotic stress decrease the crop yields by less than 10%, but abiotic stress on the other hand, can severely affect the crop yield by up to 65% of the total crop productivity (Serrano 1999). Thus abiotic stresses are one of the main challenges in expansion of planting area worldwide.

There are two main abiotic stresses that dramatically reduce plant productivity; these are drought and salinity (Zhang et al., 2000; Bray et al., 2002). These cause similar reactions in plants i.e. water stress. Water availability is the most important among the abiotic factors that have shaped and continue shaping plant evolution. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Bray et al., 2000; Lee et al., 2009). Plants need essential mineral nutrients to grow and develop. However, excessive soluble salts in the soil are harmful to most plants. In fact, no toxic substance restricts plant growth more than does salt on a world scale. Soils in arid and semi-arid regions, especially those with ineffective drainage, accumulate salt as irrigation water evaporates, leaving deposits of soluble salts. Today, salinity continues to reduce food production with perhaps the greatest threat being to sustainable irrigated agriculture (Flowers and Yeo 1995; Rengasamy 2002, 2006; Rozema and Flowers 2008; Yadav et al., 2011). An average yield of major crop plant is reduced by more than 50% (Vinocur and Altman 2005).
The loss of farmable land to salinization is in direct conflict with the needs of world’s population which is increasing exponentially, projected to increase by 1.5 billion in the next 20 yrs. (Biumwald and Grover 2006; Ismail et al., 2007). It is therefore imperative to increase the crop yield under salinity stress conditions by applying various genetic and genomic approaches. As salt stress imposes a major environmental threat to agriculture as well as plantation, forestry and afforestation effects, understanding the basic physiology and genetics of cells under salt stress is crucial for developing any transgenic strategy.

1.2 Adverse effect of salinity on plant

The effects of saline soils on plant growth have been a focus of research for nearly 100 years because salt stress is a major stress, limiting crop productivity (Fougere et al., 1991). More than 800 million ha of land throughout the world are salt-affected. It has been reported that more than 6% of the world’s total land area or 20% of the irrigated land area are affected by salinity stress according to the FAO Land and Nutrition Management Service (FAO 2008). Salinity limits crop production in arid and semi-arid regions, where soil salt content is naturally high and precipitation can be insufficient for dilution through leaching (Zhao et al., 2007). Salinity affects plant growth and development at physiological, biochemical and molecular level. Salts decrease water potential. The low osmotic potential of saline solutions hampers plant water uptake, resulting in “physiological drought”. Hence the plants become more sensitive toward salinity. It is one of the major factors causing agricultural losses, particularly in arid regions (Greenway and Mums 1980; Boyer 1982). In such circumstances, plants must decrease inner water potential so that it may uptake water continuously. The cellular and molecular responses of plants to environmental stress have been studied intensively. The direct effect of salts on plant growth may be divided into three broad categories: (i) a reduction in the osmotic potential of the soil solution that reduces plant available water, (ii) a deterioration in the physical structure of the soil such that water permeability and soil aeration are diminished, and (iii) increase in the concentration of certain ions that have an inhibitory effect on plant metabolism (Yeo 1998; Carvajal et al., 1999 and Grattan and Grieve 1999). High salinity adversely affects plant growth and development by disturbing the
intracellular ion homeostasis, which results in membrane dysfunction, attenuation of
metabolic activity and secondary effects that inhibit growth and induce cell death
(Hasegava et al., 2000). Metabolic imbalances caused by ion toxicity, osmotic stress
and nutritional efficiency under saline conditions may also lead to oxidative stress
(Zhu 2002). The stress caused by ion concentrations decreases water gradient, thus
making it more difficult for water and nutrients to move through the root
membrane. As a result, water uptake slows down and the osmotic effect spreads
from the root membrane to the internal membrane, the ion concentration inside the
plant changes the osmotic balances (Volkmar et al., 1998). Once high concentration
of salt reaches inside the plant tissues and organs, development is severely affected.
The accumulated salt causes a slower rate of extension of cells and these
compromises the dimension of the leaves (Volkmar et al., 1998). The overall effect of
salinity on plants is the final reduction of leaf size, which leads to loss of the leaf and
finally the plant. Salinity can also cause reduction in the ATPs and growth regulators
required for normal functioning in plants (Allen et al., 1994). All of these cause
adverse pleiotropic effects on plant growth and development mainly due to changes
at the molecular level (Mansour 2000) ultimately effecting physiological and
biochemical levels (Munns 2002; Yildirim et al., 2008). Therefore survival and
growth of plants in saline environments is the result of adaptive processes such as
ion transport and compartmentation, osmotic solute synthesis and accumulation
that lead to osmotic adjustment, and protein turnover for cellular repair (Munns and
Terma 1986; Poljakoff-Mayber 1998; Hong et al., 2000).

Based on their capacity to grow on high salt medium, plants are traditionally
classified as glycophytes or halophytes (Flowers et al., 1977). Halophytes are tolerant
to high concentrations of NaCl; some can withstand salts that are more than twice
the concentration of seawater. Most plants, including the majority of crop species,
are glycophytes and cannot tolerate high salinity. For glycophytes, salinity imposes
ionic stress, osmotic stress, and secondary stresses such as nutritional disorders and
oxidative stress (Zhu 2001). Sodium toxicity represents the major ionic stress
associated with high salinity. Additionally, some plant species are also sensitive to
chloride, the major anion found in saline soils. In certain saline soils, ion toxicity is
Introduction

worsened by alkaline pH. For halophytic plants that are tolerant of sodium toxicity, osmotic stress may be the main cause of growth inhibition. A generalized scheme for plant responses to abiotic stresses and mechanism of stress tolerance (Lokhande and Suprasanna 2012) is presented in figure (Fig.1).

Fig. 1.1: Generalized scheme for plant responses to abiotic stresses and mechanism of stress tolerance (adopted from Lokhande and Suprasanna 2012)
1.3 Soil salinity:

A saline soil is defined as having a high concentration of soluble salts, which affect plant growth high enough. Soil salinity is measured by its electrical conductivity (Rhoades 1996). The SI unit of electrical conductivity (EC) is dS m\(^{-1}\). 10mM NaCl has an EC close to 1dS m\(^{-1}\). For non-sensitive plants, EC measurements <4 dS m\(^{-1}\) are satisfactory. Soils with EC >4 dS m\(^{-1}\) are considered saline and plant growth may be inhibited (Pierzynski et al., 2005). According to the USDA Salinity Laboratory, saline soil is defined as having an electrical conductivity of the saturation extract (ECc) of 4dS m\(^{-1}\) or more which is equivalent to approximately 40mM NaCl and it generates an osmotic pressure of approximately 0.2MPa. The most widely accepted definition of a saline soil has been given by FAO (1996) as one that has an ECc of 4dS m\(^{-1}\) or more and soils with ECc’s exceeding 15dS m\(^{-1}\) are considered strongly saline.

1.4 Management of salinity by Plant

Plant’s tolerance or susceptibility to abiotic stresses is a complex phenomenon and plants adopt various strategies and pathways to combat stress. It is the coordinated action of various genes in different metabolic pathways that bring about the requisite phenotype and lead to plant tolerance. Plants adjust to high salt environments by activating a signal transduction system involving Ca\(^{2+}\) (Bressan et al., 1998, Epstein 1998, Serrano et al., 1999) and this leads to adaptations of growth, ion uptake and metabolism. Plants have stress-specific adaptive responses as well as responses which protect the plants from more than one environmental stress (Huang et al., 2011). Physiological, molecular and biochemical analyses have uncovered multiple mechanisms which seem to act synergistically and additively to enable plants to cope with high salinity. Salt tolerance is a response that is referred to as acclimation, phenotypic plasticity, or environmental variation (Hasegawa et al., 2000; Debat and David 2001; Zhu 2002; Munns and Tester, 2008; Taiz and Zeiger 2010). Some genetic studies revealed stress tolerance to be governed by multiple genes (Bohnert et al., 2001; Kanasaki et al., 2001).

When the plant is exposed to salinity stress, the proper function of the cell will be highly affected. Therefore plants have to adapt physiologically to these as the
local environment changes and their ability to adapt and live in a changing environment relies on tolerance to adverse growing seasons. Cell survival under stress condition directly depends on how the cell can adapt itself to the environment. In order to survive in stresses, different plant species adapt to the environment with various mechanisms. Numerous efforts have been made to study the mechanism of plant salt tolerance. To cope with the detrimental effects of salt stress, plants have evolved many biochemical and molecular mechanisms (Parida and Das 2005). Plants use three main mechanisms with which they tolerate salinity stress:

- Osmotic tolerance – the ability to maintain growth whilst under osmotic stress by various, as yet unknown, mechanisms
- Ion exclusion – reducing Na\(^+\) and Cl\(^-\) accumulation in the shoot by manipulating root ion transport processes to minimize Na\(^+\) and Cl\(^-\) delivery to the shoot
- Ion tissue tolerance – tolerating the Na\(^+\) and Cl\(^-\) that builds up in the leaf by compartmentalizing them into organelles (like vacuole) within a cell.

At the cellular level, plants adopt a wide range of responses to cope with salinity stress. The plasma membrane is mainly involved in stress signal perception, transducing into the cell, and ion and water transport. Most receptor proteins are located in the plasma membrane, and thus the plasma membrane is directly involved in stress sensing (Komatsu 2008). The tonoplast is involved in ion balance and adjustment of water content. The nucleus has a variety of functions including transcriptional regulation, signaling, and gene regulation (Hossain et al., 2012). Cellular defense mechanisms against salinity stress are mainly controlled by the expression of responsible genes and proteins. Generally, many plants are susceptible to unfavorable environmental conditions. In order to know about major gene conferring stress tolerance, it is important to understand the roles of organelles themselves in the cell. Stress-responsive genes or proteins were arranged according to the localization of the over expressed gene in the cell. Nucleus, chloroplast,
plasma membrane, ER, mitochondria, and vacuole are the organelles and compartments in which the presence of overexpressed genes was confirmed.

**Fig. 1.2:** Effect of salinity on the growth of plant

**1.5 Ion Homeostasis - Transport Determinants and Their Regulation**

NaCl is the most soluble and widespread salt and it is most abundant salt encountered by plants under salinity stress, all plants have evolved mechanisms to regulate its accumulation and to select against it in favour of other nutrient (K⁺ and NO₃⁻) Salinity entails ionic stress mainly due to Na⁺, Cl⁻, and SO₄²⁻. High levels of Na⁺ (sodicity) in soil solution can cause soil structure problems such as dispersion, and drainage problems. Excessive Na⁺ in soil can destroy soil structure, permeability and reduce water infiltration (Brady and Weil 2001). Hence Na⁺ represents the major ion causing toxicity related to high salinity. Some plant species are also sensitive to chloride, the major anion found in saline soils. High concentrations of Na⁺ disturb osmotic balance and results in physiological drought condition which prevent plant water uptake. NaCl causes rapid increase in the concentration of cytosolic free Ca²⁺ (Niu et al., 1995., Wu et al., 1996). In most plants Na⁺ and Cl⁻ are effectively excluded by roots while water is taken up from the soil (Munns 2005). Na⁺ levels should be
controlled at the entry point (Munns 2002). At high salinity, some symptoms of plant damage may be recognized, such as necrosis and leaf tip burn due to Na\(^+\) or Cl\(^-\) ions (Wahome et al., 2001). Hence sodium and chloride, usually the most prevalent ions in saline soils or water, account for most of the deleterious effects that can be related to specific ion toxicities (Levitt 1980). There is a strong correlation between salt exclusion and salt tolerance in many species (Greenway and Munns 1980; Läuchli 1984; Yeo and Flowers 1986; Munns and James 2003; Tester and Davenport 2003). Ion transporters are considered to play an important role in salt tolerance. Transport systems that facilitate compartmentation of Na\(^+\) into the vacuole are critical (Binzel et al., 1988). Transporter present in tonoplast are responsible for ion accumulation in organelle and play an important role in regulating cytosolic ion concentrations and there by homeostasis (Apse et al., 1999).

Fig. 1.3: Membrane Transporter mediating Na\(^+\), K\(^+\) and Ca\(^{2+}\) transport during salinity stress. (Redesigned from Yokoi et al., 2002)
Since NaCl is the principal soil salinity stress, a research focus has been the transport systems that are involved in utilization of Na\(^+\) as an osmotic solute (Blumwald et al., 2000; Hasegawa et al., 2000; Niu et al., 1995). Salt tolerance may be partly linked to the plant’s ability to control Na\(^+\) influx and to ensure appropriate K\(^+\) nutrition. Research of more than 30 years previously, established that intracellular Na\(^+\) homeostasis and salt tolerance are modulated by Ca\(^{2+}\) and high [Na\(^+\)] externally negatively affects K\(^+\) acquisition (Rains and Epstein 1967). Na\(^+\) competes with K\(^+\) for uptake through common transport systems and does this effectively since the [Na\(^+\)] externally in saline environments is usually considerably greater than [K\(^+\)] externally. Ca\(^{2+}\) enhances K\(^+\)/Na\(^+\) selective intracellular accumulation (Maathuis et al., 1996; Rains and Epstein 1967). Research of the last decade has defined many of the molecular entities that mediate Na\(^+\) and K\(^+\) homeostasis and given insight into the function of Ca\(^{2+}\) in the regulation of these transport systems.

The proteins involved in salt transport across the plasma membrane and the tonoplast i.e. proton pumps and Na\(^+\)/H\(^+\) antiporters have been identified. The SOS stress-signaling pathway was identified to be a pivotal regulator of plant ion homeostasis and salt tolerance (Hasegawa et al., 2000; Sanders 2000). NHX antiporters may also facilitate salt tolerance by mediating vacuolar Na\(^+\) accumulation necessary for osmotic adjustment, cell expansion, and intracellular K\(^+\) homeostasis (Jiang et al., 2010; Bassil et al., 2011; Pardo and Rubio 2011; Barragán et al., 2012). The plasma membrane Na\(^+\)/H\(^+\) antiporter SOS-11 is responsible for apoplastic efflux, and NHX type Na\(^+\)/H\(^+\) antiporters for vacuolar and endosomal compartmentalization. NHX-1 Na\(^+\) transporters, which regulate Na\(^+\) loading into the root xylem, limiting flux to and accumulation in the shoot. The high affinity K\(^+\) transporter (HKT) from wheat and low affinity cation transporter (LCT) also may be responsible for Na\(^+\) influx across the plasma membrane (Schachtman 2000; Amtmann and Sanders 1999; Blumwald et al., 2000). Little is known about the mechanistic entities that are responsible for Cl\(^-\) transport or the regulation of Cl\(^-\) homeostasis (Hedrich 1994). Diagram of the relevant transporters and Ca\(^{2+}\) dependent stress signaling pathway involved in Na\(^+\) homeostasis is shown in figure (Fig. 1.3).
1.6 Arabidopsis thaliana and other halophytes as model plant for study of salt tolerance:

The search for stress-associated genes may have been saturated at least in Arabidopsis thaliana, because it is popular as a model organism in plant biology and genetics. It has a rather small genome, only 157 Mbp (Mega base pairs) and small size of genome makes it useful for genetic mapping and sequencing with about 157 million base pairs and five chromosomes. Arabidopsis thaliana is a small annual weed of the mustard family (Brassicaceae) that is native to Europe, Asia, and northwestern Africa (Koornneef et al., 2004). Arabidopsis thaliana has one of the smallest genomes among plants. It was the first plant genome to be sequenced, completed in 2000 by the Arabidopsis Genome Initiative. So Arabidopsis thaliana is a popular tool for understanding the molecular biology of many plant traits. In the last decade, Arabidopsis thaliana, a genetic model plant, has been extensively used for unravelling the molecular basis of stress tolerance. Arabidopsis also proved to be extremely important for assessing functions for individual stress-associated genes due to the availability of knock-out mutants and its amenability for genetic transformation.

It has been hypothesized that differences in salt tolerance mechanisms between salt-sensitive glycophytes, and salt-tolerant halophytes, result from changes in the regulation of the same basic set of genes involved in salt tolerance (Zhu 2000; Zhu 2001; Xiong and Zhu 2002). It has been reported that the genetic model plant species, Arabidopsis thaliana, is relatively salt-sensitive (Gong et al., 2005). Therefore when Arabidopsis thaliana compared with other species under similar conditions of light and humidity (that is, at high transpiration rates), is found to be a salt-sensitive species. This sensitive plant may provide limited insights into mechanisms of salinity tolerance unless it is compared with tolerant relative halophytes.

Elucidation of the fundamental mechanisms underlying plant salt tolerance has historically been based on comparative analyses between halophytic and glycophytic species. In fact, genetic studies using halophytic species are virtually non-
existent (Munns and Tester 2008). In order to exploit genetically the existing resources, it is necessary to identify species that are halophytic and are either amenable to genetic analysis or exhibit characteristics of an established genetic model system. Over the last two decades the use of *Arabidopsis thaliana* as a genetic model system has advanced plant biology to new levels of understanding (Minke *et al.*, 1998, Sanders 2000; Chen *et al.*, 2004). Although *Arabidopsis*, a salt-sensitive species, can provide only limited information about mechanisms that support salinity tolerance, numerous genes involved in salt tolerance have been revealed by mutational approaches that resulted in plants with an even lower salt tolerance (Sanders 2000). Since last two decades, the major studies on molecular mechanism of salt tolerance is concentrated on glycophytes, however limited studies have been performed on halophytes. The increasing problem of global land salinization and associated multibillion dollar losses in crop production (Flowers 2004; Rengasamy 2006; Bonales-Alatorre *et al.*, 2013) require a better understanding of key physiological mechanisms conferring salinity tolerance in crops. The effective way of gaining such knowledge comes from studying halophytes.

Halophytes have always attracted the attention of plant physiologists, due to their remarkable ability to tolerate and even benefit from salt concentrations that kill most other plant species. At the very least, halophytes may provide genes that allow transgenic conference of salinity tolerance to crops. The study of the salt tolerance mechanisms of halophytic plants has emerged as an important area because these species are well-adapted to and can overcome soil salinity more efficiently than glycophytic plants (Gong *et al.*, 2005). The halophytes have a unique genetic makeup allowing them to grow and survive under salt stress conditions (Agarwal *et al.*, 2010). In order to understand the genetic bases that characterizes halophytism better, it is necessary to establish ‘halophyte genetic model systems’ (as advocated by Flowers and Colmer 2008) that can be manipulated with ease and flexibility comparable to that available for *Arabidopsis*. Such a genetic model, an *Arabidopsis*-Relative Model System (ARMS), could contribute to the identification and characterization of halophyte-specific mechanisms.
Recently, *Arabidopsis* relative, *Thellungiella halophila*, has been adopted as a model plant for salt tolerance research. This species has been claimed to be extremely tolerant to salt, but also to temperature extremes and drought (Taji et al., 2004; Gong et al., 2005; Amtmann et al., 2005). The *T. halophila* genome shares 95% identity with that of *A. thaliana* (Radyukina et al., 2007), which allows the use of most of the molecular tools available for *A. thaliana* (Karrenberg and Widmer, 2008). *Thellungiella halophila*, now promises to help in the detection of new tolerance determinants and operating pathways in a model system that is not limited to *Arabidopsis* traits or ecotype variations (Zhu et al., 2001). Comparative analysis of ion homeostasis in the two species allows the identification of ion transport pathways that are critical for salt tolerance and provides the basis for future studies into their molecular features (Wang et al., 2006). *Arabidopsis* and *Thellungiella halophila* (salt cress) were then adopted as extreme models to assess salt responses among other close relatives. Both of these plants present some limitations mainly in relation to their response to salinity. Arabidopsis is a glycophyte, whereas *Thellungiella* is a facultative halophyte. To identify candidate genetic determinants of the difference in salt tolerance between halophytes and glycophytes this requires genetically accessible halophyte models, which are not available to date, with the exception of *Thellungiella halophila*. However, more models are required, particularly because *T. halophila* is not a typical halophyte (Rozemaa and Schatb, 2013). Worldwide different halophytes are being used for the study of salt tolerance gene expression study: *Thellungiella halophila* (Oh et al., 2009) and *Puccinellia tenuiflora* (Wang et al., 2011), *Salicornia dolichostachya* (Katschnig et al., 2013), *Salicornia europaea* L., (Sulian et al., 2012), *Salicornia brachiata* (Yadav et al., 2012), *Craterostigma plantagenium*, *Mesembryanthemum crystallinum* (Bartelsa and Sunkara, 2007), *Helianthus annuus* (Kane and Rieseberg 2007) *Mimulus guttatus* (Lowry et al., 2009). Since *Thellungiella halophila* and other halophytes is not found to occur in India and particularly in the semi-arid region, an alternative has been picked up as a candidate halophyte *Lepidium sativum* L., in an effort to identify and characterize genes that enable salt tolerance. Halophyte-*Lepidium sativum* is easily grown in a Hydroponic system.
The hydroponic system is a technique of cultivation of the plant/crops in liquid nutrient medium, without soil. Hydroponics system provides plants with extra oxygen in the growth phase, which stimulates root growth. Plants that have ample oxygen in the root area are able to absorb nutrients much faster. Additionally, the nutrients in a hydroponic system are mixed with water and sent directly to the root system, whereas a plant grown in soil would have to take the time to search for the same nutrients from within the soil. Hydroponic systems offer a higher degree of control over soil-borne pests and weeds. Usually, the water used in hydroponic gardens is recycled and used repeatedly. The benefits of using a hydroponic system as compared to soil culture (geoponics) and aseptic culture on semi-solid media are tabulated in the following table (Table 1.1).

**Table 1.1**: Comparison of Geoponics and hydroponics

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Parameter</th>
<th>Geoponic</th>
<th>Agar plate</th>
<th>Our Hydroponic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Setup cost</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>2.</td>
<td>Running cost</td>
<td>A) media</td>
<td>Low</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B) equipment</td>
<td>Low</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3.</td>
<td>Foot print</td>
<td>Small</td>
<td>Small</td>
<td>Small</td>
</tr>
<tr>
<td>4.</td>
<td>Sterile culture</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5.</td>
<td>Batch variability</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>6.</td>
<td>Experimental flexibility</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>7.</td>
<td>Contamination (fungal &amp; bacterial)</td>
<td>Medium</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>8.</td>
<td>Throughput</td>
<td>High</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>9.</td>
<td>Root entanglement</td>
<td>Yes</td>
<td>Potential</td>
<td>Little &amp; manageable</td>
</tr>
<tr>
<td>10.</td>
<td>Developmental window</td>
<td>Mature plants</td>
<td>&lt;2 Week old seedling</td>
<td>Mature plants</td>
</tr>
</tbody>
</table>
including the fact that the roots of the plant have constant access to oxygen and that
the plants have access to as much or as little water as they need. These two
researchers (Hoagland and Arnon 1950) developed several formulas for mineral
nutrient solutions, known as Hoagland solution. Modified Hoagland solutions are still
used today.

1.7 *Lepidium sativum* L.: A model plant for present study

*Lepidium sativum* L. is a halophyte which belongs to brassicaceae family and close
relatives of *Arabidopsis*. *L. sativum* is reported to be tolerant to saline irrigation
water (Laborde 1997). *L. sativum* (Garden cress) is an annual herb, fast-growing,
edible plant botanically related to watercress and mustard. Seeds, leaves and roots
are economically important, however, the crop is mainly cultivated for seeds. In
some regions garden cress is known as garden peppercress, peppergrass or
pepperwort. It is also known as Asalio or chandrasur in India and it is an important
medicinal crop in India. The seeds contain a high proportion of mucilage and used for
different therapeutic purposes (Rangari 2002). *L. sativum* has been considered as
important medicinal plant since Vedic era. In various countries of Africa, *Lepidium
sativum* seeds are thought to be an elective medicinal remedy to cure respiratory
disorders, like bronchitis and asthma (Parajapati and Mehta 2006; Kloos 1976) and
have important role in gut stimulatory actions like constipation and digestion
(Najeeb-Ur-Rehman *et al.*, 2011). In South Asia, it is used in traditional medicine to
treat asthma, bronchitis, and cough and is considered useful as abortifacient,
antibacterial, aphrodisiac, diuretic, expectorant, gastrointestinal stimulant, gastro
protective, laxative, and stomachic (Baquar 1989; Duke *et al.*, 2002). In many parts of
the world seedlings of *L. sativum* are used in salads because of their pungent taste.
The main character of chandrasoor is that it can grow in any type of climate and soil
condition. *L. sativum* is known to tolerate very high NaCl concentrations. *L.
virginicum*, another species with halophytic behavior, displayed relatively low
stomatal closure when treated with salt. Inan *et al.*, (2004) reported that despite the
observed tolerance of *T. halophila* to salt at plant stage, its germination is extremely
sensitive to salt. Apparently, seeds dormancy could have been enhanced by a salty
environment as a consequence of endogenous production of ABA, which has been
proved to dramatically reduce *T. halophila, A. thaliana* and *L. sativum* germinability (Inan *et al.*, 2004; Muller *et al.*, 2006). The species belongs to same family; it is hypothesis that they share maximum genome similarity. The *A. thaliana* and *L. sativum*, belonging to the same family brassicaceae, by studying together with a different salt response, makes the study of *L. sativum* extremely interesting, and full of possibilities. This unique characteristic provides an advantage for the study of salt tolerance mechanisms.

Most of the plants accumulate salt in vacuoles, which reflects the fact that antiporter genes are necessary to maintain homeostasis in extreme salinity. *L. sativum* known to be a halophyte and can survive at higher salinity. Hence this plant may serve as a model plant to study the salt responsive genes. Moreover, there is no report in the literature about expression of gene under high salinity from *L. sativum*.

1.8 Bioinformatic tools to analyze plant salt tolerance mechanisms

In spite of evolutionary divergence and the pressures of domestication, there has been a noticeable conservation of genetic synteny between related plant species. There has been considerable interest in defining these interrelationships, from the angles of both evolutionary genetics and plant breeding (Bowers *et al.*, 2005; Devos 2005; Sorrells *et al.*, 2003; Windsor *et al.*, 2006; Xu *et al.*, 2005; Ziolkowski *et al.*, 2006; Parkin *et al.*, 2005; *et al.*, Paterson *et al.*, 2005). Molecular biologists work with DNA databases that often include entire genomes. A common requirement is to search a DNA database to find exact matches for a non degenerate or partially degenerate query. Various software programs available for such purposes. The Gene Expression Omnibus (GEO) is a public repository that archives and freely distributes high-throughput gene expression data submitted by the scientific community. GEO currently stores approximately half a billion individual gene expression measurements, derived from over 100 organisms, addressing a wide range of biological issues. These huge volumes of data may be effectively explored, queried, and visualized using user-friendly Web-based tools (www.ncbi.nlm.nih.gov/geo.). Rapidly accumulating data from plant genome sequencing and comparative genetic mapping have led to new resources for accessing and displaying these data sets (e.g.
Introduction


Typically, whole genome comparative studies are based upon the identification of genetic synteny between a model and crop species by either reference to existing sources or the development of de novo markers which target particular areas of a genome [http://www.gramene.org/cmap/]. Over the last several years, molecular models and sequence analysis have grown productive and mature. Sequence analysis aims to efficiently compare nucleotide and amino acid sequences, thereby allowing researchers to impute a gene’s function by considering evidence from homologous genes, often from different biological systems. Sequence analysis has now become an indispensable part of target identification of genes which play important role in stress.

Over the past few decades, major advances in the field of molecular biology, coupled with advances in genomic technologies, have led to an explosive growth in the biological data generated by the scientific community. The critical need to process and analyze such a deluge of data and turn it into useful knowledge has caused bioinformatics to gain prominence and importance. Bioinformatics is an interdisciplinary research area that applies techniques, methodologies, and tools in computer and information science to solve biological problems. Bioinformatics has recently played a vital role in the advancement of biological sciences.

Bioinformatics is quickly becoming the central core that integrates the many disparate bodies of data, scientific knowledge and computational infrastructure from fields as diverse as genetics, structural biology, medical and animal models of disease, imaging, engineering, etc. For example, biological imaging and shape representation (Lele et al., 2011; Tu et al., 2008), sequence analysis (Badidi et al., 2003), regulatory genomics (Dieterich et al., 2003) and alternative splicing (Modrek et al., 2002) are critical components of computational biology. Many space, time, function or interaction modeling techniques apply across the vast spectrum of scales
from genotypes to phenotypes, from the small scale of microarray imaging for
genomics, to the larger *in vivo* neuroimaging scale. Bioinformatics and high-
throughput technologies such as microarray studies allow the measure of the
expression levels of large numbers of genes simultaneously, thus helping us to
understand the molecular mechanisms of various biological processes in a cell.

To understand salt tolerance in plants, the mechanism in each aspect need to
be understood. Stress-relevant genes are ubiquitously present in the plant kingdom. Several stress associated genes have been evaluated or studies are in progress for
their contribution to drought or salt tolerance and its complete data are available
that can be access by the help of study of bioinformatics. In Bioinformatics, a high
throughput technology is used that allows detection of thousands of genes
simultaneously called as Microarray technology.

*Arabidopsis thaliana* is a popular modal plant for understanding the
molecular biology of many traits. The most up-to-date version of the *Arabidopsis
thaliana* genome is maintained by The Arabidopsis Information Resource (TAIR).
TAIR maintains a database of genetic and molecular biology data for the model
higher plant *Arabidopsis thaliana*. Plant GDB (http://www.plantgdb.org/) is a
database of molecular sequence data for all plant species with significant sequencing
efforts. The database organizes EST sequences into contigs that represent tentative
unique genes. Some of the genes which play very important role in salinity are
studied and its complete information is available and maintained in such type of
database. Few of them are:

1. **AtNHX-1**: Intracellular Na\(^+\)/H\(^+\) antiporters (*NHXs*) play important role in cellular pH
and Na\(^+\) and K\(^+\) homeostasis in all eukaryotes. *NHX* gene would be highly
conserved in terms of evolution in plant. The *Arabidopsis thaliana AtNHX-1* gene
encodes a vacuolar Na\(^+\)/H\(^+\) antiporter that are important in salt tolerance. *AtNHX-1*
may play a role in pH regulation and/or K\(^+\) homeostasis in the specialized cells.
*AtNHX-1* activity was substantially up-regulated in *Arabidopsis* by NaCl, KCl or
ABA, demonstrating that salt and ABA regulation of *AtNHX-1* expression occurs at
the transcriptional level.
Introduction

2. **AtNHX-1**: In *Arabidopsis thaliana*, the NHX-1 (Salt Overly Sensitive 1) locus is essential for Na\(^+\) and K\(^+\) homeostasis, and NHX-1 mutations render plants more sensitive to growth inhibition by high Na\(^+\) and low K\(^+\) environments. The transmembrane region of NHX-1 has significant sequence similarities to plasma membrane Na\(^+\)/H\(^+\) antiporters from bacteria and fungi. Sequence analysis of various SOS-1 mutant alleles reveals several residues and regions in the transmembrane as well as the tail parts that are critical for NHX-1 function in plant salt tolerance. NHX-1 gene expression in *Arabidopsis* is up-regulated in response to NaCl stress. This up-regulation is abated in SOS-3 or SOS-2 mutant plants, suggesting that it is controlled by the SOS-3/SOS-2 regulatory pathway.

3. **AtNHX-1**: AtNHX-1 is physiologically distinct major determinants of salinity resistance. It causes an increase in Na\(^+\) levels in the xylem sap and shoots and a concomitant Na\(^+\) reduction in roots. It encodes a potassium transporter (*NHX-1*) expressed in xylem parenchyma cells. But it is also known to transfer sodium across membrane. Mutants over-accumulate sodium in shoot tissue and have increased sodium in the xylem sap and reduced sodium in phloem sap and roots.

4. **AtCLC-c**: The *Arabidopsis* vacuolar anion transporter, AtCLC-c, is involved in the regulation of stomatal movements and contributes to salt tolerance.

Including those genes which have the major role in salt tolerance such there are so many other genes are still undiscovered which play important role in salinity which co-expressed with the known salt tolerance genes and might be putative. When analyzing genomic data, biologists can often fail to discover interesting genes for experimental analysis when dealing with hundreds of putative candidate genes.

Microarray gene-expression profiling is one of the major techniques emerging as a result of genomic research that is widely used for target gene selection. Recently, several research groups have investigated drought-mediated changes in gene expression using microarrays. Microarray datasets typically include several thousands to tens of thousands of genes with relatively a small number of samples, but many genes are irrelevant or redundant for the purpose of this study. Biologically, there are often tens to hundreds of genes significantly associated to a trait like drought and salt resistance. Hence, it is important to develop computational
methods to mine these genes based on microarray data. There might be some other genes which are co expressed with the known salt tolerant genes but not yet been considered called as putative genes. Those genes might be stress associated genes which are differentially regulated, that have not been evaluated and can be contributed to drought or salt tolerance in laboratory studies. Those genes might be located in exonic, intronic or intergenic region. Many powerful software and tools for manipulating genetic resource are available in Bioinformatics for identifying salt-tolerant germplasm. The first step towards bioinformatics applications to address the model plant the glycophyte *Arabidopsis*, contain genes that can be used to increase salt tolerance, or do halophytes have novel genes which absent in glycophytes that confer the trait of interest. This finding is crucially important to the current assumptions that modifying expression patterns of stress-inducible genes in *Arabidopsis* could increase salt and drought tolerance.

1.9 Genetic Diversity for Salt Tolerance in Plants

Salinity tolerance is the ability of a plant to grow and complete its life cycle under stressful salt conditions like NaCl or with association of other salts. Plants have stress-specific adaptive responses as well as responses which protect the plants from more than one environmental stress (Huang *et al.*, 2011). Two things are very important for the adaptation of a species under saline environment, one is control of water loss another is improved ionic balance. On the basis of tolerance level, plants are traditionally classified as glycophytes or halophytes referring to their capacity to grow on highly saline environments (Flowers *et al.*, 1977). A plant adapted to saline conditions is called a halophyte. Halophyte tolerates high concentrations of salt while the glycophytes are susceptible (Maas and Nieman 1978). Being the natural inhabitants of highly saline soils, halophytes efficiently excludes salts from their roots and leaves and some can endure salts that are more than twice the concentration of seawater. The general difference between salt tolerant plant species (halophytes) and the salt sensitive ones (non-halophytes or glycophytes) can be defined by their success to germinate and propagate under saline conditions. Halophytes have the ability to tolerate high concentrations of Na\(^+\) and Cl\(^-\) by excluding toxic ions (Greenway and Munns 1980; Jeschke 1984; Lauchli 1984). It can
usually withstand up to 3.5 M NaCl in the soil (Flowers and Colmer 2008; Flowers et al., 1977), whereas glycophytes show impaired growth and stress-evoked damage in presence of much lower salt concentrations (Dajic 2006; Greenway and Munns 1980). In glycophytes, ions are present in the roots and do not move but halophytes move these ions towards shoot and this is the way, they tolerate the toxicity of ions (Flowers et al., 1977). Most of the crop plants are glycophytes and thus fail to withstand a prolonged exposure to even mild salinity (Zhu 2001). According to Greenway and Munns (1980), salt sensitivity in non-halophytes may result from either inability of osmoregulation, or injury caused by inorganic ions which are absorbed by the cell and are not compartmentalized. Some of the common symptoms associated with glycophytes plants when grown in areas of high salinity include: osmotic stress, increased ionic stress, oxidative stress, nutritional disorders, sodium toxicity and chloride sensitivity. Glycophytes limit sodium uptake, or partition sodium in older tissues, such as leaves, that serve as storage compartments which are eventually abscised (Cheeseman 1988). Halophytes are plants that adapt themselves in saline conditions by altering their physiological and molecular mechanism (Winicov and Bastola 1997). These plants are ideal for studying the mechanisms they are known to hold high salt concentrations. Therefore, these plants can be used as model system in research studies aiming to improve the tolerance of non-halophytic plants.

1.10 Mechanism of salinity tolerance by halophytes and glycophytes

Many critical physiological mechanisms of plant salt tolerance were determined initially from studies of halophytes (Flowers et al., 1986, 2010) and some of these have been linked to salt tolerance determinants through research using genetically tractable glycophyte model plants, particularly A. thaliana (Flowers et al., 1977, 1986; Hasegawa et al., 2000; Zhu 2002, 2003; Flowers and Colmer 2008; Munns and Tester 2008). Halophytes are polyphyletic in origins; it appears to have evolved the same basic method of osmotic adjustment: accumulation of inorganic salts, mainly NaCl, in the vacuole and accumulation of organic solutes in the cytoplasm. Differences between halophyte and glycophyte ion transport systems are becoming apparent. The pathways by which Na⁺ and Cl⁻ enters to halophyte cells are not well
understood but may involve ion channels and pinocytosis, in addition to Na\(^+\) and Cl\(^-\) transporters (Edward et al., 1999). Na\(^+\) uptake into vacuoles requires Na\(^+\)/H\(^+\) antiporters in the tonoplast. Tonoplast antiporters are constitutive in halophytes, whereas they must be activated by NaCl in salt-tolerant glycophytes, and they may be absent from salt-sensitive glycophytes. Concerning halophytic plants that are tolerant of sodium toxicity, osmotic stress might be the main reason of growth inhibition. Adaption to saline environments by halophytes may take the form of salt tolerance (halotolerance) or salt avoidance. Exclusion of salts from shoot portion of the plant is a prime form of tolerance in non-halophytic plants. Halophytes have strong Na\(^+\) compartmentalization and active efflux mechanism to manage low salinity (Na\(^+\) concentration) in the cytosol. Halophytes, the natural flora of highly saline soils, are able to maintain this exclusion at higher salinities than glycophytes. For example, *Hordeum marinum*, excluded both Na\(^+\) and Cl\(^-\) until at least 450mM NaCl (Garthwaite et al., 2005). Mechanism of salt tolerance in halophytes and glycophytes has been reviewed by many workers (Bernstein and Hayward 1958; Strogonov 1964; Levitt 1972; Greenway 1973; Mass and Nieman 1978; Cramer et al., 1985). Tightly controlled uptake of Na\(^+\) and Cl\(^-\) ions is closely correlated with growth in halophytes (Inan et al., 2004).

Both glycophytes and halophytes cannot tolerate large amounts of salt in the cytoplasm and therefore under saline conditions they either restrict the excess salts in the vacuole or compartmentalize the ions in different tissues to facilitate their metabolic functions (Iyengar and Reddy 1996; Zhu 2003). Therefore, in order to successfully understand salt tolerance in plants, the mechanism in each aspect need to be understood. Traditional breeding programs trying to improve abiotic stress tolerance have had some success, but are limited by the multigenic nature of the trait. Tolerant plants could be valuable tools to dissect the extreme tolerance nature. Stress-tolerant plants have evolved certain adaptive mechanisms to display different degrees of tolerance, which are largely determined by genetic plasticity. Plants may need gradual adaptation for proper expression of genes responsible for acquisition of tolerance (Zhu 2001). More importantly, we learned that it is very likely that the extreme tolerant model plants did not acquire unique genes since stress-relevant
genes are ubiquitously present in the plant kingdom. To resist in high salinity condition plant over express the related genes to survive in such condition. Those genes are belonging to three major groups. The first group is those that are involved in signaling cascades and in transcriptional regulation. The second group is those having a role in the protection of membranes and proteins and the third group are those involved in water and ion uptake and transport (Wang et al., 2003).

Salinity tolerance comes from genes that limit the rate of salt uptake from the soil and the transport of salt throughout the plant, adjust the ionic and osmotic balance of cells in roots and shoots, and regulate leaf development and the onset of senescence. Salt tolerance genes in halophytes may have evolved from genes in glycophytes that were adapted to low levels of salt stress, a common environmental factor for most plants because Na$^+$ is one of the most abundant soil cations. Accumulation of Na$^+$ to toxic levels may not occur because of salt tolerance genes that regulate the fluxes of Na$^+$. Plants employ three different strategies to prevent and adapt to high Na$^+$ concentrations: (i) active Na$^+$ efflux, (ii) Na$^+$ compartmentalization in vacuoles, and (iii) Na$^+$ influx prevention (Niu et al., 1995; Rajendran et al., 2009). Plants adapt to environmental stresses through the expression of both stress related genes and metabolites. Numerous abiotic stress-related genes, as well as transcription factors and regulatory sequences in plant promoters, have been characterized (Agarwal and Jha 2010).

*Arabidopsis* can be expected to have salt tolerance genes that are not very different from those of halophytes. The large number of genes implicated in salinity tolerance may not be surprising, because many of the traits underlying adaptation to abiotic stress are quantitative and controlled by perhaps hundreds of genes and dozens of genetic pathways (Sreenivasulu et al., 2007 and Masle 2005). While several studies have investigated the molecular basis of salinity tolerance in *A. thaliana* (Hugovieux 2001; Zhu 2000; Kreps 2002; Yoshiba 1995), little attention has been paid to measuring naturally occurring quantitative variation in salinity tolerance and other abiotic stresses in this model species (Masle 2005). By DNA technologies, several hundred stresses induced or up regulated genes have been identified. Several stress-associated genes have been evaluated or studies are in
progress for their contribution to drought or salt tolerance in laboratory studies. However, progress in breeding for salt tolerant crops/plantations has been hampered by the lack of complete understanding of the molecular basis of salt tolerance and lack of availability of gene that confer salt tolerance.

Increased salt tolerance requires new genetic sources of this tolerance, and more efficient techniques for identifying salt-tolerant germplasm. Powerful new molecular tools for manipulating genetic resource are becoming available, but the application of the new technologies are not yet fully utilized to introduce new genes for tolerance into current cultivar/verities because the pathway for abiotic stress tolerance is not fully understood yet. Moreover studying the halophytic system is logically going to give a better understanding of the salt tolerance mechanisms at a molecular level.

1.1 Objectives

With the above background, the present was planned with following objectives to enable visualization of gene expression pattern of the key genes involved in plants response to salinity stress:-

1. To develop and standardize a hydroponics system for growing halophyte (Lepidium sativum) under various salt regimes.
2. To analyze the gene families using multiple sequence alignment algorithms for identification of regions of least homology that will be the pre-requisite for designing gene specific primers
3. To design gene specific primers for selected genes.
4. To study gene expression pattern (up-regulation, down-regulation) in the halophyte, using RT-PCR approaches under different salt concentrations, different time periods and different plant parts.