UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN

Scienze Agrarie e Ambientali

CICLO: XXVII

COORDINATORE Prof. Orlandini Simone

Physiological and genetic responses of cucumber *(Cucumber sativus L.)* to salt stress

Settore Scientifico Disciplinare AGR/03

Dottorando

Dott. **Redwan Mirvat**

Tutore

Prof. **Mancuso Stefano**

Coordinatore

Prof. **Orlandini Simone**

Anni 2013/2016

Acknowledgements

At this special moment, and in the last leg of my PhD journey, I feel grateful to what I have gained over the past five years at the university of Florence.

First of all I would like to extend my sincere thanks and appreciations to LINV family. I could not find another name more appropriate than this, because it was really a family for me, for every support, motivation and encouragement.

My special and deepest gratitude to my supervisor Prof. Stefano Mancuso for his patience, continuous support and guidance. It was a pleasure for me to be one of his PhD's students.

I cannot forget to offer my sincerest gratitude to both Elisa Azzarello and Elisa Masi for their constant help and support during my staying in the lab.

I really appreciate the great help from Francesco Spinelli for standing beside me and keeping constant communication to complete the final part of the research.

I want to say thank you to all my lab colleagues and to all friends for their positive support and for the good time I spent with them.

Finally, I would like to give my thanks to my family in Syria for the continuous encouragement and love.

Table of Contents

Aim of the thesis

Salinity is one of the most global increasing problems. It has been a factor affecting agriculture for more than 6000 years. Its harmful effects limit both growth and productivity of agricultural crops, and to some extent rising salt levels leads to environmental degradation (loss of species and biodiversity). And this problem is becoming more evident with global climate changes, that will increase the severity of drought problem, which in turn will force farmers to turn to brackish and saline water, substantially exacerbating salinity problem.

The current estimates indicate that food production should increase up to 70% by 2050 to cope with the future demand of increasing world population. In order to enhance the productivity of crops under salt stress, significant efforts have been done to understand the mechanisms underlying salt tolerance in plants. This is not a simple task, because, as it is known, salinity is a complex trait determined by a number of physiological and biochemical traits and highly influenced by environmental factors. From here stands out the

importance to develop an effective evaluation approach for screening salt tolerance genotypes, which should be quick, easy, practical, and economic.

Plants are traditionally classified as glycophytes or halophytes referring to their capacity to grow on high saline environments. Glycophytes are not adapted to high salinity concentrations. Most of the vegetable crops belong to this category, and they vary in response to salinity from very salt sensitive to moderately salt resistant. Cucumber (*Cucumis sativus* L), an important horticultural crop, has been selected as the test plant material because it is considered sensitive to salinity, especially at germination and during the seedling stage.

In the first part of this work, a screening for salt tolerance was conducted for different varieties of cucumber basing on their germination ability, and then, according to this selection, the screening was continued in order to identify different new criteria, which could be used all together as effective selection targets (chapter 2).

Going forward in this work, the attention was focused on studying plant responses under heterogeneous salinity in order to

investigate the transmission of signals among roots, using electrophysiology and genetic analysis, considering that the distribution of salts in the root environment in general is unequal (chapter 3).

Chapter 1 Introduction

"Role of Ion Transporters in Salinity Resistance in Plants"

Mirvat REDWAN, Francesco SPINELLI, and Stefano MANCUSO

A modified version of this chapter has been published in *Environmental Control in Biology*,54.1 (2016): 1-6.

1.1 Physiological effects of salinity on plants

Salinity is an increasing problem that affects vast areas of the world (irrigated, arid and semiarid areas) in particular where precipitation is insufficient to leash salts from the root zones (LELAND and EUGENE 1999). About 6% of the global land area is affected by salinity (Flowers and Yeo 1995) and salt affected soils include about one third of the world's irrigated soils presently and that portion is expanding (Robinson and Downton 1984; Chauhan *et al.* 1987).Because of the intensive irrigation practices, secondary salinization is increased in the agricultural soils. And in combination with the competition of fresh water among municipal, industrial and agricultural sectors, this problem (salinity) becomes more sever in particular with the growing water shortage problem, where more than 80 countries suffer from water shortage each year (Gleick 1993), taking into consideration that agriculture consumes more fresh water than any other human activity (Falkenmark *et al.* 1987).

A definition for saline soils adopted from FAO (1997) is those soils that have an electrical conductivity of the saturation extract (EC_e) of 4 dsm^{-1} or more. Regardless of the specific nature of cations or anions the soil was considered saline when contains excessive amounts of salts in general. However, Na^+ and Cl^- are considered the most important ions and are toxic to plants (Hasegawa *et al.* 2000). The toxicity of Na^+ is due to its ability to inhibit enzyme function more specifically either directly by binding to inhibitory sites or indirectly by displacing K^+ from activation sites (Serrano 1996). Moreover Na^+ is considered cytotoxic at cytosolic concentration in

excess of about 100Mm (Serrano *et al.* 1999). The competition between Na^+ and K^+ in the cytosol is likely to be a more critical factor in determining Na^+ toxicity than cytosolic Na^+ concentration *per se*. Salinity has a major impact on plant growth, and usually during the first several days of the stress the inhibition of growth occurs and primarily restricted to the shoot (Munns and Termaat 1986). It was found that leaf growth is more sensitive to salinity than root growth under salt stress, this can be explained or by water deficit or a specific salt toxicity in the shoot or in the root, but in case of long term salinity, large amounts of salt is brought into the shoot, especially into the leaves, by prolonged transpiration which in turn results in damaging leaves; this process must eventually limit the supply of assimilates to the growing regions and might be the main factor determining yield. Another hypothesis for the mechanism by which salinity reduces shoot growth.; One suggestion is that salinity reduces photosynthesis which in turn limits the supply of carbohydrates needed for growth (Yeo and Flowers 1985; Munns and Schachtman 1993). A second is that salinity reduces shoot growth by reducing turgor in expanding tissues which are not able to fully osmoregulate in response. A third is that roots sense salinity and down regulate shoot growth by via a long distance signal (Termaat *et al.* 1985; Munns and Termaat 1986; Rengel 1992). Fourth, a disturbance in mineral supply to the shoot, either an excess (Na^+ or Cl^-) or deficiency, might directly affect growth (Abel and MacKenzie 1964; Läuchli and Wieneke 1979; Flowers and Yeo 1981; Jeschke 1984).It was reported that high Cl^- concentration reduced photosynthetic capacity due to non-stomatal effects: there was chlorophyll degradation, and a reduction in the

actual quantum yield of PSII electron transport in barley plant (Tavakkoli *et al.* 2010). However, the contribution of Cl^- to growth reduction under salt stress is less well understood than that of Na^+ in major crops. This reflects the fact that most research on salt tolerance has focused on Na^+ with little regard to Cl^- toxicity (Teakle and Tyerman 2010). It was found also that NaCl induced disturbance in the supply of carbon to the growing zones of shoots might be associated with the increased starch accumulation in mature leaves (Munns and Schachtman 1993). For several decades significant efforts have been done to understand all mechanisms underlying salt tolerance in order to enhance the productivity of salt sensitive crops, but progress is still limited (Cuartero *et al.* 2006b; Panta *et al.* 2014) most likely due to the complexity of salt tolerance mechanisms (Munns and Tester 2008).Growth reduction in response to salinity is the result of various effects as mentioned before, including diminished water uptake due to root- and shoot-related physiological changes, reduced carbon fixation due to stomatal closure or specific ion toxicity, increased energy costs for protective processes such as osmotic adaptation and ion exclusion, and growth limitations originating from nutritional imbalances (Munns and Tester 2008). Despite the complexity of processes and mechanisms involved in the responses described above, it is clear that the ability of a plant to maintain a high cytosolic K^+/Na^+ ratio is a critical factor in salt tolerance (Shabala and Cuin 2008). As a result, the major efforts of researchers and breeders have been aimed for many decades at improving this ratio by minimizing and preventing Na^+ uptake and transport to shoot, which would in turn also avoid specific Na^+

toxicity (Shabala and Cuin 2008). However, despite some positive results under controlled conditions, there are currently no reports that confirm that higher Na^+ extrusion from plant roots may result into superior salinity tolerance (Shabala 2013). Consequently, it has recently been hypothesized that Na^+ exclusion from the roots could actually aggravate salt stress by increasing the osmotic and ionic imbalance in the root-zone (Adem *et al.* 2014). On the other hand, evidence among several cereal crop species now indicates that the ability of a cell to retain K^+ is at least as important as a plant's ability to exclude or compartmentalize toxic Na^+ (Chen *et al.* 2007; Shabala and Cuin 2008; Shabala and Pottosin 2014).

1.2 K^+ transport in plant tissues under salt stress

Potassium (K) is an essential macroelement in plants, as it is in every living organism. In its ionic form, it is the major inorganic cation in the cell cytoplasm where it plays a role in basic functions such as maintaining the turgor of the whole cell, controlling the turgor of guard cells and stomatal movements at the leaf surface, controlling cell membrane polarization, electrical signalling and osmoregulation. More than 50 enzymes are activated by potassium and sodium cannot substitute in this role (Bhandal and Malik 1988), so high levels of sodium or high $Na^+\backslash K^+$ ratio can disrupt various enzymatic processes in the cytoplasm, and from here we can imagine the gravity of the excessive amount of sodium in the soil, that Na^+ can compete with potassium for binding sites essential for cell functions. Moreover, protein synthesis could be disrupted by elevated concentrations of sodium.

Channels and transporters present on the plasma membrane transfer K^+ in the plant cell against its concentration gradient. The mechanism mediating K^+ uptake depends on the K^+ concentration outside of the cell: if its concentration is low, transporters facilitate K^+ uptake, whereas K^+ uptake is carried out by K^+ channels when the extracellular concentration is high (Gupta and Huang 2014). As reported in previous studies K^+ acquisition from soils is mainly mediated by K^+ transporters and channels, such as those of the High Affinity Potassium Transporters (HKT) family, HAK/KT/KUP family and Shaker Inward Rectifying Potassium Channels (AKT1-like K^+ channels) (Shabala 2003; Aleman *et al.* 2011; Wang and Wu 2013; Very *et al.* 2014). Sodium has a strong inhibitory effect on K^+ uptake by cells, presumably by interfering with transporters in the root plasma membrane such as the Shaker type K^+ channels (KAT1 and AKT1 form the predominant inward K^+ conductance observed in plant plasma membranes). Such channels generally have a high K^+/Na^+ selectivity and are generally regarded not to play a significant role in Na^+ (Schachtman and Schroeder 1994; Amtmann and Sanders 1998). However, a more recent work suggests that the picture is more complex and there may be ecophysiological variations in this respect. (Wang *et al.* 2006)used pharmacological approach to characterize Na^+ uptake in the halophyte *Suaeda maritime* and concluded that the low-affinity Na^+ uptake pathway in this species resembles an AKT1 channel. Similarly, Kader and Lindbergh (Kader and Lindberg 2005) provide evidence that K^+ channels mediate substantial Na^+ influx in a salt-sensitive rice cultivar but not in a tolerant one. In both cases the conclusions are derived from applying channel blockers and inhibitors

which can be notoriously nonspecific, but these findings do suggest that K^+ channels are potential pathways for root Na^+ influx. In addition, the study by (Wang *et al.* 2006) suggests that basic processes such as Na^+ uptake may be considerably different in halophytes and such diversity could be an important contributor to salt tolerance. However, the scarcity in data from halophytes in this respect forms a large hindrance in testing this hypothesis. AKT1-like channels are considered the main channel components that mediate K^+ influx into root cells in many plant species; these channels include AKT1 in *Arabidopsis thaliana,* a major inward K^+ channel expressed primarily in *A. thaliana* roots and localized to the plasma membrane of epidermal cells (Sentenac *et al.* 1992; Lagarde *et al.* 1996; Hirsch *et al.* 1998). It has also been reported that the transcript levels of AKT1 are regulated by external sodium concentration, and it has been shown that *A. thaliana* plants over-expressing PutAKT1(an AKT1-type K^+ channel investigated in *Puccinellia tenuiflora*) have an increased K^+ content and enhanced salt tolerance compared to wild-type plants under salt stress (Ardie *et al.* 2010). The other type of K^+ transporters is HKTs (high affinity potassium transporters); they are basically carrier type proteins that mediate K^+. Members of the HKT gene family were initially described as high affinity K^+ transporters, but at the same time are Na^+-specific transporters. In addition to HKTs, other carriers have been implicated in Na^+ uptake. Some members of the high-affinity K^+ uptake transporter family HAK/KUP/KT may transport Na^+ with low affinity in the presence of high Na^+ : K^+ ratios (Pardo 2004).

1.3 Uptake and accumulation of Na^+ (Na^+ Transport) and mechanisms of tolerance

In order to better understand ionic homeostasis in plants under salt stress, identification of Na^+ transport pathways was as comprising a key gap, not only that but also the regulation of Na^+ transport across the plasma and vacuolar membranes comprises a critical factor in determining the specific manner in which plant cells handle extra cellular Na^+ loads (Niu *et al.* 1995). In general living cells in both low and high salt environments try to balance passive influx of Na^+ with Na^+ efflux, either across the plasma membrane back into the apoplast or across the tonoplast into the vacuole. For salt tolerance in addition to the considerable energy required for Na^+ efflux, time is an important factor because the rate of Na^+ uptake will determine how quickly Na^+ reaches toxic levels inside the cell. So it is clear that under high salt conditions the limitation of Na^+ influx into root cells is fundamental and the balance of Na^+ influx with Na^+ export from the cytoplasm back into the apoplast is one way of reducing the Na^+ load (sometimes termed 'futile cycling')(Britto and Kronzucker 2006; Malagoli *et al.* 2008). Futile cycling occurs to varying degrees in all plants investigated so far with 78–98% of Na^+ taken up transported back into the environment (Kronzucker *et al.* 2006; Wang *et al.* 2006; Malagoli *et al.* 2008).

The importance of Na^+ export from root cells for salt tolerance is evident in the salt over-sensitivity of mutants that are impaired in the plasma membrane Na^+/H^+ antiporter SOS1 (salt overly sensitive)(Banuelos *et al.* 1996; Shi *et al.* 2000). This system seems to

be similarly crucial in salt-sensitive and salt tolerant species (Oh *et al.* 2007). A second strategy for removing Na^+ from the cytoplasm is to compartmentalize it in the vacuoles. Na^+ uptake into the vacuole also requires energy, but has a dual benefit in saline conditions; it avoids Na^+ build-up in the apoplast (Oertli 1968) and enhances the intracellular solute potential thereby contributing to turgor adjustment For example, halophytes exhibit a marked ability for Na^+ accumulation, and their tolerance relies on controlled uptake and compartmentalization of Na^+, K^+ and Cl^- and the synthesis of organic compatible solutes to prevent Na^+ cytotoxicity. Several classes of Na^+ transporters have been identified such as the sodium-proton exchanger (NHX-),SOS1and HKT1. They have demonstrated significant involvements in Na^+ sequestration in vacuoles or extrusion from cells or circulation for the alleviation of sodium stress under saline conditions; for example, the Na^+/H^+ antiporter SOS1 mediates the efflux of Na^+ from root cells into the soil or into the cortical apoplast (Banuelos *et al.* 1996; Shi *et al.* 2000; Rus *et al.* 2004).To date, SOS1are the best characterized class of transporters attributed with Na^+ exclusion from the cytosol across the plasma membrane. It was found in different plant species such as Arabidopsis and tomato a significant increase in salt sensitivity and the tendency to accumulate high amounts of Na^+ in the Knockout/Knockdown plants of corresponding SOS1 genes compared with wild types under high salt concentration (Banuelos *et al.* 1996; Shi *et al.* 2000; Oh *et al.* 2009). Together with HKT and CHX-type transporters, it also provides a means for Na^+ transport into and out of the xylem (Shi *et al.* 2002; Hall *et al.* 2006; Huang *et al.* 2006; Munns *et al.* 2006; Byrt *et al.*

2007). Much less is known about the transporters responsible for Na^+ uptake into root epidermal and cortical cells. Whereas some HKT transporters change their K^+ and Na^+ selectivity depending on the ionic conditions, similar to multi-ion channel pores (Schachtman and Schroeder 1994; Rubio *et al.* 1995; Gassmann *et al.* 1996; Horie *et al.* 2001), the only HKT transporter encoded in the *Arabidopsis* genome, AtHKT1, was found to be more Na^+ selective (Gao-Uozumi *et al.* 2000) and it was also found that when the encoding genes of some of these transporters is overexpressed, the salinity tolerance is improved (He *et al.* 2005; Agarwal *et al.* 2013). So Na^+ influx into plant roots can occur through ion channels or other membrane transport proteins that facilitate passive diffusion of Na^+ across the plasma membrane in most cases through K^+ pathways (HKT1), but the main pathway for Na^+ uptake in high salt concentrations is through non selective cation channels (Maathuis and Amtmann 1999a; Rus *et al.* 2001; Maser *et al.* 2002) which -nonselective cation channels- considered to be partially sensitive to calcium, since the entry of Na^+ into roots was inhibited in the presence of calcium (Tester and Davenport 2003). Even of their physicochemical similarities, it is K^+ rather than Na^+ that is essential to plant life, this means that plants in saline habitats have acquired mechanisms that allow the selective uptake of K^+ in the face of considerable competition from Na^+ (Maathuis and Amtmann 1999b). Plant cells employ primary active transport, mediated by H^+-ATPase, and secondary transport mediated by channels and co-transporters in order to maintain high concentrations of K^+ and low concentrations of Na^+ in the cytosol. The overall sub cellular compartmentation of Na^+ into the vacuole and other organelles, such as mitochondria and

plastids, also lowers Na^+ concentration in the cytoplasm, and at the same time contributes to osmotic adjustment, in order to maintain water uptake under salt conditions. The AtNHX family of Na^+/H^+ antiporters which localized in the tonoplast membrane here plays an important role in Na^+ compartmentation using the H^+ gradient as a driving force across the membrane under salinity stress (Blumwald 2000; Bassil *et al.* 2012). The first functionally-characterized member of this gene family, AtNHX1, contributes to Na^+ and monovalent cation sequestration in plant vacuoles, and the role of these vacuolar NHXs in Na^+ compartmentation has been strongly supported by the fact that *Arabidopsis atnhx1* mutant exhibited Na^+ sensitivity and less vacuolar Na^+/H^+ antiporter activity (Apse *et al.* 2003). Furthermore, also overexpression of genes encoding vacuolar AtNHX1 was shown to increase salt tolerance in a range of plant species with accompaniment increase in tissue Na^+ (Apse *et al.* 1999; Zhang *et al.* 2001; Agarwal *et al.* 2013).

So we can summarize with a simple model for mechanisms of Na^+ absorption, recirculation, and extrusion by different classes of Na^+ channels/transporters; Na^+ influx is mediated by HKT transporters (Gao-Uozumi *et al.* 2000; Maser *et al.* 2002; Sunarpi *et al.* 2005); excessive Na^+ in the cytosol is partially transported out of cells by SOS1 antiporters (Shi *et al.* 2000), and partially sequestered in vacuoles by AtNHX1 topnoplast antiporters (Apse *et al.* 1999). AtHKT1.1, and OsHKT1.5 are present in the plasma membrane of xylem parenchyma cells, and mediate unloading of Na^+ from xylem vessels into xylem parenchyma cells, thus protecting leaves from Na^+

over accumulation and Na^+ damage (leaf Na^+ exclusion) (Berthomieu *et al.* 2003; Ren *et al.* 2005; Sunarpi *et al.* 2005).

Salt stress has effects on plant growth; it reduces soil water potential leading to osmotic stress, it induces ion imbalance in cells, especially lower concentrations of K^+, Ca^{2+}, and $NO3^-$, and it causes ion (Na^+ and/or Cl^-) toxicity. Mechanisms of salinity tolerance can be classified into three categories: firstly, osmotic tolerance, which is regulated by long distance signals that reduce shoot growth and is triggered before shoot Na^+ accumulation; secondly, ion exclusion, where Na^+ and Cl^- transport processes in roots reduce the accumulation of toxic concentrations of Na^+ and Cl^- within leaves; and finally, tissue tolerance, where high salt concentrations are found in leaves but are compartmentalized at the cellular and intracellular level (especially in the vacuole); in other words, plants can reduce ion toxicity by reducing the accumulation of toxic ions in the leaf blades (Na^+ and Cl^- exclusion), and\or by increasing their ability to tolerate the salts that they have failed to exclude from the shoot, such as by compartmentation into vacuoles (tissue tolerance: involve the removal of Na^+ from the cytosol and compartmentation of it in the vacuole before the ion has a detrimental effect on cellular process (Tester and Davenport 2003; Plett *et al.* 2010). However, it is observed that there are many mechanisms of salinity tolerance, and many of these can be present in a particular plant. To date, there is neither evidence that these mechanisms are mutually exclusive (e.g. ion exclusion prevents tolerance to the 'osmotic phase' of salt toxicity), nor that a particular plant is committed to only one strategy (e.g. a plant may have ion

exclusion as its primary tolerance mechanism at moderate salinity, but it can switch to tissue tolerance mechanism as its main tolerance strategy when the exclusion processes are 'swamped' at high salinity). It is possible that some tolerance mechanisms are more effective in particular circumstances. For example, Na^+ exclusion may be more effective in conditions of higher salinity (Munns *et al.* 2012), whereas 'osmotic tolerance' may be more important in moderately saline conditions. Interactions with other abiotic stresses, such as low water availability, are also likely to be important.

1.4 NaCl sensing and genes defense activation

It is still unclear the sensing mechanism of salt in plant cell. The dual nature of salt stress, ionic and osmotic, brings a two sensory modality. For example, a high salt concentration in soil produces a hyperosmotic stress at root level. These two modalities are distinct from each other, because there are some responses specific for salt, other for purely osmotic stress (Deinlein *et al.* 2014). Nevertheless salt receptor(s) have not been known yet, histidine kinase receptor protein HK1 from *Arabidopsis thaliana* could be a good candidate for salt receptor. It was demonstrated that HK1 can complement in yeast the loss of the osmoreceptor SLN1 (Urao *et al.* 1993) and the overexpression or loss of function of this protein led to a drought associated phenotype (Tran *et al.* 2007). This osmosensor is coupled with calcium channel, because after salt exposure, there is a rapid increased of Ca^{2+} ions in cytosol (Ismail *et al.* 2014). High cytosolic levels of calcium ions trigger a reactive oxygen species (ROS) production by activating respiratory burst oxidase homolog F

(RBOHF) (Julkowska and Testerink 2015). RBOHF plays a pivotal role in salt response and more in general in abiotic stress response, indeed during pathogen attack the ROS production is due by respiratory burst oxidase homolog D (Montillet and Hirt 2013).One of the main functions of ROS signalling is the regulation of defence mechanisms against biotic threats and acclimation to abiotic stress conditions (Mittler *et al.* 2004; Torres 2010). ROS balance is disturbed during biotic and abiotic stresses by either enhancing ROS generating or reducing ROS scavenging abilities (Steffens *et al.* 2013). Molecular evidences suggest that ROS acts as a secondary signals during stress response by regulating and modifying gene expression. In particularly ROS influence the gene expression by modifying transcription factors at nucleus level (Apel and Hirt 2004). Moreover, high level of cytosolic ROS induces mitogen activated protein kinase (MAPK) such as MAPK3/6 and MAPK9/12;such as phosphorylation cascade has as a final target transcription factors in nucleus (Kovtun *et al.* 2000). Other kinases may be involved in transcription factor induction under salt stress, in particular calcium dependent-protein kinases (CLDPK) (Harmon *et al.* 2000; Boudsocq and Sheen 2013) and calcineurin B-like proteins (CBLs) with CBL-interacting protein kinases (CIPKs) (Weinl and Kudla 2009). At nuclear level many transcription factors are activated, like MYB20. It was demonstrated that MYB20 overexpressed lines are more tolerant to salt than knock-out lines (Cui *et al.* 2013). Other very important transcription factors are those belonging to the class of ABA responsive element-binding proteins (AREB1) and ABA insensitive 5 (ABI5), which activate a gene for acclimation and also for plasma

membrane ion channel and Respiratory Burst Oxidases Homolog F (RBOHF)(Umezawa *et al.* 2013). Activation of acclimation genes array leads to an accumulation of organic osmolytes, such as proline, glycine betaine, sugar alcohols, polyamines, and proteins from the late embryogenesis abundant (LEA) super family. These molecules play a pivotal role in maintaining low intracellular osmotic potential, to preventing the osmotic damage under salt stress (Verslues *et al.* 2006), in particularly the proline is involved in cell proliferation and cell death (Szabados and Savoure 2010). Also glycine betaine is proposed as a protectant of membrane structure and major enzyme (Guinn *et al.* 2011). In the last years it has emerged the idea that also chromatin modifications, referred to as epigenetic modifications, contribute to the adaptation potential of plants to different environmental stresses (Zhu *et al.* 2008). Several researches showed that modifications on chromatin are involved in the resistance responses of plants to salt stress in the same generation as the stress occurs. In particular, hyperosmotic priming was reported for Arabidopsis plants that have been treated with mild salt stress in the seedling stage, followed by cultivation under normal media salt free (Sani *et al.* 2013). During Na^+ stress-free period, no differences between pretreated plants and control plants were detected. Subsequently, after an additional salt stress application, the pretreated group accumulated less Na^+ and thus was more tolerant. This phenotype was attributed to epigenetic histone modifications that mainly affected expression of resistance transcription factors. Moreover the salinity changes methylation level of chromatin, specifically a target region of transcription factors (Song *et al.* 2012).

1.5 Cucumber (*Cucumis sativus* L.) and salinity

Cucumber is a popular fruit vegetable consumed worldwide and it has moderate sensitivity to salinity meaning that growth and productivity is restricted by high salt conditions (Mather and Jinks 2013). Cucumber belongs to the botanical family of cucurbitaceae, commonly known as cucurbits and gourds, that includes several economically important cultivated plants, beside cucumber (*C. sativus* L.), such as melon (*C. melo*L.), watermelon (*Citrullus lanatus* Thunb.Matsum. &Nakai) and squash and pumpkin (*Cucurbita* spp.). Agricultural production of cucurbits uses 9 million hectares of land and yields 184 million tons of vegetables, fruits and seeds annually (http://faostat.fao.org). The cucurbit family also displays a rich diversity of sex expression, and the cucumber has frequently served as a primary model system for sex determination studies (Guo *et al.* 2010). The cucurbits are also model plants for the study of vascular biology, as both xylem and phloem sap can be readily collected for studies of long-distance signaling events. Despite the agricultural and biological importance of cucurbits, knowledge of their genetics and genome is currently very limited. This is also true in the case of cucumber salt tolerance. In fact, despite genotypic variation in salt tolerance in cucumber has already been confirmed (Malik *et al.* 2010; Tiwari *et al.* 2011), the genetics of salt tolerance is poorly understand in this species (Tiwari *et al.* 2011), mainly due to the complexity of salt tolerance mechanisms (Maas and Poss 1989; Munns and Tester 2008). In order to take the first step towards the understanding of the genetics of cucumber salt tolerance, it is necessary to investigate and

develop an efficient and accurate selection traits protocol, in order to be able to predict salt tolerance of cucumber at different growth stages, in particular at seedling stage because it is relatively the most critical and sensitive stage. A clear understanding of the mechanisms of tolerance against salt stress in cucumber may contribute significantly to the improvement of the crop salt tolerance. In fact, the potential of genetic improvement of salt tolerance in cucumber is feasible if the gene action of superior parents is fully understood and a suitable breeding program is then selected (Dashti *et al.* 2012). Reduced emergence and expansion of leaves are generally accepted as adaptive mechanisms of plants exposed to osmotic stress due to salinity (Vaario *et al.* 2011). It was found that a combination of some traits (such as higher membrane stability, lower Na^+/K^+ ratio, higher osmotic concentration, selective uptake of useful ions and prevention of over accumulation of toxic ions) contributes to salt stress tolerance in cucumber (Tiwari *et al.* 2010). Accordingly, these traits would be useful selection criteria during salt stress breeding in cucumber. Currently, there is no proper established criterion of screening for salt tolerance in cucumber (Tiwari *et al.* 2010). Therefore, there is an urgent need for the development of rapid screening techniques and proper selection method to study salt tolerance.

As mentioned, the understanding of physiological, biochemical and genetic mechanisms is fundamental for a successful breeding program, in order to select the desired traits among the different genetic backgrounds (Munns *et al.* 2006). Furthermore, a more satisfying knowledge on biochemical and physiological aspects of salt stress tolerance mechanism in cucumber not only helps in identifying

the important criteria for salt tolerance and chalking out effective breeding programs, but also helps in cloning genes involved in salt tolerance and in the development of transgenic, as demonstrated in transgenic tobacco (Tarczynski *et al.* 1992). However, development of simple replicable and reliable criteria for screening cucumber under salinity has remained elusive. Leaf and other growth parameters may serve as useful selection criterion for salt tolerance in cucumber at seedling stage if the inheritance pattern is known. In cucumber, high salinity reduced photosynthetic rate, stomatal conductance and increased inter-cellular carbon dioxide (Xia *et al.* 2009), meanwhile in other plants high salinity decreased inter-cellular carbon dioxide, and this contradiction is probably due to many factors (salt level and duration of exposition, genotypes and species)(Colla *et al.* 2006; He *et al.* 2009). Decreased of cucumber plant growth (dry weight) under salt stress is due to reduction in photosynthesis rate because of both stomatal and non stomatal factors (high intercellular CO_2 concentration Ci) (Degl'Innocenti *et al.* 2009; Stepien and Johnson 2009; Pompelli *et al.* 2010; Silva *et al.* 2010). The high Ci observed in plants may be associated with damage of photosynthetic apparatus and cell membrane (Xia *et al.* 2009; Huang *et al.* 2011). It has been reported that salinity also causes degradation or inhibition of chlorophyll (Chl) biosynthesis in cucumber plants: the observed decrease in Chl content in the cucumber plants grown under saline conditions may be attributed to both an increased degradation and inhibited synthesis of that pigment (Sultana *et al.* 1999; García-Sánchez *et al.* 2002; Kere *et al.* 2016). Moreover, salinity is shown to increase sodium content Na^+, Na^+/K^+ ratio, proline accumulation, to

reduce sugar and phenol content, and to decrease potassium content, membrane stability index MSI in cucumber leaves under increasing salt stress decreases as well. In cucumber (Martinez *et al.* 1994; Botía *et al.* 2005) and in melon (Víllora *et al.* 1998), the similar trends of increase in proline and soluble sugars with the increase in salt concentration was reported. However, proline accumulation cannot be used as a sole criterion for salt tolerance, as it also accumulates under other stresses such as high temperature, drought and starvation (Hong *et al.* 2000).

Taking all the previous responses into account and trying to compare between salt tolerant and salt sensitive genotypes we can say that the key to salt tolerance in plants is the ability to comprise successfully between osmotic adjustment, ion nutrition, maintenance of energy pool and restriction of Na^+ in order to maintain a low Na^+:K^+ ratio in the cytosol of cells which is a crucial aspect of survival for a plant in saline environment. In general halophytes accumulate higher Na^+ levels in their leaves, than do glycophytes (Flowers *et al.* 1977). Salt tolerance involves tissues and whole plant integration of many different transport processes as compartmentation of ions and synthesis of organic osmolytes at cellular level.

References

Abel, G. H., and A. J. MacKenzie. 1964. Salt tolerance of soybean varieties (Glycine max L. Merrill) during germination and later growth. *Crop Science* 4 (2):157-161.

Agarwal, P. K., P. S. Shukla, K. Gupta, and B. Jha. 2013. Bioengineering for salinity tolerance in plants: state of the art. *Molecular biotechnology* 54 (1):102-123.

Apel, K., and H. Hirt. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373-399.

Apse, M. P., G. S. Aharon, W. A. Snedden, and E. Blumwald. 1999. Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science* 285 (5431):1256-1258.

Apse, M. P., J. B. Sottosanto, and E. Blumwald. 2003. Vacuolar cation/H+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1, the Arabidopsis vacuolar Na+/H+ antiporter. *Plant Journal* 36 (2):229-239.

Banuelos, G. S., A. Zayed, N. Terry, L. Wu, S. Akohoue, and S. Zambrzuski. 1996. Accumulation of selenium by different plant species grown under increasing sodium and calcium chloride salinity. *Plant and Soil* 183 (1):49-59.

Bassil, E., A. Coku, and E. Blumwald. 2012. Cellular ion homeostasis: emerging roles of intracellular NHX Na/H antiporters in plant growth and development. *J Exp Bot* 63 (16):5727-5740.

Berthomieu, P., G. Conejero, A. Nublat, W. J. Brackenbury, C. Lambert, C. Savio, N. Uozumi, S. Oiki, K. Yamada, F. Cellier, F. Gosti, T. Simonneau, P. A. Essah, M. Tester, A. A. Very, H. Sentenac, and F. Casse. 2003. Functional analysis of AtHKT1

in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance. *Embo Journal* 22 (9):2004-2014.

Blumwald, E. 2000. Sodium transport and salt tolerance in plants. *Current Opinion in Cell Biology* 12 (4):431-434.

Boudsocq, M., and J. Sheen. 2013. CDPKs in immune and stress signaling. *Trends in Plant Science* 18 (1):30-40.

Britto, D. T., and H. J. Kronzucker. 2006. Futile cycling at the plasma membrane: a hallmark of low-affinity nutrient transport. *Trends in Plant Science* 11 (11):529-534.

Byrt, C. S., J. D. Platten, W. Spielmeyer, R. A. James, E. S. Lagudah, E. S. Dennis, M. Tester, and R. Munns. 2007. HKT1;5-like cation transporters linked to Na+ exclusion loci in wheat, Nax2 and Kna1. *Plant Physiology* 143 (4):1918-1928.

Chauhan, J., Z. J. Hawrysh, C. Ko, and S. Ko. 1987. Taste Perception of Salt in Young, Old, and Very Old Adults. *Annals of the New York Academy of Sciences* 510:222-223.

Cui, M. H., K. S. Yoo, S. Hyoung, H. T. K. Nguyen, Y. Y. Kim, H. J. Kim, S. H. Ok, S. D. Yoo, and J. S. Shin. 2013. An Arabidopsis R2R3-MYB transcription factor, AtMYB20, negatively regulates type 2C serine/threonine protein phosphatases to enhance salt tolerance. *Febs Letters* 587 (12):1773-1778.

Deinlein, U., A. B. Stephan, T. Horie, W. Luo, G. H. Xu, and J. I. Schroeder. 2014. Plant salt-tolerance mechanisms. *Trends in Plant Science* 19 (6):371-379.

Falkenmark, M., A. K. Biswas, H. Hori, T. Ishibashi, G. Kovacs, P. Rogers, and H. I. Shuval. 1987. Water-Related Limitations to Local Development. *Ambio* 16 (4):191-200.

Flowers, T., P. Troke, and A. Yeo. 1977. The mechanism of salt tolerance in halophytes. *Annual review of plant physiology* 28 (1):89-121.

Flowers, T., and A. Yeo. 1981. Variability in the resistance of sodium chloride salinity within rice (Oryza sativa L.) varieties. *New Phytologist* 88 (2):363-373.

Gao-Uozumi, C. X., N. Uozumi, E. Miyoshi, K. Nagai, Y. Ikeda, T. Teshima, K. Noda, T. Shiba, K. Honke, and N. Taniguchi. 2000. A novel carbohydrate binding activity of annexin V toward a bisecting N-acetylglucosamine. *Glycobiology* 10 (11):1209-1216.

Gassmann, W., F. Rubio, and J. I. Schroeder. 1996. Alkali cation selectivity of the wheat root high-affinity potassium transporter HKT1. *Plant Journal* 10 (5):869-882.

Gleick, P. H. 1993. Water and Conflict - Fresh-Water Resources and International Security. *International Security* 18 (1):79-112.

Guinn, E. J., L. M. Pegram, M. W. Capp, M. N. Pollock, and M. T. Record. 2011. Quantifying why urea is a protein denaturant, whereas glycine betaine is a protein stabilizer. *Proceedings of the National Academy of Sciences* 108 (41):16932-16937.

Hall, D., A. R. Evans, H. J. Newbury, and J. Pritchard. 2006. Functional analysis of CHX21: a putative sodium transporter in Arabidopsis. *J Exp Bot* 57 (5):1201-1210.

Harmon, A. C., M. Gribskov, and J. F. Harper. 2000. CDPKs - a kinase for every Ca2+ signal? *Trends in Plant Science* 5 (4):154-159.

Hasegawa, M., R. Bressan, and J. M. Pardo. 2000. The dawn of plant salt tolerance genetics. *Trends in Plant Science* 5 (8):317-319.

He, C. X., J. Q. Yan, G. X. Shen, L. H. Fu, A. S. Holaday, D. Auld, E. Blumwald, and H. Zhang. 2005. Expression of an arabidopsis vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. *Plant and Cell Physiology* 46 (11):1848-1854.

Horie, T., K. Yoshida, H. Nakayama, K. Yamada, S. Oiki, and A. Shinmyo. 2001. Two types of HKT transporters with different properties of Na+ and K+ transport in Oryza sativa. *Plant Journal* 27 (2):129-138.

Huang, Y. Z., G. P. Zhang, F. B. Wu, J. X. Chen, and Y. P. Xiao. 2006. Interaction of salinity and cadmium stresses on antioxidant enzymes, sodium, and cadmium accumulation in four barley genotypes. *Journal of Plant Nutrition* 29 (12):2215-2225.

Ismail, A., S. Takeda, and P. Nick. 2014. Life and death under salt stress: same players, different timing? *J Exp Bot* 65 (12):2963-2979.

Jeschke, W. D. 1984. Effects of transpiration on potassium and sodium fluxes in root cells and the regulation of ion distribution between roots and shoots of barley seedlings. *J Plant Physiol* 117 (3):267-285.

Julkowska, M. M., and C. Testerink. 2015. Tuning plant signaling and growth to survive salt. *Trends in Plant Science*.

Kovtun, Y., W. L. Chiu, G. Tena, and J. Sheen. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proceedings of the National Academy of Sciences of the United States of America* 97 (6):2940-2945.

Kronzucker, H. J., M. W. Szczerba, M. Moazami-Goudarzi, and D. T. Britto. 2006. The cytosolic Na+ : K+ ratio does not explain salinity-induced growth impairment in barley: a dual-tracer

study using K-42(+) and Na-24(+). *Plant Cell and Environment* 29 (12):2228-2237.

Läuchli, A., and J. Wieneke. 1979. Studies on growth and distribution of Na+, K+ and Cl− in soybean varieties differing in salt tolerance. *Zeitschrift für Pflanzenernährung und Bodenkunde* 142 (1):3-13.

Leland, E., and V. Eugene. 1999. Crop Response and Management of Salt-Affected Soils. *Handbook of Plant and Crop Stress*:169.

Maathuis, F. J., and A. Amtmann. 1999a. K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. *Annals of Botany* 84 (2):123-133.

Maathuis, F. J. M., and A. Amtmann. 1999b. K+ nutrition and Na+ toxicity: The basis of cellular K+/Na+ ratios. *Annals of Botany* 84 (2):123-133.

Malagoli, P., D. T. Britto, L. M. Schulze, and H. J. Kronzucker. 2008. Futile Na+ cycling at the root plasma membrane in rice (Oryza sativa L.): kinetics, energetics, and relationship to salinity tolerance. *J Exp Bot* 59 (15):4109-4117.

Maser, P., M. Gierth, and J. I. Schroeder. 2002. Molecular mechanisms of potassium and sodium uptake in plants. *Plant and Soil* 247 (1):43-54.

Montillet, J.-L., and H. Hirt. 2013. New checkpoints in stomatal defense. *Trends in Plant Science* 18 (6):295-297.

Munns, R., R. A. James, and A. Lauchli. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *J Exp Bot* 57 (5):1025-1043.

Munns, R., and D. P. Schachtman. 1993. Plant-Responses to Salinity - Significance in Relation to Time. *International Crop Science I*:741-745.

Munns, R., and A. Termaat. 1986. Whole-Plant Responses to Salinity. *Australian Journal of Plant Physiology* 13 (1):143-160.

Munns, R., and M. Tester. 2008. Mechanisms of salinity tolerance. In *Annu Rev Plant Biol*. Palo Alto: Annual Reviews, 651-681.

Niu, J., B. K. Rao, P. Jena, and M. Manninen. 1995. Interaction of H-2 and He with Metal Atoms, Clusters, and Ions. *Physical Review B* 51 (7):4475-4484.

Oertli, J. 1968. Extracellular salt accumulation a possible mechanism of salt injury in plants. *Agrochimica* 12 (5):461-&.

Oh, D. H., Q. Q. Gong, A. Ulanov, Q. Zhang, Y. Z. Li, W. Y. Ma, D. J. Yun, R. A. Bressan, and H. J. Bohnert. 2007. Sodium stress in the halophyte Thellungiella halophila and transcriptional changes in a thsos1-RNA interference line. *Journal of Integrative Plant Biology* 49 (10):1484-1496.

Oh, D. H., E. Leidi, Q. Zhang, S. M. Hwang, Y. Z. Li, F. J. Quintero, X. Y. Jiang, M. P. D'Urzo, S. Y. Lee, Y. X. Zhao, J. D. Bahk, R. A. Bressan, D. J. Yun, J. M. Pardo, and H. J. Bohnert. 2009. Loss of Halophytism by Interference with SOS1 Expression. *Plant Physiology* 151 (1):210-222.

Ren, C. M., J. W. Pan, W. Peng, P. Genschik, L. Hobbie, H. Hellmann, M. Estelle, B. Gao, J. R. Peng, C. Q. Sun, and D. X. Xie. 2005. Point mutations in Arabidopsis Cullin1 reveal its essential role in jasmonate response. *Plant Journal* 42 (4):514-524.

Rengel, Z. 1992. Modeling Magnesium Uptake from an Acid Soil .4. Depletion of Magnesium, Calcium, and Potassium from Soluble and Exchangeable Phase. *Communications in Soil Science and Plant Analysis* 23 (1-2):165-174.

Robinson, S. P., and W. J. S. Downton. 1984. Potassium, sodium, and chloride content of isolated intact chloroplasts in relation to

ionic compartmentation in leaves. *Archives of biochemistry and biophysics* 228 (1):197-206.

Rubio, F., W. Gassmann, and J. I. Schroeder. 1995. Sodium-Driven Potassium Uptake by the Plant Potassium Transporter Hkt1 and Mutations Conferring Salt Tolerance. *Science* 270 (5242):1660-1663.

Rus, A., B. H. Lee, A. Munoz-Mayor, A. Sharkhuu, K. Miura, J. K. Zhu, R. A. Bressan, and P. M. Hasegawa. 2004. AtHKT1 facilitates Na+ homeostasis and K+ nutrition in planta. *Plant Physiology* 136 (1):2500-2511.

Rus, A., S. Yokoi, A. Sharkhuu, M. Reddy, B. H. Lee, T. K. Matsumoto, H. Koiwa, J. K. Zhu, R. A. Bressan, and P. M. Hasegawa. 2001. AtHKT1 is a salt tolerance determinant that controls Na+ entry into plant roots. *Proceedings of the National Academy of Sciences of the United States of America* 98 (24):14150-14155.

Sani, E., P. Herzyk, G. Perrella, V. Colot, and A. Amtmann. 2013. Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. *Genome Biology* 14 (6).

Schachtman, D. P., and J. I. Schroeder. 1994. Structure and Transport Mechanism of a High-Affinity Potassium Uptake Transporter from Higher-Plants. *Nature* 370 (6491):655-658.

Serrano, R. 1996. Salt tolerance in plants and microorganisms: Toxicity targets and defense responses. *International Review of Cytology - a Survey of Cell Biology, Vol 165* 165:1-52.

Serrano, R., F. A. Culianz-Macia, and V. Moreno. 1999. Genetic engineering of salt and drought tolerance with yeast regulatory genes. *Scientia Horticulturae* 78 (1-4):261-269.

Shi, H. Z., M. Ishitani, C. S. Kim, and J. K. Zhu. 2000. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proceedings of the National Academy of Sciences of the United States of America* 97 (12):6896-6901.

Shi, H. Z., F. J. Quintero, J. M. Pardo, and J. K. Zhu. 2002. The putative plasma membrane Na+/H+ antiporter SOS1 controls long-distance Na+ transport in plants. *Plant Cell* 14 (2):465-477.

Song, Y. G., D. D. Ji, S. Li, P. Wang, Q. Li, and F. N. Xiang. 2012. The Dynamic Changes of DNA Methylation and Histone Modifications of Salt Responsive Transcription Factor Genes in Soybean. *Plos One* 7 (7).

Sunarpi, T. Horie, J. Motoda, M. Kubo, H. Yang, K. Yoda, R. Horie, W. Y. Chan, H. Y. Leung, K. Hattori, M. Konomi, M. Osumi, M. Yamagami, J. I. Schroeder, and N. Uozumi. 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na+ unloading from xylem vessels to xylem parenchyma cells. *Plant Journal* 44 (6):928-938.

Szabados, L., and A. Savoure. 2010. Proline: a multifunctional amino acid. *Trends in Plant Science* 15 (2):89-97.

Termaat, A., J. B. Passioura, and R. Munns. 1985. Shoot Turgor Does Not Limit Shoot Growth of Nacl-Affected Wheat and Barley. *Plant Physiology* 77 (4):869-872.

Tester, M., and R. Davenport. 2003. Na+ tolerance and Na+ transport in higher plants. *Annals of Botany* 91 (5):503-527.

Tran, L. S. P., T. Urao, F. Qin, K. Maruyama, T. Kakimoto, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in

Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 104 (51):20623-20628.

Umezawa, T., N. Sugiyama, F. Takahashi, J. C. Anderson, Y. Ishihama, S. C. Peck, and K. Shinozaki. 2013. Genetics and Phosphoproteomics Reveal a Protein Phosphorylation Network in the Abscisic Acid Signaling Pathway in Arabidopsis thaliana. *Science Signaling* 6 (270).

Urao, T., K. Yamaguchi-Shinozaki, S. Urao, and K. Shinozaki. 1993. An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *The Plant Cell* 5 (11):1529-1539.

Verslues, P. E., M. Agarwal, S. Katiyar-Agarwal, J. H. Zhu, and J. K. Zhu. 2006. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant Journal* 45 (4):523-539.

Wang, B., R. J. Davenport, V. Volkov, and A. Amtmann. 2006. Low unidirectional sodium influx into root cells restricts net sodium accumulation in Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana. *J Exp Bot* 57 (5):1161-1170.

Weinl, S., and J. Kudla. 2009. The CBL–CIPK Ca2+-decoding signaling network: function and perspectives. *New Phytologist* 184 (3):517-528.

Yeo, A. R., and T. J. Flowers. 1985. The Absence of an Effect of the Na/Ca Ratio on Sodium-Chloride Uptake by Rice (Oryza-Sativa-L). *New Phytologist* 99 (1):81-90.

Zhang, H. X., J. N. Hodson, J. P. Williams, and E. Blumwald. 2001. Engineering salt-tolerant Brassica plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proceedings of the National Academy of Sciences of the United States of America* 98 (22):12832-12836.

Zhu, J., J. C. Jeong, Y. Zhu, I. Sokolchik, S. Miyazaki, J. K. Zhu, P. M. Hasegawa, H. J. Bohnert, H. Shi, D. J. Yun, and R. A. Bressan. 2008. Involvement of Arabidopsis HOS15 in histone deacetylation and cold tolerance. *Proceedings of the National Academy of Sciences of the United States of America* 105 (12):4945-4950.

Chapter 2

"Potassium fluxes and reactive oxygen species production as potential indicators of salt tolerance in *Cucumis sativus* L."

Mirvat Redwan, Francesco Spinelli, Lucia Marti, Matthias Weiland, Emily Palm, Elisa Azzarello and Stefano Mancuso

Department of Plant, Soil and Environmental Science, University of Florence, Viale delle Idee 30, 50019 Sesto Fiorentino, Florence Italy.

Corresponding author: stefano.mancuso@unifi.it

Keywords: Salt tolerance, K^+ fluxes and ROS production.

A modified version of this chapter has been published in *Functional Plant Biology*43.11 (2016): 1016-1027.

2.1 Background information

2.1.1 Screening for salt tolerance in cucumber (*Cucumis sativus* L.)

Salt tolerance is a multifactorial trait and for several decades significant efforts have been done to understand all mechanisms underlying salt tolerance in order to enhance the productivity of salt sensitive crop; Nevertheless; progress is still limited most likely due to the complexity of salt tolerance mechanisms. In view of the complexity of salt tolerance and its great variation among plant species and context, it is difficult to identify single criteria, which could be used as effective selection targets. Plant breeders have successfully improved salinity tolerance of some crops in recent decades, using artificial selection and conventional breeding approaches (vigor or seed yield as the main selection criteria), although molecular biology approaches are currently being intensively pursued for achieving this goal. Selection may be more convenient and practicable if the crop possesses distinctive indicators of salt tolerance at the whole plant, tissue or cellular level. It is most meaningful if physiological and biochemical indicators for individual species are determined rather than generic indicators. Thus, there is a need to determine the underlying biochemical mechanisms of salinity tolerance so as to provide plant breeders with appropriate indicators. A large body of evidence indicates that salt tolerance involves the ability of cells to retain potassium ions which isat least as important as a plant's ability to exclude or compartmentalize toxic Na^+, and to

regulate ROS production and synthesize new molecules to cope with osmotic stress.

Despite the complexity of processes and mechanisms involved in the responses described above, it is now clear that the ability of a plant to maintain a high cytosolic K^+/Na^+ ratio is a critical factor in salt tolerance and as a result, the major efforts of researchers and breeders have been aimed for many decades at improving this ratio by minimizing and preventing Na^+ uptake and transport to shoot, which would in turn also avoid specific Na^+ toxicity (Shabala and Cuin 2008).

In this chapter, screening for salt tolerance in cucumber plants (*Cucumis sativus*) was conducted using several different physiological and genetic approaches. In the first phase six different cultivars of cucumber were screened for salt tolerance under 100Mm NaCl; then, two different cultivars (Parys, sensitive and Polan, tolerant), were selected based on their germination capability under salt stress, in order to continue the screening for salt tolerance as a second phase of the work by using several different physiological and genetic approaches . Thus, K^+ fluxes from roots were measured, as an immediate response to salinity, and the expression level of Inward Rectifying Potassium Channel (AKT1) was investigated as well. ROS production, the level of Respiratory Burst Oxidase Homolog F (RBOHF) gene, and the induction of Ethylene Responsive Factor 109 (ERF109) transcription factor after salt treatment were also examined, in both cultivars, as indicators for salt tolerance. Basing on results, we suggest that root ability to retain K^+, a higher level of RBOHF and a

strong induction of ERF109, should all be considered important components for salt tolerance in *Cucumis sativus*.

2.2 Potassium fluxes and Reactive Oxygen Species production as potential indicators of salt tolerance in *Cucumis sativus* L

2.2.1 Abstract

Salt stress, among different abiotic stresses, has a high impact on crop yield. Salt tolerance is a multifactorial trait that involves the ability of cells to retain potassium ions, regulate ROS production, and synthesize new molecules to cope with osmotic stress. In the present work two different cultivars of *Cucumis sativus* (Parys, sensitive and Polan, tolerant), were selected based on their germination capability under 100 mM sodium chloride. The capacity of these two cultivars to tolerate salt stress was analysed using several different physiological and genetic approaches. K^+ fluxes from roots, as an immediate response to salinity, showed a higher ability of cv Polan to maintain K^+ compared to cv Parys, according to the expression level of Inward Rectifying Potassium Channel (AKT1). ROS production was also investigated in both cultivars, and in particular a higher basal ROS level was observed in cv Polan than in cv Parys. Concurrently, an increased basal level of Respiratory Burst Oxidase Homolog F (RBOHF) gene was also found, as well as a strong induction of Ethylene Responsive Factor 109 (ERF109) transcription factor after salt treatment in cv Polan. Our data suggest that root ability to retain K^+, a higher level of RBOHF and a strong induction of ERF109, should all be considered important components for salt tolerance in *Cucumis sativus* L.

2.2.2 Introduction

Global demand for food is growing rapidly due to an increasing world population. Current estimates indicate that food production would have to increase by up to 70% by 2050 to keep pace with future demands (Panta *et al.* 2014). Suitable resources for future agricultural expansion are also limited due to competing land and water uses for human consumption and non-food (e.g., biofuel) crop production (Beddington 2010; DeHaan *et al.* 2010). Among the many abiotic stresses limiting agricultural productions, salinity has been a factor affecting agriculture for more than 6000 years and is now a major economic concern in industrialized agriculture with current global annual losses around US$27 billion and rising (Cheeseman 2015). To complicate matters further, it is logical to predict that the acuteness of this problem will be aggravated by global climate change that will increase the frequency and severity of drought stress and, thus, our reliance on irrigation in many regions worldwide (Panta *et al.* 2014). Concurrently, it is also expected that good quality water will be increasingly reserved for drinking and urban use, and thus farmers will need to turn to brackish and saline water substantially exacerbating salinity problems.

Salinity affects almost every aspect of plant physiology and biochemistry, significantly reducing plant growth and crop yield (Cuartero *et al.* 2006a). In particular, among different abiotic stresses, soil and water salinity are major constraints reducing crop productivity in many areas of the world (Arzani 2008). Most

horticultural crops are glycophytes as they evolved in ecosystems with low soil sodium levels (Cheeseman 2015).

For several decades significant efforts have been done to understand the mechanisms underlying salt tolerance in order to enhance the productivity of salt sensitive crops, but progress is still limited due to the complexity of the problem (Munns and Tester 2008). Growth reduction in response to salinity is the result of various effects, including diminished water uptake, reduced carbon fixation, increased energy costs for protective processes such as osmotic adaptation and ion exclusion, and growth limitations originating from nutritional imbalances (Munns and Tester 2008). Despite the complexity of processes and mechanisms involved in the responses described above, some critical aspects of the problem, such as the ability of the plant to maintain a high cytosolic K^+/Na^+ ratio, have been clarified (Shabala and Cuin 2008). Thus, the major efforts of researchers and breeders have been aimed at improving this ratio by minimizing and preventing Na^+ uptake and transport to shoot, which would in turn avoid specific Na^+ toxicity (Shabala and Cuin 2008). However, despite some positive results under controlled conditions, there are currently no reports confirming that higher Na^+ extrusion from plant roots may result in superior salinity tolerance (Shabala 2013). Consequently, it has recently been hypothesized that Na^+ exclusion from the roots could actually aggravate salt stress by increasing the osmotic and ionic imbalance in the root-zone (Adem *et al.* 2014). On the other hand, several lines of evidence now indicates that the ability of a cell to retain K^+ is at least as important as a plant's

ability to exclude or compartmentalize toxic Na^+ (Chen *et al.* 2007; Shabala and Cuin 2008; Shabala and Pottosin 2014). K^+ has a pivotal role in maintaining the turgor of the whole cell. Channels and transporters present on the plasma membrane transfer K^+ into the plant cell against its concentration gradient. The mechanism mediating K^+ uptake depends on the K^+ concentration outside of the cell: if its concentration is low, transporters facilitate K^+ uptake, whereas K^+ uptake is carried out by K^+ channels when the extracellular concentration is high (Munns and Tester 2008).

As reported in previous studies K^+ acquisition from soils is mainly mediated by K^+ transporters and channels, such as the High Affinity Potassium Transporters (HKT family), the HAK/KT/KUP family and the Shaker Inward Rectifying Potassium Channels (AKT1-like K^+ channels) (Shabala 2003; Aleman *et al.* 2011; Wang and Wu 2013; Very *et al.* 2014). AKT1-like channels are considered the main channel components that mediate K^+ influx into root cells in many plant species; these channels include AKT1 in *Arabidopsis thaliana*,a major inward K^+ channel expressed primarily in roots and localized to the plasma membrane of epidermal cells (Sentenac *et al.* 1992; Lagarde *et al.* 1996; Hirsch *et al.* 1998). It has also been reported that the transcript levels of AKT1 are regulated by external sodium concentration, and that *A. thaliana* plants over-expressing AKT1 have an increased K^+ content and enhanced salt tolerance compared to wild-type plants under salt stress (Ardie *et al.* 2010).

Salt stress is one of the factors that triggers the production of Reactive Oxygen Species (ROS) and causes an increase of these

compounds in plant cells (Apel and Hirt 2004). One of the main functions of ROS signalling is the regulation of defence mechanisms against biotic threats and acclimation to abiotic stress conditions (Mittler *et al.* 2004; Torres 2010). In this context it is also important to shed light on the involvement of some integral plasma membrane proteins, such as the Respiratory Burst Oxidases (RBOHs). It was recently reported that Respiratory Burst Oxidase Homologue F (RBOHF) can regulate the activity of potassium channels, improving K^+ homeostasis (Ma *et al.* 2011; Tran *et al.* 2013). The generation of ROS as a secondary messenger in the transduction pathway in plants under abiotic stress results in the expression of multiple stress responsive genes or transcription factors (TF) (Huang *et al.* 2011). Among these transcription factors, Ethylene Response Factor 109 (ERF 109, also known as RRTF1) is strongly activated under biotic stress and under salt treatment; the molecular mechanisms and the signalling pathway activated by this TF still remains unclear to date (Matsuo *et al.* 2015). It was previously reported that an intracellular increase of ROS level can produce a variation in the sugar content within a plant cell (Keunen *et al.* 2013). In addition, the main mechanism used by plants to counteract water loss under salt stress is the synthesis of compatible solutes; this includes amino acids and the accumulation of carbohydrates such as sugars (e.g., glucose, fructose, fructans, and trehalose) and starch that are involved inosmo-protection, carbon storage and scavenging of reactive oxygen species. It was observed in *A. Thaliana* that a high level of raffinose, produced by galactinol synthase (GOLS), led to an improved osmo-tolerance during salt stress (Sun *et al.* 2013).

Among key horticultural crops, cucumber (*Cucumis sativus L)* is an important species for cultivation worldwide, and is generally considered moderately salt sensitive (Kere *et al.* 2013). For instance, a salinity of 5 dS m^{-1} (approximately 50 mM NaCl) was found to reduce both shoot and root growth, by *ca.* 50% in three-week old seedling exposed to NaCl stress for 2 weeks, although reductions in growth were already visible with 2 dS m^{-1} (ca. 20 mM NaCl)(Khan *et al.* 2013). In stressed cucumber plants, sodium chloride related injuries have been associated with decreased plant growth and chlorophyll content, impaired gas exchange changes in chlorophyll fluorescence and increased membrane permeability and oxidative damage (Chartzoulakis 1994; Zhu *et al.* 2004; Stepien and Klobus 2006; Zhu *et al.* 2008; Khan *et al.* 2013). Given the lack of clear and consistent criteria for screening cucumber under salinity, the main objective of this work was to evaluate the potential of rapid K^+ flux measurement for use as a rapid screening test on seedlings. Six different cucumber cultivars were screened under salt stress, and from among these, cv Parys and cv Polan were selected on the basis of sensitivity and tolerance, respectively. We investigated their performance in term of K^+ fluxes following the addition of NaCl and in combination with physiological assessments of different parameters, including plant growth and the ion content of plant tissues. Moreover, we associated these measurements with gene modulation of ion channel AKT1 in both cultivars; the response of AKT1 to external saline conditions in *Cucumis sativus* still has to be investigated. ROS content was also evaluated to better understand the role of these compounds in the establishment of the salt tolerance. We then correlated these findings

to the gene expression level of RBOHF, the NADPH oxidase involved in ROS production under abiotic stress (Baxter *et al.* 2014). Finally, we evaluated the gene expression of two stress related genes, GOLS 1 and ERF 109.

2.2.3 Materials and Methods

2.2.3.1 Seeds germination assay

This experiment was conducted to evaluate the germination of 6 commercial cucumber cultivars (Odys, Parys, Polan, Marketmore, Ashly and Chinese Slogan) under salt stress. Three replicates of 25 seeds of each were used. Seeds were surface-sterilized and placed in petri dishes (12) cm*(12) cm on filter paper soaked with the appropriate treatment solution (deionized water for the control and 100mM NaCl in the treatment). The lids were sealed with parafilm and the dishes were kept in a dark germination chamber at 26˚C for seven days. A seed was considered to have germinated when the radicle emerged, so from that point, germinated seeds were counted daily at a specific time. Observations were made daily. Mean daily germination (MDG) was calculated as MDG = FGP / d , where FGP is the final germination percent and d is the test period. Daily germination speed (DGS) is the inverse of mean daily germination and was calculated as: DGS = 1 /MDG (Ashkan and Jalal 2013).

2.2.3.2 Salt treatment

Two cucumber varieties Polan and Parys were selected to perform this experiment on the base of their germination parameters. After germination, plants were transferred to an aerated nutrient solution (Hoagland): (mM) 0.5 KNO_3, 0.5 $CaNO_3$, 0.33 $MgSO_4$, 0, 0.3 NH_4H2PO_4; and (μM): 20 FeEDTA, 2.3 H_3BO_3, 2 $MnSO_4$, 2 $ZnSO_4$, 0.5 $CuSO_4$, 0.5 Na_2MoO_4. A quarter-strength nutrient Hoagland solution was used for the first week, a half-strength solution for the second week and a full-strength solution for the remaining time of the experiment. Plants were grown in a naturally-lit glasshouse with an average 18/28°C night/day temperature and an average relative humidity of 60%. The nutrient solution was replaced weekly and the pH of the nutrient solution was adjusted to 5.8 using KOH. Three weeks after transferring plants to the glasshouse, uniform plants were selected and divided into two groups according to variety, and then further divided into four salt treatment groups (control, 50, 100, 200 mM NaCl) of six replicates each.

2.2.3.3 Ion flux measurement

The experiment consisted of 10 replicates and focused on the response to 100 mM NaCl. Two-week old intact seedlings were used for the measurements. After germination, seedlings were transferred into 25% nutrient solution (Hoagland) for one week prior to the measurement of ion fluxes and then were used for ion flux measurements. Net K^+ Fluxes were measured non-invasively on roots

of cucumber seedlings using the Vibrating Probe system (VIP, University of Florence, Italy). Microelectrodes were fabricated and calibrated following the methods previously described (Mancuso *et al.* 2000). Two hours prior to the measurements, seedlings of the two selected varieties were placed in measuring chambers with their roots immobilized in the horizontal position and submerged in 4 ml basal salt media (BSM: 0.1 mM $CaCl_2$, 0.2 mM KCl) bath solution. Basal K^+ fluxes were then measured for 5 min and subsequently 0.7 ml of BSM with 1 mM sodium chloride was added to chamber's solution in order to have final concentrations of 100 mM NaCl. Net K^+ fluxes were monitored until stable values were obtained ,which occurred after at least 40 min.

2.2.3.4 ROS detection

ROS detection was carried out using an upright Leica laser-scanning confocal microscope SP5 (Leica Microsystems Wetzlar GmbH, Germany) equipped with a 40× oil-immersion objective, the probe used in the experiments was 2′,7′-Dichlorofluorescein (DCF-DA, Sigma Aldrich). This particular dye was chosen as it is considered to be a very specific indicator for intracellular ROS production in root cells (Rosenkranz *et al.* 1992). After germination, seedlings were transferred in Hoagland solution (25%) for two weeks and then used to perform the measurement on confocal microscope. Roots were treated with 100 mM NaCl for 3 hours, after which they were washed with deionized water and then incubated for 2 min in a solution of 5 µM (DCF-DA). The same procedure was followed for

the control but without the addition of salt. After incubation, the samples were mounted in a water solution on a slide and observed. The excitation wavelength was set at 494±1nm, and emission was detected at 522±5nm. ROS content in seedling roots was quantified after compiling the projection from 10 confocal sections of 0.5 μm each and from the apex along the entire root. The quantification was expressed in relative units using LCS Lite software from Leica.

2.2.3.5 Gene expression analysis

Three-week old seedlings were used for sampling after 3h of the treatment with 100 mM NaCl. Samples from both control and treatment roots of the two cultivars were directly frozen in liquid nitrogen and then stored at -80 C° until the RNA extraction process could be performed. RNA was extracted using Tryzol reagent (Life Technology) according to the manufacturer's instructions. The amount of RNA was measured using a Tecan Infinite 200 Spectrophotometer (Mannedorf, Switzerland). RNA was treated with RQ1 DNase (Promega), and first-strand cDNA was obtained using Improm II reverse transcriptase (Promega). Primers were designed using Primer 3 software (Koressaar and Remm 2007; Untergasser *et al.* 2012). Gene sequences were retrieved in cucumber databases (cucumber.genomics.org.cn). The sequences of the primers are as follows: ACT3 actin 3 Fw, TTCTGGTGATGGTGTGAGTC; ACT3 actin 3 Rv, GGCAGTGGTGGTGAACATG; AKT1 Fw, CTGTTCGTACAAAGCGATTG; AKT1 Rv, TCCAACAAAACTCCTTCCAT; RBOHF Fw,

GAGGCATTGTAGCAGGAGTT; RBOHF Rv, GTGGCCAACATTTCAACATA; Galactinol synthase 1-like Fw, TACAAGCCCATCTCCTCGGA; Galactinol synthase 1-like Rv, AGTGAACAGGTCCAGCTTCG; ERF109-like Fw, ACCCACCCAATTTTCCCTCC; ERF109-like Rv, ACTCCAACGCCGCTTTATCA.

Real Time qPCR was performed using a RotorGene 6000 (Quiagen). cDNA (corresponding to 5 ng of total RNA) was amplified using SSo Advance Universal Sybr Green Supermix (BioRad) and 0.5 µM of each primer. Data analysis was done using LinRegPCR software. Expression level of gene was referred to Actin 3 (Act3) as housekeeping gene and was determined using a modification of the Pfaffl method (Pfaffl 2001), as reported in Ferrari et al. 2006 and expressed in arbitrary units.

2.2.3.6 Determination of fresh and dry mass

A subset of plants was used for destructive measurements of fresh weight and dry weight on day zero (six plants), and the remaining plants were processed after eight days of treatment. Individual plants were separated into roots, leaves and stems. Roots were thoroughly rinsed three times with water and excess water removed by blotting the roots with paper towels. Fresh mass of all plant tissues was recorded immediately and their dry masses recorded after one week in a drying oven 70°C.

2.2.3.7 Leaf gas exchange parameters and chlorophyll fluorescence

Net photosynthetic rate, stomatal conductance, intercellular CO2 concentration and chlorophyll fluorescence were measured on young fully expanded leaves in both cultivars under 100 mM NaCl due to the dramatic observed reduction in both fresh and dry total mass at this salt concentration. The Li-6400 XT open gas exchange system (Li-Cor Inc.) was used for all measurements. Leaf gas exchange measurements were taken on all plants from each treatment at a relative humidity of 40–50%, a reference CO2 concentration of 400 mmolmol^{-1} , a leaf chamber temperature at 25 ±1˚C and a PAR of 1500 mmol m^{-2} s $^{-1}$. These measurements were carried out 1 day before the treatment (0 mM NaCl), the first day after the treatment (100 mM NaCl) and on the day before the final harvest (100 mM NaCl). Using the integrated fluorescence chamber head (Li-6400–40) of the Li-6400 XT open gas exchange system, we measured maximum quantum yield via chlorophyll fluorescence on the same leaves used for gas exchange measurements at the end of the night period (i.e. dark adapted for at least 11 h). The minimum fluorescence level in the dark-adapted state (F0) was measured using a modulated pulse, and the dark-adapted maximum fluorescence (Fm) was measured after applying a saturating actinic light pulse of 7000 mmol m^{-2} s $^{-1}$. The maximum quantum yield was calculated as Fv/Fm, where Fv = Fm – F0 (Maxwell and Johnson 2000).

2.2.3.8 Determination of potassium and sodium concentrations in plant tissues

Dried plant tissues (leaves, stems and roots) were ground separately, and K^+ and Na^+ concentrations were obtained after digesting the samples in 0.5 M HNO3 by shaking the mixture in vials for 48 h in the dark at room temperature. Diluted extracts were analyzed for K^+ and Na^+ concentrations by using a Digiflame DV710 (NT Laboratory) according to the manufacturer's instructions.

2.2.4 Statistical analyses

Statistical analyses were conducted using GraphPad Prism ver. 5.0 software (GraphPadInc, www.graphpad.com). One-way ANOVA or Student's t-test, depending on the dataset, were used to identify the overall significant differences among treatments. Unless otherwise stated, the significance level was $P \leq 0.05$.

2.2.5 Results

2.2.5.1 Screening of salt tolerance in cucumber varieties through Seed Germination Assay

Both the germination rate and mean daily germination of all cucumber cultivars decreased with the treatment with 100 mM NaCl, although an increase in the daily germination speed was observed. Variations in seed germination were recorded in all tested cucumber cultivars (Table 1). A significant reduction in germination rate was

detected in Parys, which achieved a final germination rate of 8%, followed by Odys (56%) in 100 mM NaCl; however, the other cucumber cultivars were successful in maintaining seed germination above 80% (Table 1). On the basis of the results of this test, we considered Parys as the most susceptible and Polan as the most tolerant for all other experiments.

Table 1: Effect of salt treatment on seed germination.

Cucumber cultivars	Control			Salt treatment (100 mM)		
	%Germination	MDG	DGS	%Germination	MDG	DGS
Odys	92a	13.14a	0.076a	56b	8b	0.12b
Marketmore	92a	13.14a	0.076a	88a	12.6a	0.09c
Parys	96a	13.71a	0.073a	8c	1.1c	0.9a
Polan	100a	14.29a	0.07a	92a	13.1a	0.08c
Chinese Slogan	100a	14.29a	0.07a	88a	12.6a	0.09c
Ashly	96a	13.71a	0.073a	80a	11.4a	0.09c

Among cultivars, the values followed by the same letter are not significantly different at α=0.05. Mean Daily Germination (MDG), Daily Germination Speed (DGS) were calculated.

2.2.5.2 Effect of salinity on photosynthesis and plant growth

Leaf net photosynthetic rate and stomatal conductance decreased in both cultivars after treatment with NaCl, whereas intracellular CO2 concentration increased after 1 and 7 days of

treatment in both Polan and Parys (Fig. 1). A significant reduction was observed in both photosynthesis and stomatal conductance after 1 day of the treatment with 100 mM NaCl in both cultivars compared with the 0 mM NaCl (Fig. 1a, b). Moreover, the difference after 1 day in 100 mM was significant between the cultivars. Polan maintained a higher photosynthetic rate and stomatal conductance than Parys. However, after 7 days of the treatment with 100 mM NaCl, both photosynthesis and stomatal conductance decreased dramatically, which may explain the dramatic decrease in final biomass compared with the control in both cultivars. The maximum quantum yield decreased with increased NaCl concentration in both cultivars without any significant difference between the cultivars (data not shown).

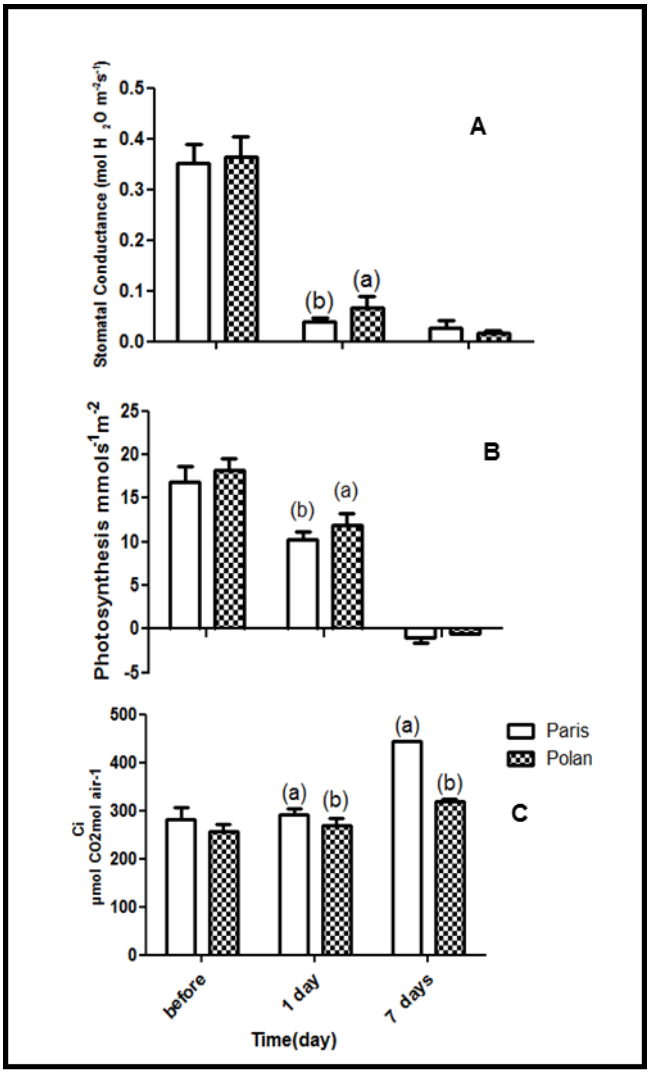


Fig. 1. (a) Stomatal conductance, (b) Net CO2 assimilation and (c) CO2 concentration in internal air space in two varieties of *Cucumis sativus* plants grown with 100 mM NaCl concentrations in the nutrient solution after 1 and 7 days of the treatment. Values are means ±s.e. (n = 6). A t-test was conducted and the different letters indicate statistically significant differences at $P < 0.05$.

Fresh and dry mass declined in both cultivars with increasing NaCl concentrations in the nutrient solution. A significant reduction was detected in the fresh mass of Parys compared with that of Polan in the 50 mM NaCl treatment; however, the fresh mass was further reduced in response to 100 mM NaCl and the difference between the cultivars disappeared (Fig. 2b). Similarly, with increasing NaCl concentration in the root zone, dry mass declined in both varieties, but with 50 mM NaCl, Polan maintained a higher dry mass than Parys. At 100 mM NaCl, no significant differences in the reduction of dry mass were detected between the varieties (Fig. 2d).

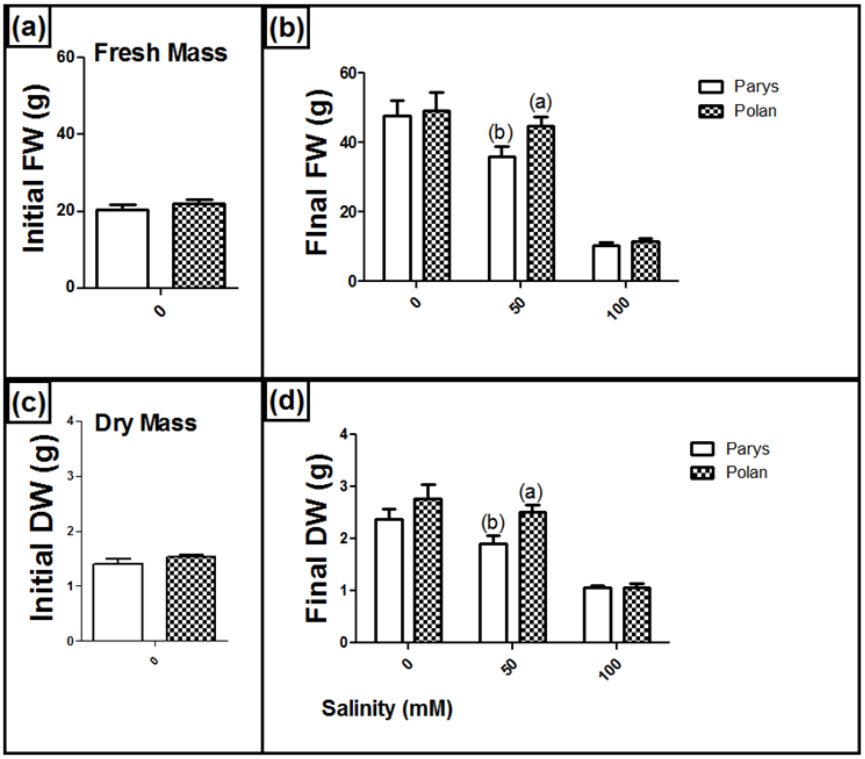


Fig. 2. Initial (a) FW and (c) DW, and total (b) FW and (d) DW in two *Cucumis sativus* varieties grown with increasing NaCl concentrations in the root zone after 1 week. Values are mean ±s.e. (n = 6). A t-test was conducted and the different letters indicate statistically significant differences at $P < 0.05$.

2.2.5.3 Polan accumulated higher amount of sodium in the tissue with less potassium efflux.

Unsurprisingly, Na^+ content increased in all plant tissues (roots, stems and leaves) with increasing NaCl concentrations (Fig. 3a–c). At 50 and 100 mM NaCl, significant differences were seen for root and stem Na^+ concentrations between cultivars, since Polan accumulated a higher amount of Na^+ in different tissues than Parys (Fig. 3a–c). K^+ concentrations decreased in the different plant tissues in both cultivars between the control plants (Fig. 3d–f). Significant differences between the cultivars under salt stress were found for K^+ concentrations in root and stem tissues with 50 and 100 mM NaCl: Polan was able to maintain a higher concentration of K^+ in root and stem tissues than Parys (Fig. 3d, e).

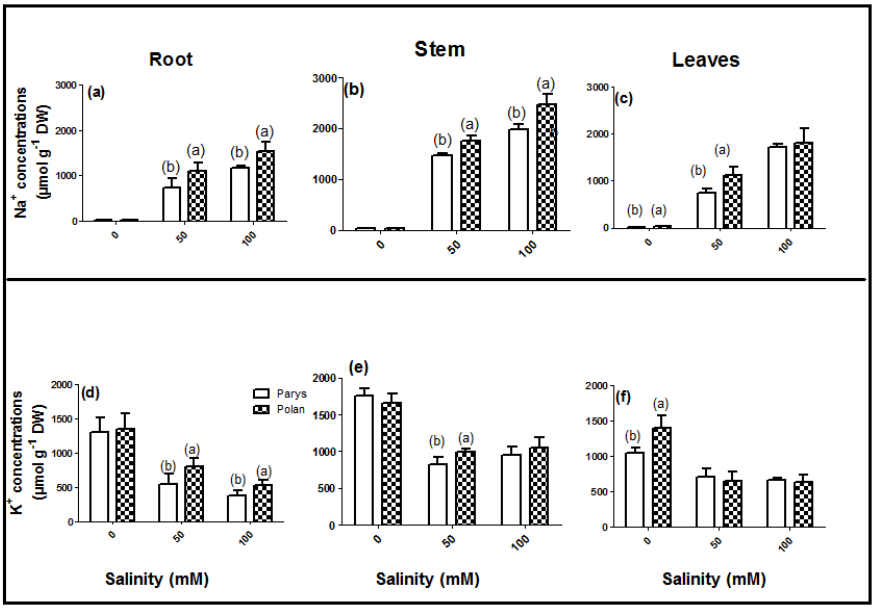


Fig. 3. Effects of salinity on Na^+ concentration in (a) roots, (b) stems and (c) leaves, and on K^+ concentration in (d) roots, (e) stems and (f) leaves of two varieties of Cucumis sativus plants grown with increasing NaCl concentrations in the root zone for 1 week. Values are means ±s.e. (n = 6). A t-test was conducted and the different letters indicate statistically significant differences at $P < 0.05$.

Based on the observed differences in tissue K^+ content, we analyzed the K^+ fluxes in the roots of cucumber seedlings exposed to 100 mM NaCl. Immediately, salinity treatment caused a significant K^+ efflux in both cultivars, which gradually recovered over the next 20–30 min (Fig. 4a). However, the immediate K^+ efflux in Parys was almost 2.3-fold higher than in Polan; after 30 min of treatment, K^+ efflux in Parys was almost fourfold greater than that in Polan (Fig. 4a), with a significant statistical difference. These data clearly indicate

Polan's greater ability to retain K^+ in the roots following NaCl stress. To assess the mechanism underlying the ability of Polan to maintain a higher level of K^+ in the tissue, we analyzed the expression of the A. thaliana homologue gene AKT1 in the roots of cucumber by real-time qPCR. As reported in Fig. 4b, the basal gene expression level of AKT1 is significantly higher in Polan than in Parys. After 3 h of treatment with salt, the expression of the gene was not affected in either cultivar compared with the control.

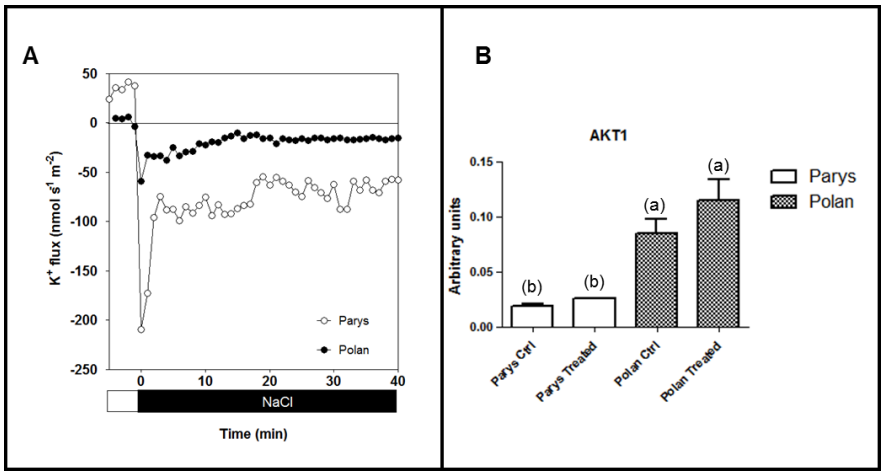


Fig. 4. (a) Net K^+ fluxes in the mature root zone of two cultivars of cucumber (Cucumis sativus) seedlings in response to 100 mM NaCl concentrations in the nutrient solution in the first 40 min following the addition of salt stress. (b) Expression of AKT1.was analyzed by real-time quantitative PCR in cucumber roots treated for 3 h with water (control) or 100 mM of salt. The CsACT3 gene was used as a reference. Letters in histogram bars represent statistical groupings using a post hoc test, where

bars with different letters are significantly different (Tukey, $P < 0.05$). Error bars represent the s.e.m.

2.2.5.4 Polan has a higher ROS content in tissue

Salinity stress also induces an oxidative response (Baxter et al. 2014). We demonstrated differences in ROS production in both cultivars (Parys and Polan); these data suggest different sensitivities to high NaCl concentration. DCF-DA, coupled with confocal microscopy analysis, is a reliable tool for realtime detection of increased intracellular concentrations of ROS (Ashtamker et al. 2007). We observed that in the cucumber cultivar Polan, there is higher ROS accumulation in both control and treated plants than in Parys (Fig. 5a–c). In order to corroborate confocal data, the expression level of the RBOHF gene, one of the NADPH oxidase enzymes involved in ROS production under abiotic stress (Baxter et al. 2014) was also analyzed. Indeed, ROS content within the cells of Polan was matched by the high expression level of RBOHF in both control and treated plants. We did observe an increase in the expression of RBOHF in treated Parys plants, although it was not as high as in Polan (Fig. 5d).

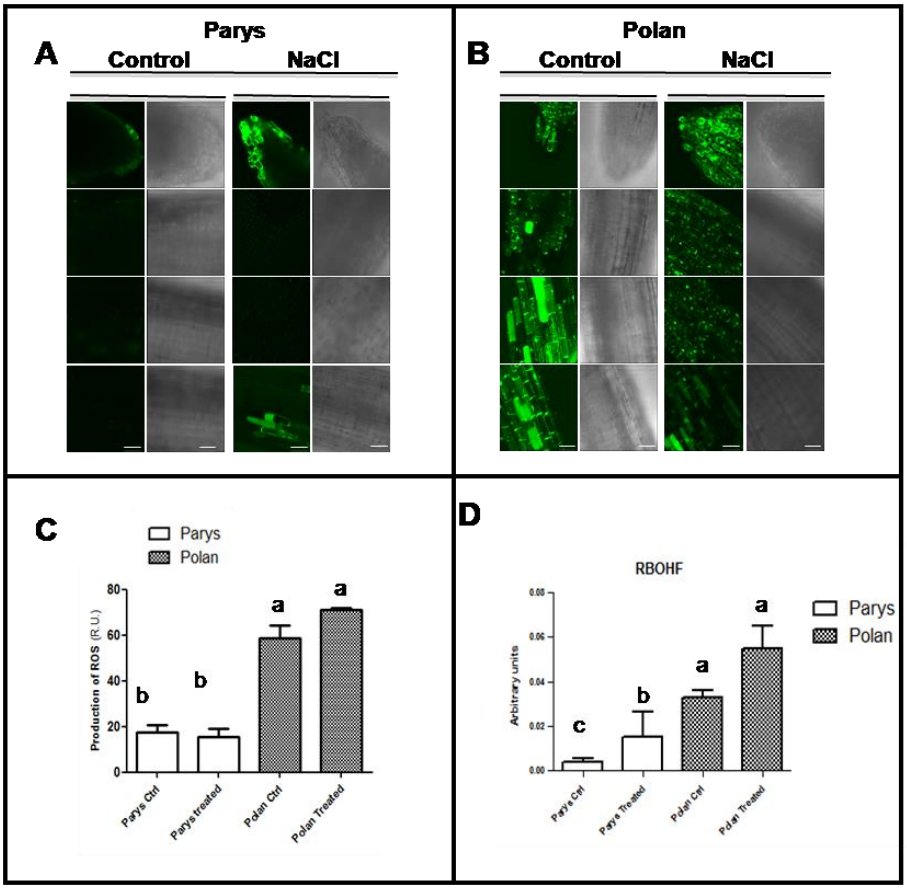


Fig. 5. Reactive oxygen species (ROS) and salt tolerance in cucumber seedling roots. (a, b) 2,7 -Dichlorofluorescein staining was used to detect peroxides generated in rsponse to 3 h treatment of 100 mM of salt by confocal microscopy analysis, either in (a) cv. Parys or in (b) Polan. Scale bar = 50mm. (c) ROS content in seedling roots was quantified after compiling the projection from 10 confocal sections of 0.5 mm each and from the apex along the entire root. The graph shows fluorescence across the sections quantified in relative units using LCS Lite software (Leica). (d) The expression level of the gene RBOHF, the NADPH oxidase involved in ROS production under abiotic stress. Expression of RBOHF was analyzed by real-time quantitative PCR in cucumber roots treated for 3 h with water (control)

or 100 mM of salt. The CsACT3 gene was used as a reference. Letters in histogram bars represent statistical groupings using a post hoc test, where bars with different letters are significantly different (Tukey's, $P < 0.05$). Error bars represent the s.e.m.

2.2.5.5 EFR109 and GOLS1-like are induced in cv Polan

In A. thaliana, salt treatment induces an array of genes that may be involved in salt tolerance. In particular, GOLS1 is a key gene involved in raffinose synthesis and an overexpression of this gene leads to improved tolerance to salt treatments (Sun et al. 2013). According to previous data (Ma et al. 2006), we selected two genes with high fold induction (over sevenfold) after 3 h of treatment with salt in A. thaliana. In particular, we selected GOLS1-like, the homologue of A. thaliana GOLS2 and the homologue of ERF109. Cucumber seedlings of both varieties were treated with 100 mM NaCl for 3 h at room temperature showed an increase of GOLS1-like (Fig. 6a), indicating that the treatment induces this particular gene to the same extent as in A. thaliana. The modulation of the other strongly activated gene, ERF109, was also monitored after 3 h of treatment. As expected, we observed an increase in the RNA level of ERF109 in Polan and a much lower induction in Parys (Fig. 6b).

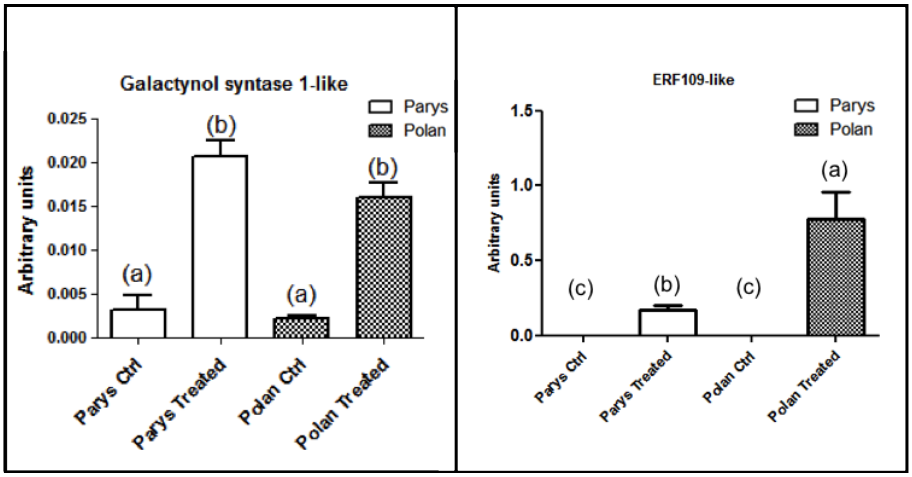


Fig. 6. Expression of (a) GOLS1-like and (b) ERF109 was analyzed by real-time quantitative PCR in cucumber roots treated for 3 h with water (control) or 100 mM of salt. The CSACT3 gene was used as a reference. Letters in histogram bars represent statistical groupings using a post hoc test, where bars with different letters are significantly different (Tukey's, $P < 0.05$). Error bars represent the s.e.m.

2.2.6 Discussion

Intense human agricultural practices over time and excessive watering of fields have increased soil salinity worldwide, creating one of the leading factors limiting yield. Salt stress has two main effects on plant physiology: ionic and osmotic (Adem et al. 2014). Under ionic stress, the cytosolic K^+ : Na^+ ratio is critical; to overcome salt stress, plants can use two ways to improve this ratio: (i) a reduction in Na^+ uptake or transporting it to the shoot tissues, or (ii) by increasing the ability of cells to retain K^+ (Shabala and Cuin2008). In this study, we screened different commercially available cucumber cultivars in

order to identify potential salt tolerance traits, such as K^+ retention ability and gene activation. Cucumber has been classified as a salt-sensitive species especially at the seedling stage (Stepien and Kłbus 2006; Duan et al. 2008). Previous studies described that germination rate can be used as reliable tool to determine the salt tolerance potential of plants, concurrently with other parameters (Ashkan and Jalal 2013). Moreover, seedling survival is a criterion upon which a large number of genotypes can be efficiently screened for salt tolerance. By using this approach, we evaluated the salt tolerance of six different cultivars of *Cucumis. sativus*. In general, under salt conditions, we observed a decrease in the germination rate; however, among these cultivars, Polan had the highest germination rate and Parys had the lowest (Table 1). The data suggest that among the set of cultivars tested here, Polan is the most salt-tolerant and Parys is the most salt-sensitive; based on this criterion, these two cultivars were selected for further experiments. Short-term NaCl stress inhibited dry mass accumulation in both cucumber cultivars in a dose-dependent manner (Arzani 2008; Zhu et al. 2008; Kere et al. 2013). However, 50 mM NaCl highlighted some key differences between the two cultivars, with cv. Parys emerging as more salt sensitive than Polan. (Fig. 2d). On the other hand, 100 Mm NaCl caused a dramatic decrease in both the fresh and dry mass compared with the initial mass after 1 week of the treatment in both cultivars. Salt stress quickly affected shoot water relations in both studied cultivars, independently of their salt tolerance, as shown by the substantial decline in stomatal conductance within the first 24 h of treatments (Fig. 1a). It is possible that the dramatic decline in FW at 100 mM NaCl was due to severe water

stress and this is supported by the early decline in stomatal conductance. In accordance with the view that salt stress initially imposes an osmotic stress and the ionic component becomes important only later (Munns 2005); after 24 h of treatment, the decline in stomatal conductance was far greater than that found for photosynthetic rate. This result supports the view that photosynthesis was initially limited by CO2 diffusion as a consequence of low leaf stomatal conductance (Medrano et al. 2002). Following this initial decline in gas exchange parameters, the data indicate that the photosynthetic apparatus of the cultivar Polan at 100 mM NaCl performed better than that of Parys, as in Polan, there was only a small decline in the net photosynthetic rate during the first 24 h (Fig. 1b). However, in Parys, prolonged NaCl stress completely abolished leaf photosynthetic rates, which coincided with substantial increases in internal CO2 concentrations (Fig.1c). This would suggest that although the initially decline in photosynthesis rate was associated with low stomatal conductance rates, NaCl subsequently impaired the leaf photosynthetic machinery. Measurements of osmotic potential and water potential (not performed here) would have provided further clarification as to whether the decline was in fact due to water stress at the higher salt concentration.

We then estimated Na^+ and K^+ content in different tissues of both cultivars, in order to evaluate both Na^+ accumulation in the different tissues and K^+ leakage. Na^+ content increased in both cultivars under all treatment conditions. Surprisingly, Polan showed a higher Na^+ content compared with Parys; however, it has been

previously reported that tolerant varieties can also accumulate salt in leaves (Kere et al. 2013). In addition, the lower rate at which the K^+ content declined in Polan in response to increasing NaCl concentrations suggests better K^+ homeostasis in this cultivar (Fig. 3). In addition, the content of Na^+ in the stem was generally higher than that in the roots and leaves; this result is consistent with that found in an experiment evaluating the response of two different cucumber cultivars with differing salt tolerance (Zhu et al. 2008). There, it was also found that stem Na^+ concentrations were always higher than those measured in roots and leaves, which led to the speculation that for those cultivars, stems might play an important role in preventing the entry of Na^+ into the leaf. In the present work, significant differences in leaf, stem and root Na^+ concentrations were observed between cultivars. In particular, Na^+ contents in the roots and stems increased significantly with increasing NaCl concentrations in the root zone, independently of the salt tolerance in both cultivars; interestingly, Polan accumulated higher Na^+ than Parys in the different tissues. These results therefore indicate that Na^+ leaf translocation from the roots and stems was not the mechanism behind the different salt tolerance between cultivars. On the other hand, in both cultivars, we observed a decrease in K^+ concentration, but Parys had higher K^+ leakage than Polan. This means that the latter had a better ability to retain K^+ in its tissues. Ion fluxes, measured in the roots by using a noninvasive vibrating probe system under 100 mM NaCl, confirmed that the amount of K^+ leakage from plant roots provided a rapid method of identifying differences in salt tolerance in these cultivars (Fig. 4a). In both cultivars, 100 mM NaCl induced an instantaneous

K^+ efflux from the epidermal cells in the mature region and a gradual decline in K^+ efflux in the 30 min following salt addition. In accordance with the previous results, the cultivar Parys emerged as the most susceptible to salt stress, with the highest rates of efflux immediately after salt exposure and in the 30 min following the imposition of the stress. Surprisingly, the magnitude of the difference in K^+ efflux after 30 min of salt stress between both cultivars reached almost ninefold, a difference much wider than what we observed for any of the other physiological parameters measured. These strong and rapid increases in K^+ efflux following salt stress could have been associated with: (i) Na^+ -induced membrane depolarization that favoured K^+ leakage via the outward-rectifying depolarization activated K^+ channel (Shabala and Cuin 2008; Jayakannan et al. 2013) or (ii) increases in ROS production, which activates nonselective cation channels (Demidchik et al. 2003; Demidchik and Maathuis 2007; Jayakannan et al. 2013). On the other hand, one of the main mechanisms of K^+ influx is through AKT1. Even though AKT1 is sensitive to membrane hyperpolarization, much evidence in the literature underlines that higher levels of AKT1 transcripts confer better tolerance to salt stress and a higher capability to retain K^+ in the root tissues (Chen et al. 2013; Chakraborty et al. 2016). For that reason, we investigated the expression level of AKT1 by real-time qPCR, showing us that this gene is not induced by treatment per se but that of Polan displayed a higher expression level of AKT1 as well as in the control (Fig. 4b). Taken together, these data suggest that Polan has a better ability to retain K^+ via the AKT1 channel. It has been reported that plant tissues generate ROS under salt treatment in both

roots and leaves (Mittler 2002; Miller et al. 2008), and they are secondary messengers in stress signaling (Huang et al. 2012). Using confocal microscopy, we showed that Polan had higher ROS cellular root content than Parys in both control and treated plants (Fig. 5c). This means that Polan plants have a higher basal ROS level, which is confirmed by the relative gene expression of the cucumber homologue RBOHF (Fig. 5d). In fact, RBOFH is one of the NADPH oxidases involved in ROS production needed in order to decrease some phytotoxic effects triggered by salt stress (Ma et al. 2011). In accordance with previously described data (Adem et al. 2014), the transcription level of RBOHF did not change between the treatment and the control. Nevertheless, the basal level of gene expression was higher in Polan than in Parys; taken together, these data suggest that ROS play a relevant role in the salt tolerance of the cultivar Polan. Therefore, we investigated the relative expression level of the homologue ERF109, for which has been shown that its expression is strictly regulated by salt treatment and that is directly correlated with ROS production (Winter et al. 2007; Matsuo et al. 2015). Surprisingly, we observed that basal ERF109 expression was the same in both cultivars, contrary to that observed in the previous analysis of the RBOHF gene (Fig. 6a). This could suggest that ERF109 is not directly linked to the activation of RBOHF in our experimental conditions. Moreover, ERF109 has been shown to be involved in biotic and abiotic responses by ROS accumulation systemically in roots and shoots (Winter et al. 2007; Matsuo et al. 2015; Matsuo and Oelmüller 2015). ERF109 is also highly conserved among angiosperms but its physiological role still remains unknown. Because high salt

concentrations also induce osmotic stress in plants (Krasensky and Jonak 2012), we focused our attention on the gene GOLS1, which plays a key role in raffinose biosynthesis and is mainly involved in relieving the osmotic stress induced by salt. Sun et al. (2013) demonstrated that overexpression of one member of the GOLS family in A. thaliana enhanced tolerance to high salinity and osmotic stresses. On the basis of our real-time qPCR analysis, we observed an induction of GOLS1 to the same extent in both cultivars (Fig. 6b), indicating that this gene alone is not enough to ensure salt stress tolerance. Field screening remains one of the main tools used to ultimately confirm or define salt tolerance in most crops, but this technique has its limitation in terms of the time required and the ever-changing environment (Chen et al. 2007). Given these limits, screening techniques are required in the initial phases of salt tolerance. Several potential criteria or traits have been proposed, ranging from Na^+ exclusion to survival (Kere et al. 2013). The present study clearly shows that K^+ efflux from the mature root zone of young seedlings following NaCl stress is a reliable indicator of salinity tolerance in cucumber seedlings, whereas Na^+ exclusion from the leaves, a typical criterion used for salt tolerance screening in horticultural crops, did not provide any significant information for the evaluation of salt sensitivity in the different cucumber cultivars. Our findings are consistent with other studies that show that ROS production could be a reliable indicator for assessing salt tolerance in plants (Adem et al. 2014; Ben Rejeb et al. 2015); moreover, ROS can also regulate ion channel activity and gene expression (Rodríguez and Taleisnik 2012). As we observed, RBOHF may have played a major role in Polan's salt tolerance through the

regulation of K^+ channels (e.g. AKT1), probably because of the proximity of these two proteins and the fact that they belong to the same subcellular compartment (i.e. the plasma membrane) (Ma *et al.* 2011). Salt tolerance is a reputedly complex trait that involves the regulation and coordination of several different physiological responses, and requires the modulation and expression of several hundred different genes. Among the transcription factors, ERF109 is one of the most interesting because it is strongly induced under salt stress but its physiological role remains unclear to date. Further studies should address the possible link between the ERF109 transcription factor and salt tolerance and adaptation (Matsuo et al. 2015). Nevertheless, it was recently suggested that up to 80% of the genetic variability may be attributed to just one physiological trait, namely, cells' ability to prevent NaCl-induced K^+ loss (Shabala and Pottosin 2014). This hypothesis is based on the view that K^+ efflux can actually be viewed as the sum of all the different factors that control intracellular K^+ homeostasis, which is a prerequisite for the optimal operation of plants' metabolic machinery and overall performance. Therefore, considering that roots are the first tissues exposed to rootbased abiotic stresses and their early responses influences the overall plant response (Zhu et al. 2008; Pandolfi et al. 2012; Bazihizina et al. 2014), it becomes clear why the roots' ability to retain K^+ when exposed to salt stress should be considered as one of the key component for plant stress tolerance (Chen et al. 2007).

2.2.7 Conclusions and remarks

Our findings showed that salt stress significantly induced different responses in the two cultivars studied here (Parys and Polan), pointing to K^+ efflux as the main pivot in the orchestration of the salt tolerance. In the present study, we used the inherent genetic diversity existing within cucumber cultivars to identify tolerant genotypes and to determine if a higher biomass accumulation under salt stress is preferentially associated with the ability of shoots and roots to retain K^+ after exposure to salt. In addition, we observed a higher AKT1 level, which could be involved in the retention of K, in Polan than in Parys. Moreover, high levels of intracellular ROS and higher basal level of the RBOHF gene in Polan suggest a link between RBOHF and ROS production in a steady state. The data collected here also show clearly that transcription factor ERF109 does not induce the expression of RBOHF.

References

Adem, GD, Roy, SJ, Zhou, M, Bowman, JP, Shabala, S (2014) Evaluating contribution of ionic,osmotic and oxidative stress components towards salinity tolerance in barley. *BMC Plant Biol***14**, 113.

Aleman, F, Nieves-Cordones, M, Martinez, V, Rubio, F (2011) Root K+ Acquisition in Plants: The Arabidopsis thaliana Model. *Plant and Cell Physiology***52**, 1603-1612.

Apel, K, Hirt, H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.***55**, 373-399.

Ardie, SW, Liu, SK, Takano, T (2010) Expression of the AKT1-type K+ channel gene from Puccinellia tenuiflora, PutAKT1, enhances salt tolerance in Arabidopsis. *Plant Cell Reports***29**, 865-874.

Arzani, A (2008) Improving salinity tolerance in crop plants: a biotechnological view. *In Vitro Cellular & Developmental Biology-Plant***44**, 373-383.

Ashkan, A, Jalal, M (2013) Effects of Salinity Stress on seed germination and seedling vigor indices of two Halophytic Plant Species (Agropyron elongatum and A. pectiniforme). *International Journal of Agriculture and Crop Sciences***5**, 2669.

Ashtamker, C, Kiss, V, Sagi, M, Davydov, O, Fluhr, R (2007) Diverse subcellular locations of cryptogein-induced reactive oxygen species production in tobacco bright yellow-2 cells. *Plant Physiology***143**, 1817-1826.

Baxter, A, Mittler, R, Suzuki, N (2014) ROS as key players in plant stress signalling. *J Exp Bot***65**, 1229-1240.

Bazihizina, N, Taiti, C, Marti, L, Rodrigo-Moreno, A, Spinelli, F, Giordano, C, Caparrotta, S, Gori, M, Azzarello, E, Mancuso, S (2014) Zn2+-induced changes at the root level account for the increased tolerance of acclimated tobacco plants. *J Exp Bot***65**, 4931-4942.

Beddington, J (2010) Food security: contributions from science to a new and greener revolution. *Philosophical Transactions of the Royal Society B-Biological Sciences***365**, 61-71.

Ben Rejeb, K, Benzarti, M, Debez, A, Bailly, C, Savouré, A, Abdelly, C (2015) NADPH oxidase-dependent H2O2 production is required for salt-induced antioxidant defense in Arabidopsis thaliana. *J Plant Physiol***174**, 5-15.

Chartzoulakis, KS (1994) Photosynthesis, Water Relations and Leaf Growth of Cucumber Exposed to Salt Stress. *Scientia Horticulturae***59**, 27-35.

Cheeseman, JM (2015) The evolution of halophytes, glycophytes and crops, and its implications for food security under saline conditions. *New Phytol***206**, 557-70.

Chen, ZH, Zhou, MX, Newman, IA, Mendham, NJ, Zhang, GP, Shabala, S (2007) Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. *Functional Plant Biology***34**, 150-162.

Cuartero, J, Bolarin, MC, Asins, MJ, Moreno, V (2006) Increasing salt tolerance in the tomato. *J Exp Bot***57**, 1045-1058.

DeHaan, LR, Weisberg, S, Tilman, D, Fornara, D (2010) Agricultural and biofuel implications of a species diversity experiment with native perennial grassland plants. *Agriculture Ecosystems & Environment***137**, 33-38.

Demidchik, V, Maathuis, FJM (2007) Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. *New Phytologist***175**, 387-404.

Demidchik, V, Shabala, SN, Coutts, KB, Tester, MA, Davies, JM (2003) Free oxygen radicals regulate plasma membrane Ca2+ and K+-permeable channels in plant root cells. *Journal of Cell Science***116**, 81-88.

Duan, CG, Wang, CH, Guo, HS (2008) Delayed resistance to Cucumber mosaic virus mediated by 3'UTR-derived hairpin RNA. *Chinese Science Bulletin***53**, 3301-3310.

Hirsch, RE, Lewis, BD, Spalding, EP, Sussman, MR (1998) A role for the AKT1 potassium channel in plant nutrition. *Science***280**, 918-921.

Huang, G-T, Ma, S-L, Bai, L-P, Zhang, L, Ma, H, Jia, P, Liu, J, Zhong, M, Guo, Z-F (2011) Signal transduction during cold, salt, and drought stresses in plants. *Molecular Biology Reports***39**, 969-987.

Jayakannan, M, Bose, J, Babourina, O, Rengel, Z, Shabala, S (2013) Salicylic acid improves salinity tolerance in Arabidopsis by restoring membrane potential and preventing salt-induced K loss via a GORK channel. *J Exp Bot***64**, 2255-2268.

Kere, GM, Guo, QW, Shen, J, Xu, J, Chen, JF (2013) Heritability and gene effects for salinity tolerance in cucumber (Cucumis sativus L.) estimated by generation mean analysis. *Scientia Horticulturae***159**, 122-127.

Keunen, ELS, Peshev, D, Vangronsveld, J, Van Den Ende, WIM, Cuypers, ANN (2013) Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant, Cell & Environment***36**, 1242-1255.

Khan, A, Lang, I, Amjid, M, Shah, A, Ahmad, I, Nawaz, H (2013) Inducing Salt Tolerance on Growth and Yield of Sunflower by Applying Different Levels of Ascorbic Acid. *Journal of Plant Nutrition***36**, 1180-1190.

Koressaar, T, Remm, M (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics***23**, 1289-1291.

Krasensky, J, Jonak, C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J Exp Bot***63**, 1593-1608.

Lagarde, D, Basset, M, Lepetit, M, Conejero, G, Gaymard, F, Astruc, S, Grignon, C (1996) Tissue-specific expression of arabidopsis AKT1 gene is consistent with a role in K+ nutrition. *Plant Journal***9**, 195-203.

Ma, L, Zhang, H, Sun, L, Jiao, Y, Zhang, G, Miao, C, Hao, F (2011) NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na+/K+ homeostasis in Arabidopsis under salt stress. *J Exp Bot*

Ma, S, Gong, Q, Bohnert, HJ (2006) Dissecting salt stress pathways. *J Exp Bot***57**, 1097-107.

Mancuso, S, Papeschi, G, Marras, AM (2000) A polarographic, oxygen-selective, vibrating-microelectrode system for the spatial and temporal characterisation of transmembrane oxygen fluxes in plants. *Planta***211**, 384-389.

Matsuo, M, Johnson, JM, Hieno, A, Tokizawa, M, Nomoto, M, Tada, Y, Godfrey, R, Obokata, J, Sherameti, I, Yamamoto, YY, Bohmer, FD, Oelmuller, R (2015) High REDOX RESPONSIVE TRANSCRIPTION FACTOR1 Levels Result in Accumulation of Reactive Oxygen Species in Arabidopsis thaliana Shoots and Roots. *Molecular Plant***8**, 1253-1273.

Matsuo, M, Oelmüller, R (2015) REDOX RESPONSIVE TRANSCRIPTION FACTOR1 is involved in age-dependent and systemic stress signaling. *Plant Signal Behav***10**, e1051279.

Medrano, H, Escalona, JM, Bota, J, Gulias, J, Flexas, J (2002) Regulation of photosynthesis of C-3 plants in response to progressive drought: Stomatal conductance as a reference parameter. *Annals of Botany***89**, 895-905.

Miller, G, Shulaev, V, Mittler, R (2008) Reactive oxygen signaling and abiotic stress. *Physiol Plant***133**, 481-9.

Mittler, R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science***7**, 405-410.

Mittler, R, Vanderauwera, S, Gollery, M, Van Breusegem, F (2004) Reactive oxygen gene network of plants. *Trends in Plant Science***9**, 490-498.

Munns, R (2005) Genes and salt tolerance: bringing them together. *New Phytologist***167**, 645-663.

Munns, R, Tester, M (2008) Mechanisms of salinity tolerance. In 'Annu Rev Plant Biol.' Vol. 59 pp. 651-681. (Annual Reviews: Palo Alto)

Pandolfi, C, Mancuso, S, Shabala, S (2012) Physiology of acclimation to salinity stress in pea (Pisum sativum). *Environmental and Experimental Botany***84**, 44-51.

Panta, S, Flowers, T, Lane, P, Doyle, R, Haros, G, Shabala, S (2014) Halophyte agriculture: Success stories. *Environmental and Experimental Botany***107**, 71-83.

Pfaffl, MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research***29**,

Rodríguez, AA, Taleisnik, EL (2012) Determination of Reactive Oxygen Species in Salt-Stressed Plant Tissues. In 'Plant Salt Tolerance: Methods and Protocols.' (Eds S Shabala, AT Cuin.) pp. 225-236. (Humana Press: Totowa, NJ)

Rosenkranz, AR, Schmaldienst, S, Stuhlmeier, KM, Chen, W, Knapp, W, Zlabinger, GJ (1992) A microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescin-diacetate. *J Immunol Methods***156**, 39-45.

Sentenac, H, Bonneaud, N, Minet, M, Lacroute, F, Salmon, JM, Gaymard, F, Grignon, C (1992) Cloning and Expression in Yeast of a Plant Potassium-Ion Transport-System. *Science***256**, 663-665.

Shabala, S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. *Annals of Botany***92**, 627-634.

Shabala, S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Ann Bot***112**, 1209-21.

Shabala, S, Cuin, TA (2008) Potassium transport and plant salt tolerance. *Physiol Plant***133**, 651-69.

Shabala, S, Pottosin, I (2014) Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. *Physiol Plant***151**, 257-279.

Stepien, P, Klobus, G (2006) Water relations and photosynthesis in Cucumis sativus L. leaves under salt stress. *Biologia Plantarum***50**, 610-616.

Sun, Z, Qi, X, Wang, Z, Li, P, Wu, C, Zhang, H, Zhao, Y (2013) Overexpression of TsGOLS2, a galactinol synthase, in Arabidopsis thaliana enhances tolerance to high salinity and osmotic stresses. *Plant Physiology and Biochemistry***69**, 82-89.

Torres, MA (2010) ROS in biotic interactions. *Physiol Plant***138**, 414-429.

Tran, D, El-Maarouf-Bouteau, H, Rossi, M, Biligui, B, Briand, J, Kawano, T, Mancuso, S, Bouteau, F (2013) Post-transcriptional regulation of GORK channels by superoxide anion contributes to increases in outward-rectifying K plus currents. *New Phytologist***198**, 1039-1048.

Untergasser, A, Cutcutache, I, Koressaar, T, Ye, J, Faircloth, BC, Remm, M, Rozen, SG (2012) Primer3—new capabilities and interfaces. *Nucleic Acids Research***40**, e115.

Very, AA, Nieves-Cordones, M, Daly, M, Khan, I, Fizames, C, Sentenac, H (2014) Molecular biology of K+ transport across the plant cell membrane: What do we learn from comparison between plant species? *J Plant Physiol***171**, 748-769.

Wang, Y, Wu, WH (2013) Potassium Transport and Signaling in Higher Plants. *Annual Review of Plant Biology, Vol 64***64**, 451-476.

Winter, D, Vinegar, B, Nahal, H, Ammar, R, Wilson, GV, Provart, NJ (2007) An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *Plos One***2**, e718.

Zhu, J, Jeong, JC, Zhu, Y, Sokolchik, I, Miyazaki, S, Zhu, JK, Hasegawa, PM, Bohnert, HJ, Shi, H, Yun, DJ, Bressan, RA (2008) Involvement of Arabidopsis HOS15 in histone deacetylation and cold tolerance. *Proceedings of the National Academy of Sciences of the United States of America***105**, 4945-4950.

Zhu, ZJ, Wei, GQ, Li, J, Qian, QQ, Yu, JQ (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (Cucumis sativus L.). *Plant Science***167**, 527-533.

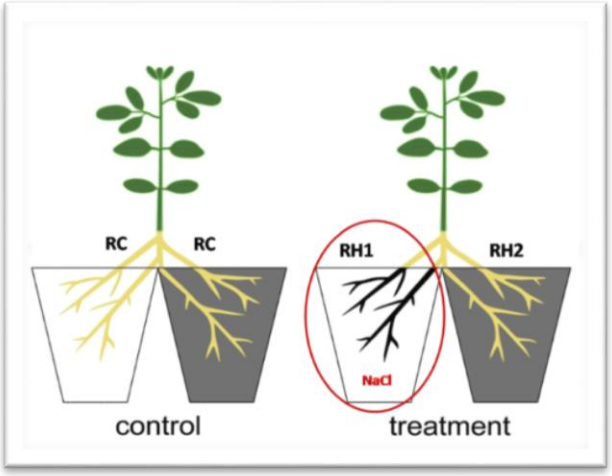
Chapter 3

Heterogeneous Salinity and Plant electrophysiology as a tool to monitor their responses

Mirvat Redwan, Francesco Spinelli, Elisa Masi, Lucia Marti, Elisa Azzarello and Stefano Mancuso

Department of Plant, Soil and Environmental Science, University of Florence, Viale delle Idee 30, 50019 Sesto Fiorentino, Florence Italy.

Corresponding author: stefano.mancuso@unifi.it



Ready to be submitted.

3.1 Background

3.1.1 Plant responses to heterogeneous stress conditions

Soil salinity is one of the most widespread abiotic stresses and constitutes a stringent factor limiting plant productivity (Lu *et al.* 2003). Plant response to salt stress can differ greatly depending on environmental factors in the soil. One of these factors is the distribution of salts in the root environment (Meiri and Plaut 1985; Sonneveld and De Kreij 1999). Soil salinity is often heterogeneous in saline fields, therefore most studies have focused on plant response to non-uniform salinity in the root zone (Meiri and Plaut 1985; Bazihizina *et al.* 2009; Dong *et al.* 2010). Interestingly, it was noticed that plants under heterogeneous salinity grew better. For example, when roots were subjected to salinity, the water use of the whole plant decreased as NaCl concentrations in the medium increased (Shani *et al.* 1993; Bazihizina *et al.* 2009), while non-uniform salinity soil conditions determined opposite results, suggesting that the low salinity side of roots could partially compensate the reduced water uptake by the high salinity side (Shani *et al.* 1993; Bazihizina *et al.* 2009)**.**

The improvement in plant growth under non uniform salinity was attributed to the increased root growth and water uptake in the low salinity root portion, reduced Na^+ accumulation, enhanced Na^+ efflux from the low salinity and increased K^+ concentration and K^+/Na^+ ratio in leaves (Flores *et al.* 2002; Tabatabaie *et al.* 2003; Bazihizina *et al.* 2009; Kong *et al.* 2011). Accordingly, altered

physiological aspects and improved plant growth and yield by unequal salt distribution in either controlled or field environment have been reported in many plants like cotton, tomato, cucumber (Sonneveld and De Kreij 1999). Unequal distribution of salts can be accurately realized with a split root system, in which the root system is divided into two or more equal portions and each portion is irrigated with varied concentrations of NaCl (Shani *et al.* 1993; Zhu and Ito 2000; Messedi *et al.* 2004; Lycoskoufis *et al.* 2005; Bazihizina *et al.* 2009; Dong *et al.* 2010). Yet cucumber plants (*Cucumis sativus* L.) responses to unequal salt distribution in the root zone is little studied.

In this chapter the objective is to investigate the transmission of signals among roots under unequal salt distribution on cucumber plants using electrophysiological and genetic analysis.

3.1.2 Plant electrophysiology

For a long time, plants were thought to be living organisms with limited abilities of moving and sensing (Trewavas 2003), with the exception only for some plants with rapid and/or purposeful movements such as *Mimosa pudica* (also called the sensitive plant),*Drosera* (sundews), *Dionea muscipula* (flytraps) and tendrils of climbing plants. Interestingly these peculiar plants attracted the attention of researchers who found them not only to be equipped with various mechano-receptors that exceeded the sensitivity of a human

finger, but also with the ability to trigger action potentials (APs) that linked to these movements. Electrical signaling in plants is a major system to transmit information over long distances throughout the different organs; and plant electrophysiology has attracted researchers since the eighteenth century (Bertholon 1783; Burdon-Sanderson *et al.* 1873; Darwin 1875; Bose 1926). Rapid plant responses to environmental changes are associated to electrical excitability and signaling, using the same electrochemical pathways to drive physiological responses, characterized by continuous growth. Two different types of electrical signals have been reported in plants: AP (Fromm 2006),which is a rapid propagating electrical pulse, travelling at a constant velocity and maintaining a constant amplitude, and VP (slow wave or "variation potential"), corresponding to a long range of a variation pulse (Stahlberg *et al.* 2006), which varies with the intensity of the stimulus, and its amplitude and speed decreases with increasing distance from its generation site (Davies 2006).

Electrical pulses can be monitored in plants as signals, which are transmitted through excitable phloematic cell membranes, enabling the propagation of electrical pulses in the form of a depolarization wave (Dziubińska *et al.* 2001; Fromm and Lautner 2007). At the onset of a change in the environmental conditions, plants respond rapidly at the site of occurrence whilst bioelectrical pulses are distributed throughout the entire plant, from roots to shoots and vice versa. The plants' need to respond rapidly to environmental stress factors can explain the reason why they have developed pathways for electrical signal transmission (Fromm and Lautner

2007). Plants' action potentials (APs) and variation potentials (VPs) are fast and accurate physiological reactions to specific abiotic and biotic stimuli and expressed by means of real-time detectable signals (Datta and Palit 2004; Lautner *et al.* 2005; Gil *et al.* 2008; Volkov *et al.* 2009; Oyarce and Gurovich 2011). Electrochemical excitation in plant tissues is caused by ionic fluxes through ion channels and electrogenic pumps embedded in the cell plasma membrane (Hedrich and Schroeder 1989; Sten-Knudsen 2002; Blatt 2008). As an example, The K^+ transporting ATPase enables the onset of different ion concentrations (electrical charge) on the intracellular and extracellular sides of the membrane (Maathuis and Sanders 1997). Also when ion channels get opened or closed at one point in the membrane, a local and transient change in the membrane potential is produced, which causes an electric current to flow rapidly to other points in the membrane and eventually, to the plasma membrane of surrounding cells. Plant plasma membranes always maintain a potential, the cell interior being more negative than the exterior, arising mainly from the activity of electrogenic pumps. The stable state of cells is called the resting potential which varies from -20mV to -200 mV according to cell type and plant species. Depolarization of plasma membrane occurs when ion channels start to open and close and cell interior voltage rises, or becomes more negative. Change in trans–plasma membrane potential creates a wave of depolarization, which affects the adjoining resting plasma membranes, thus generating an impulse. Once initiated, these impulses can propagate to adjacent excitable cells (van Bel and Ehlers 2003; Volkov *et al.* 2011), through plasmodesmataon short distances, and through plant phloematic tissue

on long distances (Ksenzhek and Volkov 1998; Volkov 2000, 2006; Volkov *et al.* 2011). The plasma membrane plays two important roles. First, allows a cell to function as a battery, providing power to operate the variety of electrogenic pumps embedded in its lipid bilayer (of the plasma membrane). Second, plasma membrane takes a place in transmitting signals between different parts of a cell or to other plant cells, tissues or organs in electrically excitable cells.

The technique used in this work for the measurement of electrical signals was noninvasive recording at the level of root surface, the Multi Electrode Array (MEA) system. In principle, a MEA system is a two-dimensional arrangement of voltage probes designed for extracellular stimulation and monitoring of electrical activity of electrogenic cells. Its main advantage is linked to the possibility to carry out noninvasively multisite recording and stimulation, and also the possibility of long time recording (up to weeks) with extremely high spatio-temporal resolution. The core components of this system are represented buy (1) a cell-culture dish (biochip) with an embedded microelectrodes array, (2) a multichannel extracellular recording setup based on a high input impedance multichannel electrometer, (3) an electrical stimulator, and finally, (4) a data acquisition system. For recording, any kind of samples (e.g. entire or sliced roots) is placed on a MEA biochip. Biochip contains 60 TiN(Titanium nitride) microelectrodes (diameter 10-30μm); electrodes are arranged in matrix (e.g., 8x8 or 6x10), with varied interelectrode spacing (e.g., 100 or 500μm). MEA technique was first used to detect the electrical activity from electrogenic cells of human

and animals (Hampson*et al.*,1999; Friedman, 2002), but recently thanks to (Masi *et al.*, 2009) this technique has been well used for the detection of electrical activity in different plant tissues.

The second technique used was vibrating probe, which consists of ion-selective microelectrodes used to measure ion gradients across membranes. Ion selective microelectrodes are proposed to measure ion concentration gradients, between two positions in solution outside the sample surface, and to use those gradients to calculate the net fluxes of ions of interest crossing the membrane. This techniques have a lot of advantages, first of all it is not destructive (for the plants) allowing in-situ measurements of net ion fluxes in physiologically "realistic" conditions. Second, it provides high spatial resolution since the electrode tip is about 2-3 μm in diameter and in some cases (when ionophores- which are specific molecules sense with high selectivity and form relatively stable complexes with the ion of interest- has a high signal to noise ratio as H^+) could be reduced to 0.8-1 μm, which makes it possible to measure net ion fluxes from single cells (Babourina *et al.* 2000; Shabala *et al.* 2001) or protoplasts derived from plant cells (Shabala and Newman 1998; Tyerman *et al.* 2001), thus acquiring information about spatial distribution and functional expression of specific ion transporters. Third, measurements have high temporal resolution; thus, insights into very early and fast events in terms of seconds associated with plant responses to environmental changes can be achieved. Fourth, the vibrating probe technique offers the possibility to measure the kinetics of fluxes of several ions simultaneously, and essentially at the same spot, which is important in

understanding the underlying ionic mechanisms of cell adaptive responses.

3.2 Investigation of root signaling under heterogeneous salt stress: A case study for *Cucumis sativus L*

3.2.1 Abstract

To sense, respond and adapt to the constantly changing environmental conditions, plants have developed sophisticated signaling mechanisms. The aim of this study is to investigate plant signaling under heterogeneous salt conditions. In particularly a split root system was established and one half root apparatus (HR1) was treated with salt. The impact of the salt on the electrical signals as the initial responses of the plant to exterior stimulus was measured with a Multi Electrode Array (MEA) system in the non-stressed half roots (HR2). Both duration and amplitude of the electrical signals recorded increased, while in the control (unstressed) remains similar. In order to identify the nature of the signal traveling from HR1 to HR2, H^+ and K^+ fluxes were measured in HR2 by using ion-selective microelectrodes with the vibrating probe technique. A net potassium influx was observed after 40 minutes of the treatment while no change in proton flux was detected. The increased potassium concentration in HR2 was also confirmed by fluorescent dye potassium-binding benzofuranisophthalate (PBFI) compared with the control roots. By corona sodium green (fluorescent dye) and confocal microscopy we confirmed that changes in electrical signals and fluxes are not associated with the simple apoplastic diffusion of sodium from HR1 to HR2. To further investigate the transmission of signals among roots, the activation of salt stress marker genes were examined in the split

roots system. The salt marker gene probed (CsGOLS1 like) was up-regulated in both portions of the root system after 3 and 4 h of the treatment with salt. A strong induction of Ethylene Responsive Factor 109 (ERF109) transcription was observed in the portion of root (HR1) after 3 hours of the salt treatment, meanwhile the aspartate oxidase was found to be down regulated.

3.2.2 Introduction

In contrast to animals, plants are sessile organisms, thus they have evolved complex mechanisms to adapt to the challenges of their surroundings, integrating many signals in order to achieve appropriate developmental and physiological responses. One key feature of plants adaptation to a spatially ever-changing environment is that, although they are permanently exposed to numerous abiotic and biotic stresses in a localized manner, their responses are elicited throughout the plant (Choi *et al.* 2014). In particular this phenomenon occurs in the soil environment, where nutrients and toxic substances are heterogeneous distributed throughout the profile. For example saline soils are frequently characterized by a marked spatial patchiness, containing, over relatively short distances, patches with salinities ranging from close to zero to several times the concentration of seawater (Bazihizina *et al.* 2012a). Nevertheless, the presence of vegetation in areas with soil salinities several fold higher than seawater (Yakir and Yechieli 1995) may suggest that the inherent variability in the salinity of the soil elicits specific physiological response in the plant (i.e. preferential nutrient and water uptake from the least saline side)(Bazihizina *et al.* 2012a, 2012b) that plays an important role in maintaining plant function under field conditions; as a confirmation, such concentrations when applied uniformly in controlled experiments, are usually lethal, even for halophytes (Flowers and Colmer 2008).

As roots are the first plant tissues to encounter biotic and abiotic stress in the soil profile, root emitted signals are key in the

regulation of plant physiology and growth in response to localized stresses. A well-known example is that of the stomatal regulation under drought conditions, in which roots water status has been hypothesized to be signalled to the shoot through hydraulic (i.e. changes in plant hydraulic conductance) and/or non-hydraulic (chemical and electrical) signals (Comstock 2002). Despite the nature of the root emitted signals is still unclear, there is a large body of evidence that clearly indicates that roots in the drying side (i.e. stressed side) do produce a signal that will then modify the physiological responses in the unstressed portions of the plants (Schachtman and Goodger 2008). Indeed, under heterogeneous soil moisture, the excision of roots in the dry soil caused increases in stomatal conductance and/or in leaf elongation rates, suggesting that root-derived signals from the stressed side do affect stomatal conductance or leaf elongation rates (Saab and Sharp 1989; Gowing *et al.* 1990). Similarly, under saline conditions, split-root studies have shown that roots in the saline portion do indeed affect plant physiology (Bazihizina *et al.* 2009; Bazihizina *et al.* 2012b). As for heterogeneous soil moisture, in plants exposed to heterogeneous salinity, stomatal conductance declined to 61% of that of plants in uniform 10mM NaCl despite a substantial root proliferation on the low-salt side (Bazihizina *et al.* 2012b). Given that these declines in stomatal conductance under heterogeneous salinities were not associated with detectable changes in leaf water status nor in increases in total soluble sugars in leaf tissues, it is unlikely that both sugars or leaf water status acted on stomata through negative feedback; instead, these results support the hypothesis that, as for partial root-drying,

also under heterogeneous salinities signals from the stressed roots are involved in the stomatal regulation of the shoot.

Plants have been proposed to have a sensory network that uses electrical signals moving through defined cell types to rapidly transmit information between distant sites within the organism (Fromm and Lautner 2007).In addition, given that changes in membrane potentials and the associated ion fluxes, and thus the membrane biogenic electrical potentials, are the first cellular responses to several environmental stimuli or stresses (Spalding 2000; Knight and Knight 2001), it is likely that electrical signals play a key role in the perception and propagation of stress signals. As in excitable animal tissues, plants have action potentials (APs) that propagate electrical information through the excitable membranes allowing the information to be transmitted from cell to cell (Lautner *et al.* 2005; Felle and Zimmermann 2007). APs, spike-like changes of the membrane potential, are in fact, generally self-amplifying signals dependent on voltage-gated ion channels and capable of propagating through any living cells sharing common membranes (Stahlberg and Cosgrove 1997; Pyatygin *et al.* 2008).

The generation of APs is a characteristic of all plant cells, and has been found to occur spontaneously in synchronized bursts in roots (Masi *et al.* 2009). In addition several environmental stresses (both abiotic and biotic) have also found to trigger the release and propagation of APs (Felle and Zimmermann 2007; Masi *et al.* 2009). For example, the addition of 50 mM KCl to the open end of a barley leaf or to the root system has been found to trigger APs, which

propagated with a velocity of 20–30 cm min^{-1} (Felle and Zimmermann 2007). Although propagation of APs along plant organs is well established (Fromm and Bauer 1994; Mancuso 1999; Dziubińska *et al.* 2001; Davies 2004) their roles in a possible plant-wide communication network remain poorly understood. Nevertheless, given their propagation speed and their widespread occurrence in plant cells, APs propagation represent a strong candidate for plant sensing and communication in the presence of a localized stress under heterogeneous soil conditions. To determine whether electrical signalling might act in systemic signalling, and more specifically in root to root signalling under heterogeneous salinities, we conducted several time-course experiments with cucumber seedlings using different electrophysiological techniques. The modulation of important genes involved in the response to salinity, for example, galactinol synthase which is responsible of the production of raffinose, that led to an improved osmotolerance during salt stress in the model plant *A. thaliana*, was also investigated (Sun *et al.* 2013). The modulation of the most important transcription factors such as ethylene response factor 109 (*ERF109*, also known as *RRTF1*), which is strongly activated under biotic stress and under salt treatment, was investigated as well. Finally modulation of a pivotal enzyme in ROS production, as aspartate oxidase, was investigated.

3.2.3 Materials and methods

3.2.3.1 Plant material and growth conditions

Cucumber seeds (*Cucumis sativus* L.) were surface sterilized and germinated in filter paper rolls and dipped in a solution with 0.5

mM $CaSO_4$. Filter paper rolls were maintained in vertical position, incubated at 26 °C for 48 to 72 h in the dark .After roots reached a length around 3 cm,root apices were cut with sterile razor blade in order to promote the formation of lateral roots. Each seedling was then transferred for three weeks into a 50 mL vial with half-strength nutrient solution which consisted of (mM): 0.5 KNO_3, 0.5 $CaNO_3$, 0.33 $MgSO_4$, 0.3 NH4H2PO_4; and (µM): 20FeEDTA, 2.3 H_3BO_3, 2 $MnSO_4$, 2 $ZnSO_4$, 0.5 $CuSO_4$, 0.5 Na_2MoO_4. The nutrient solution was adjusted to pH 5.8, using 1 M KOH. Seedlings were grown in environmentally controlled chambers (26/26°C and 12/12 h day/night), with an average photosynthetically active radiation at shoot height of 900 µmol m^{-2} s^{-1}. Solutions were changed weekly. Seedlings were ready to use three weeks after transferring to the nutrient solution.

3.2.3.2 Recording of the electrical activity

In order to determine whether there is a root-to-root communication under heterogeneous salt stress conditions, we conducted a split-root experiment with the MEA setup and three weeks old cucumber seedlings. This experiment consisted of six treatments with six replicates in a completely randomized block design, comprising positive (both sides of the root system receive salt) and negative (without salt addition) controls and the four treatments where one side of the root system of seedlings was exposed to increasing NaCl concentration (50-100-200 and 1000mM NaCl).Prior to the measurement, intact seedlings were transferred to a working

solution (BSM, 0.5 mM KCl and 0.2 mM $CaCl_2$) in a typical split-root setup and left to adapt to the new conditions for at least 2 h. In details, roots of each three-week old seedling were divided into two portions (split); one portion (HR1),dipped in a small container, received the salt treatment while the other portion, unstressed (HR2) was fixed onto the MEA (biochip) to allow the measurements of electrical activity. Seedlings were always maintained in vertical position using toothpicks and parts of the root not completely immersed in the working solution (BSM), were covered with agar solution 0,2%to avoid the dehydration. Action potentials (APs) were recorded in the half unstressed portion (HR2) of the root system fixed onto the (MEA). MEA measurement started after at least 10 minutes from sample (split-root) preparation; the electrical activity was then recorded for 5 minutes prior to the treatments and for the following 45 minutes (as preliminary experiments showed that all electrical activities generally ceased after 45-60 minutes).The treatments consisted of the replacement of HR1 solution with NaCl enriched BSM solution (to reach the final NaCl concentrations of 50, 100, 200 and 1000 mM), or with working BSM solution for control plants. Electrical recording was carried out following the method detailed in Masi *et al.* 2009. In brief, one intact root was gently positioned on a 6 x 10 multielectrode array with interelectrode distance of 500 μm and electrode diameter of 30 μm (Multi Channel Systems® MCS, Reutlingen, Germany), and sticked to the biochip surface using an adhesive water permeable and water resistant tape (3M Micropore SurgicalTape) that mediated close and flat adhesion of the root to the MEA surface, allowing in the meantime superfusion of the tissue

(Fig.A). The recording area generally covered the transition-elongation zone and part of the mature zone of the root apex, and photographs were taken after the recording sessions to confirm the localization of the recording electrodes. During the recordings, the working solution and ambient temperature was maintained at 26 ± 1°C.

Fig.A. Split-root for cucumber seedling; the left portion HR2 is fixed onto the MEA biochip, the right portion HR1 is immersed in the BSM solution with salt treatment.

3.2.3.3 Confocal microscopy

Confocal microscopy observation on three week-old cucumber seedlings treated with corona sodium green (fluorescent dye by Invitrogen) was performed in order to investigate sodium movements through the roots in the split root system under salt stress.Presence of Na^+ in non-treated roots (HR2) was determined 3h after NaCl addition in the opposite root portion (HR1), using the fluorescent dye Corona

sodium green. The CoroNa Green indicator allows spatial and temporal resolution of Na^+ concentrations selectively in the presence of physiological concentrations of other monovalent cations, excited by visible light and exhibits an increase in fluorescence emission intensity upon binding of Na^+ (ex λ /em λ = 492/516 nm) with just little shift in wavelength.

This experiment consisted of three treatments with six replicates in a completely randomized block design, including the negative and positive controls and split-root treated samples. Meanwhile for the electrical activities we monitored the root responses to different NaCl concentrations, for all remaining experiments,one salt concentration was used 200 mM NaCl (because with a time course we were able to detect obvious changes in action potential waveforms in particular under 200mM NaCl). For adaptation seedlings were transferred into BSM solution and split root was performed 2h before applying both the salt stress in one portion of the root system and the florescent dye (10µM) in the other half. After 3h from dye treatment, parts of roots were cut with razor, washed with deionized water and mounted on a slide in a water solution for confocal microscopy observation. Confocal imaging was performed using an upright Leica laser-scanning confocal microscope SP5 (Leica Microsystems Wetzlar GmbH, Germany) equipped with a 40× oil-immersion objective.

3.2.3.4 Ion flux measurements by vibrating probe technique

Three-week old intact seedlings were used in order to perform the measurements. This experiment consisted of 10 replicates and

focused on the response to 200 mM NaCl treatment. Each seedling was placed in a custom-built chamber, provided with two separate volumes, thus allowing the division of the root system in two portions; in each part of the chamber, roots were immobilized in horizontal position, and dipped in 3.2 mL of BSM solution (Fig.B). As for the MEA set-up, to avoid dehydration of the root portion not covered by the working solution, roots were covered with a solution with 0.2% agar, which prevented their dehydration for all the duration of the experiment. Plants were allowed to adapt to the new conditions for two hour prior to the measurement. Net K^+ and H^+ fluxes were measured on roots using a vibrating probe non-invasive system (University of Florence, Italy). Details on fabrication and calibrations of microelectrodes were described previously in (Mancuso *et al.* 2000). Basal K^+ and H^+ fluxes from roots not exposed directly to salt stress were measured for 15 min and then 0.8 mL of BSM with 1 M NaCl was added to the other part of chamber's solution in order to have final concentrations of 200 mM NaCl. Net K^+ and H^+ fluxes were monitored for up to 4h after salt treatment until stable values were obtained in the root portion not directly exposed to the salt stress. Ion fluxes in control plants, where only BSM was added in the other root portion, were also monitored.

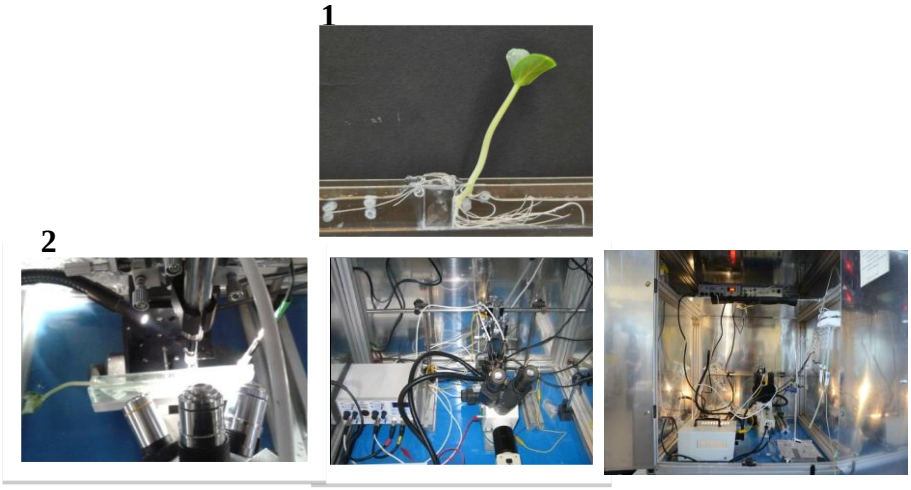


Fig.B. (1) Cucumber seedling -split root- in the measuring chamber, roots were immobilized in horizontal position, and dipped in 3.2 mL of BSM solution. (2) Vibrating Probe setup. Microelectrodes measuring ion fluxes

3.2.3.5 Detecting K^+ distribution by fluorescence microscopy

In this experiment three-week old cucumber seedlings were used in the split root system in order to detect potassium distribution at the root level during the time of exposure to salt stress.

Plants were placed in multi-well plates, and each single plant had its roots placed in two different wells for the differential application of salt and fluorescent probes potassium binding benzofuranisophthalate (PBFI). The roots of a single plant were divided into two portions, allowing the application of salt stress to one of the two root portions as described in previous experiments. After application of salt at different concentrations (0,10,50,100,200Mm NaCl) in one half of the root system (HR1), dye was added (10µM) to the other half (HR2). After 3h of exposure to salt, roots with dye from

both control and treatments were cut and rinsed in deionized water for 10 min; then, cut samples were mounted in a water solution on a slide and observed. Florescence microscopy was used to observe the distribution of K^+ in the root epidermal cells. The excitation wavelength was set at 340/390 nm, and emission was detected at 500nm. Fig C.

Fig C: Cucumber seedlings placed in multi-well plates with the root system for each seedling divided in two portions and each portion placed in different wells for the differential application of salt and fluorescent probes potassium binding benzofuranisophthalate (PBFI).

3.2.3.6 Monitoring of leaf turgor and stomatal conductance rates

Using the above described split-root setup, we determined whether the addition of salt in one root-half led to a detectable hydraulic signal in the shoot using the ZIM-probes setup. Prior to the measurements, root system of each plant was divided in two almost equal portions in hydroponic system, and then plants were left to adapt to the new conditions for at least 2 h, and then we monitored leaf turgor for the following 4 h after the salt addition (with a final NaCl concentration of 200 mM). Control plants where both sides were either left in a control solution (0 mM NaCl, negative control) or were exposed to a saline solution (positive control) were also monitored for 4 h. The measuring principle of the magnetic leaf patch clamp pressure probe (commercial name: ZIM-probe) is described in detail elsewhere (Zimmermann *et al.* 2008; Zimmermann *et al.* 2010). Briefly, an intact leaf is positioned between the two pads of the probe (diameter 10 mm), each of which is connected with magnets. The probe measures the pressure transfer exerted by the two magnets through the leaf patch, which is assumed to be in hydraulic contact with the surrounding unclamped leaf tissue, and proportional to the leaf turgor. A pressure sensor that is integrated in one of the pads senses the output pressure signal; a controller (ZIM-transmitter, ZIM Plant Technology GmbH, Hennigsdorf, Germany) transmits data to a computer for storage and following analysis. For the setup of the ZIM-probes, the clamp pressure that is exerted by the two magnets onto the leaf patch was adjusted in each leaf by varying the distance between

the two magnets in order to have an initial pressure of 20 KPa (optimal range is between 10 and 30 kPa). Concurrently, leaf gas exchange parameters were determined using the open gas exchange system Li-6400 (LiCor Inc., Lincoln, NE, USA); stomatal conductance was determined on young fully expanded leaves at ambient RH (40–50%), reference CO_2 of 400 µmolmol^{-1}, flow rate of 400 µmol s^{-1}, chamber temperature at 25°C and PAR of 300 µmol m^{-2} s^{-1}.

3.2.3.7 Gene expression analysis

Seedlings of three weeks old were used for samplings after 3h of the treatment with 200mMNaCl using the split-root setup described above. Samples from both sides of roots of control and treatment were directly frozen in liquid nitrogen and then stored at -80 °C until RNA extraction process. Samples were homogenized with a TissueLyser II (Rapid Cell and Tissue Homogenizer), then total RNA was extracted with Tryzol reagent (Life Technology) according to the manufacturer's instructions. The quantity of RNA was measured using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). For all samples, total RNA (1 µg) was treated with RQ1DNase (Promega), and first-strand cDNA was obtained using Improm II reverse transcriptase (Promega). Primers were designed using Primer 3 software (Koressaar and Remm 2007; Untergasser *et al.* 2012). Gene sequences were retrieved in cucumber databases (cucumber.genomics.org.cn). The sequences of the primers are as follows: CsACT3

(*Cucumis sativus* actin 3)

Fw,
TTCTGGTGATGGTGTGAGTC;CsACT3Rv,GGCAGTGGTGGTG
AACATG; AKT1 Fw, CTGTTCGTACAAAGCGATTG; AKT1 Rv,

TCCAACAAAACTCCTTCCAT; GOLS1-likeFw,
TACAAGCCCATCTCCTCGGA; GOLS1-like

Rv, AGTGAACAGGTCCAGCTTCG; ERF109-like Fw,
ACCCACCCAATTTTCCCTCC;

ERF109-like Rv, ACTCCAACGCCGCTTTATCA.Aspartate oxidase (AOX) Fw 5'-TTTTGCGATTGATTTGCTTA-3' Aspartate oxidase (AOX) Rv 5'-CCACCTGAGGCAAGTAGAGT-3'

Real Time qPCR was performed using a RotorGene 6000 (Quiagen). cDNA (correspondingto 5 ng of total RNA) was amplified using SSo Advance Universal Sybr Green Supermix (BioRad)and 0.5 μM of each primer. Data analysis was done using LinRegPCR software. Expression level ofgene was referred to Actin 3 (CsACT3) as housekeeping gene and was determined using a modification of the Pfaffl method (Pfaffl 2001), as reported in (Ferrari *et al.* 2006) and expressed in arbitrary units.

3.2.4 Results

3.2.4.1 APs waveforms were altered in the HR2

Consistent with previous studies (Zimmermann and Felle 2009), the addition of NaCl locally applied to one root portion altered

APs waveforms in the non-treated root portion, indicating a propagating electrical signal moving away from the point of stress application (Fig.1). In general the APs firing rate (i.e. spike frequency) tended to decline with time in all treatments, which has previously been indicated as an intrinsic property in plant cells (Fig. 2), and in most plants no spikes were detected after 40-45 minutes after the stress addition. The only exception was observed in roots treated with 1 M NaCl, whose spike rate significantly increased compared to control plants; 1M NaCl is an extremely toxic concentration that probably led to strong membrane depolarization at the point of stress application which could explain the increase in spike firing rate, that was not observed in the lower but more physiologically viable NaCl concentrations (Fig. 2).To determine the characteristics of individual extracellularly recorded spikes in all treatments, their shape (i.e. duration and amplitude) was analysed in several replicated trials. Both parameters remained unvaried in control replicates, although tending to decline with time. In details, during the first 40 minutes after the addition of BSM, spike amplitude ranged from approximately an average of 45mV to an average of 35mV (Fig. 3), and spike duration from an average of 26 ms to an average of 18 ms. By contrast, adding NaCl in the opposite root portion, significantly determined an increase in both spike duration and amplitude (Fig. 3), independently of the salinity treatment considered. A significant increase in spike duration was observed after 30 minutes of treatments meanwhile spike amplitude significantly increased after 35 minutes of treatments (Fig. 3).

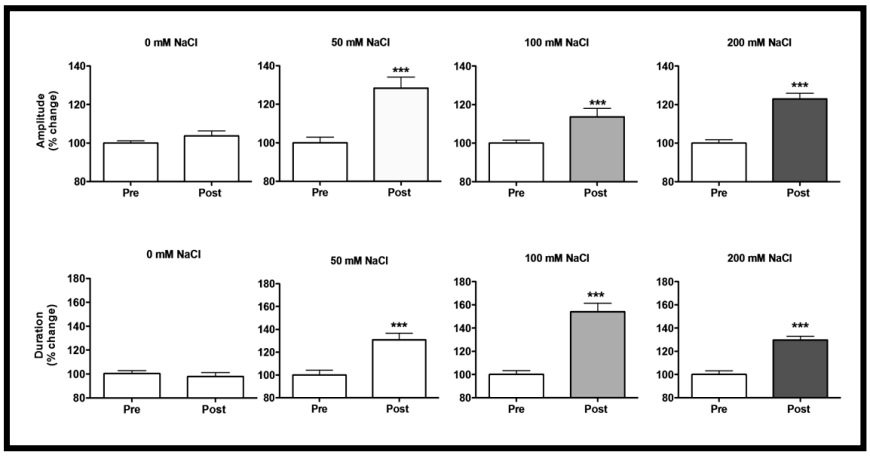


Fig. 1. Description of the spikes shape (amplitude and duration) in a root before (5 minutes) and after (45 minutes) applying BSM or NaCl in half of the root system. Values are mean values ± SE (n=6).

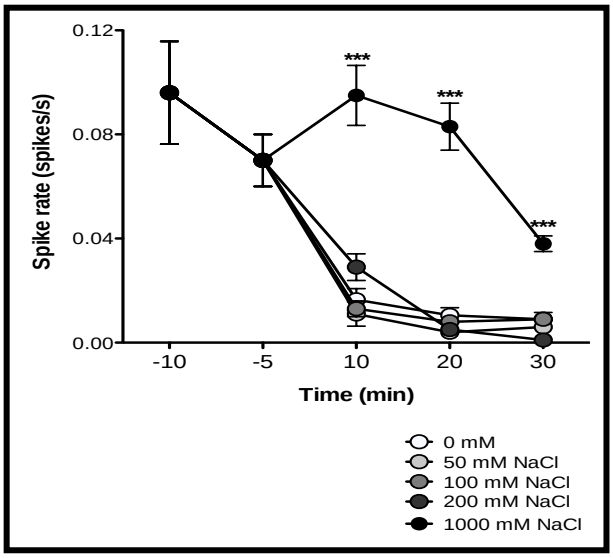


Fig. 2. Spike rate in a root before and after applying BSM or NaCl in half of the root system.

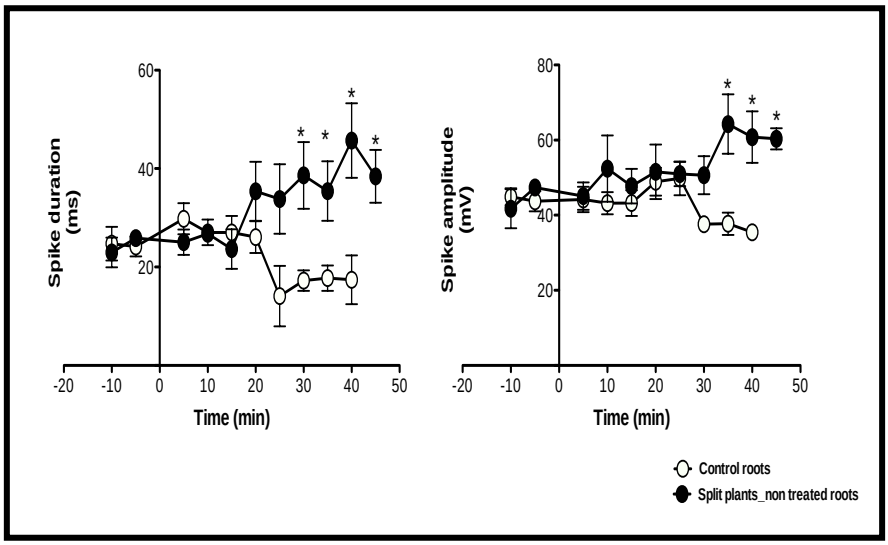


Fig. 3. Time course changes in action potential waveform following the localised addition of salt stress 200mM NaCl in the root-zone: (a) spike duration and (b) spike amplitude. Values are mean values ± SE (n=6).

3.2.4.2 Altered APs in the HR2 was not associated with Na^+ translocation

Confocal microscopy data suggested that the observed changes in electrical signals in the portion of the root system not directly exposed to salt were not associated with simple apoplastic diffusion of Na^+ from the treated roots to the non-treated ones due to a wick effect, as it is unlikely that in the time frame considered there would have been substantial Na^+ retranslocation via the phloem. The intensity of the fluorescent dye (Corona sodium green) was much higher in the portion of the root system directly exposed to salt stress (HR1) compared to HR2; in addition, no difference were observed in the intensity of the dye between HR2 and both portions of the negative

control root system, which confirm the fact that Na^+ did not affect the altered APs in HR2 (Fig.4).

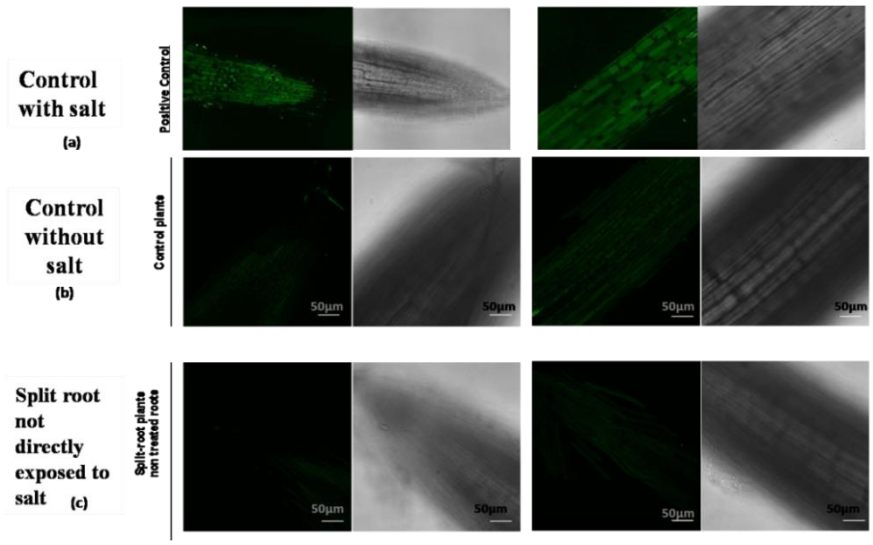


Fig. 4. Root Na content in the positive control roots (a) in the negative control roots (b) and in non-treated roots of split-root plants (c) after 3 h from the addition of 200 mM NaCl in the other root portion.

3.2.4.3 K^+ uptake rate was increased in the HR2 but no change in H^+ fluxes

The alteration of APs waveform in response to NaCl localised stress is likely to affect the property of biological membranes and/or of the ion channels in the non-stressed root half (HR2).

So it was important to conduct the measurements of K^+ and H^+ fluxes in the root zone of cucumber seedlings under heterogeneous salinity using vibrating probe technique. Approximately one hour after applying NaCl to one root portion and after the alteration of APs waveforms, there were significant increases in K^+ uptake rates in the non-stressed root half (HR2), meanwhile no evident change in H^+ flux was observed after salt treatment (Fig. 5 and 6).

Moreover, to confirm this result and to determine whether the addition of NaCl in one root half affected root ion distribution in the other half, K^+ compartmentation was monitored, using the fluorescent dyepotassium-binding benzofuranisophthalate (PBFI), in the root epidermal cells following NaCl addition in the opposite root portion. Even after 3h of localised salt treatment, there was a substantial increase in K^+ concentration in the non-treated roots (HR2) compared with control roots, where all the root system was NaCl free (Fig.7). K^+ concentrations increased in all portion of the root apex, from the meristematic zone to the mature zone.

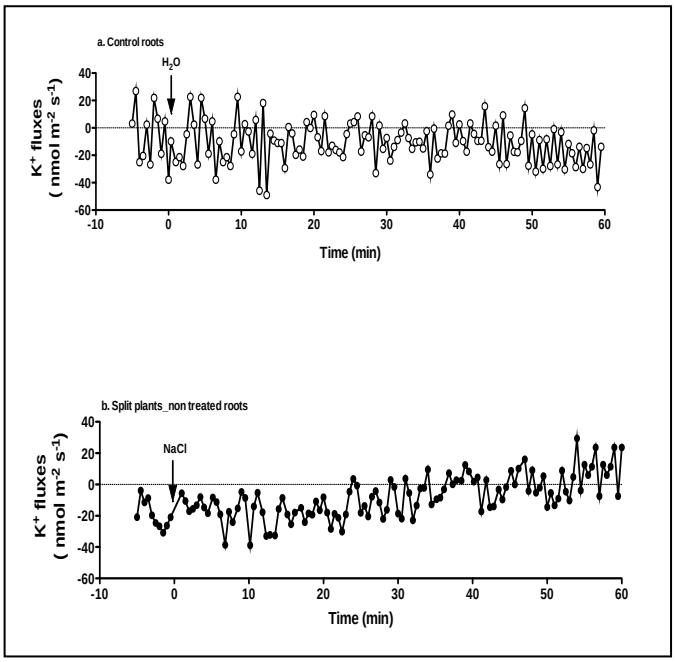


Fig. 5. K^+ fluxes in a representative control roots and in non-treated root of a split-root plant following the localised addition of NaCl.

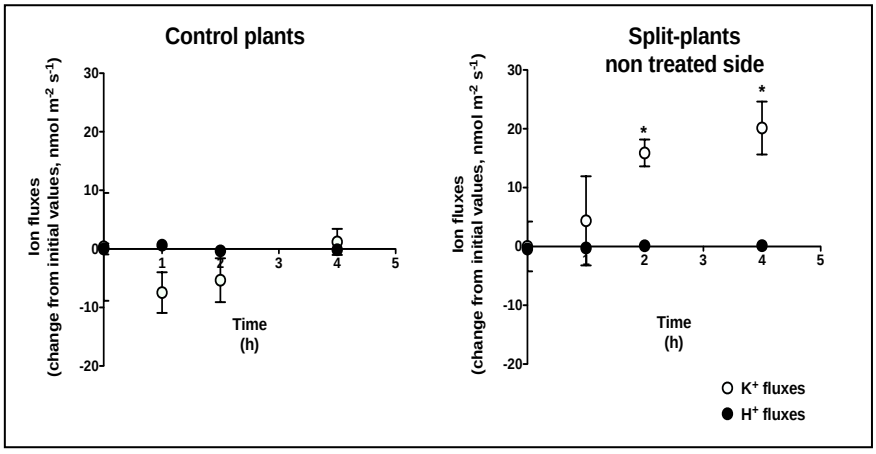


Fig.6.Temporal changes in ion fluxes (K^+ and H^+) in control roots and in non-treated roots of split-root plants after the addition of NaCl that resulted in a final NaCl concentration of 200 mM.

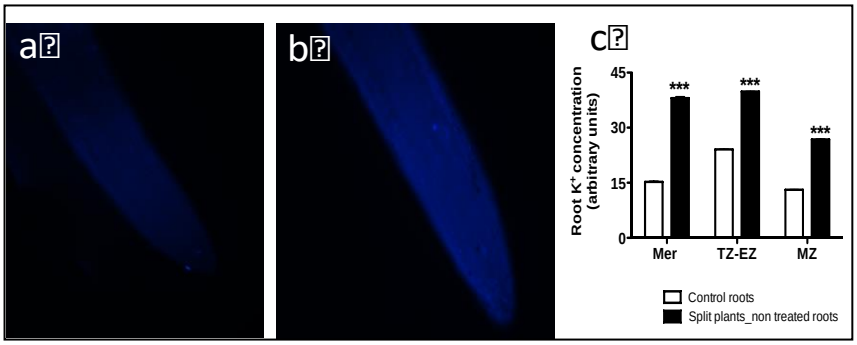


Fig.7. Temporal changes in root K^+ content in control roots (a) and in non-treated roots of split-root plants (b) after the addition of 200 mM NaCl. In (c) the quantification of root K^+ concentration was made with the ImageJ software and the fluorescence intensity of the PBFI dye indicate the K^+ content in the roots. Values are mean ± SE (n=3).

3.2.4.4 Reduction in stomatal conductance but not in leaf turgor

The addition of salt stress (NaCl 200mM) in one side of the root system reduced significantly stomatal conductance in the plants with split root system, and this reduction occurred in the first 15min of the treatment (Fig.8). Surprisingly, no changes between control (no salt) and split-root treatment (200 mM NaCl) were found regarding leaf turgor, meanwhile a significant and quick reduction was observed in the positive control where the whole root system was treated with salt (Fig.9).

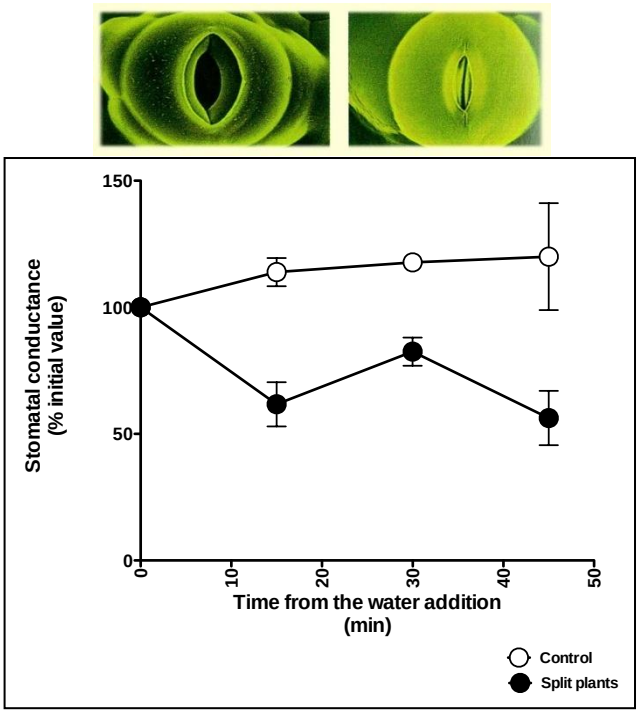


Fig. 8. Changes in stomatal conductance rates in young fully expanded leaves of control and split-root plants, with only one root portion exposed to 200 mM NaCl. Values are mean ± SE (n=4).

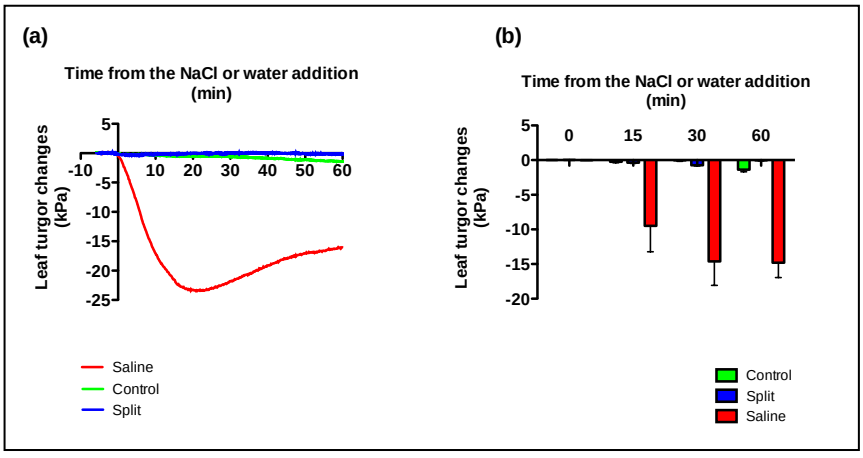


Fig.9. (a) Temporal changes in leaf turgor (kPa) compared with initial values in a representative control, salt treated and in split-root plants, with only one root half exposed to 200 mM NaCl. In (b) mean leaf

turgor changes in control, salt treated and in split-root plants, with only one root half exposed to 200 mM NaCl stress (200 mM), after 0, 15, 30 and 60 minutes of treatments. Values are mean ± SE (n=4).

3.2.4.5 GOLS1-like and ERF109 were induced but Aspartate Oxidize was down regulated after the treatment with salt

In order to investigate the transmission of signals among the roots under heterogeneous salt stress, the activation of an array of genes that may be involved in salt tolerance in *A. thaliana* was also investigated in cucumber plants treated with 200mM NaCl for three hours at room temperature. In particular, galactinolsynthase 1 (GOLS1), which is a key gene involved in raffinose synthesis and an overexpression of this gene leads to an improved tolerance to salt treatments (Sun *et al.* 2013), was investigated. According to previous data (Ma *et al.* 2006), three genes were selected: GOLS1-like, the homolog of *A. thaliana*; GOLS2, the homolog of the transcription factor ERF 109; and the gene that encode Aspartate oxidase which catalyzes the first irreversible step in the *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD).

Results showed an increase of the expression level of GOLS1-like in both sides of the split root system after the treatment with salt (Fig 10) compared with negative control, indicating that the treatment induces this particular gene to the same extent as in *A. thaliana*.

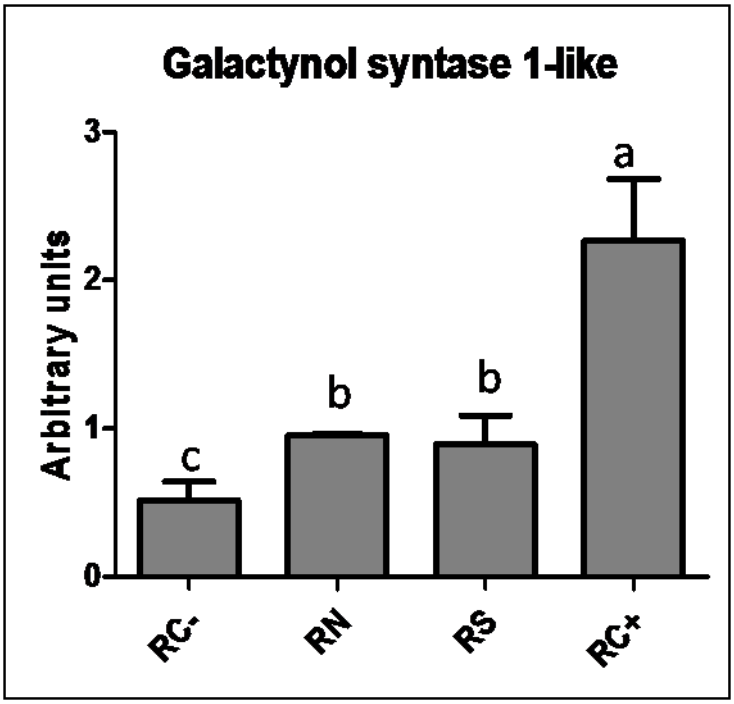


Fig. 10.Expression of CsGOLS1 like was analyzed by RT-qPCR in cucumber split roots treated for 3 h with water (control) or 200 mM of salt. The *CsACT3* gene was used as reference.

Observing the modulation of the other strongly activated gene, ERF 109, as expected, a significant increase in the RNA level was found only in the HR1 and in the roots of positive control (Fig 11a).The opposite phenomenon was observed for Aspartate oxidize; in fact, the expression level was down regulated in the portion of the root directly exposed to the salt (HR1) and in the positive control compared to (HR2) and the negative control (Fig. 11b).

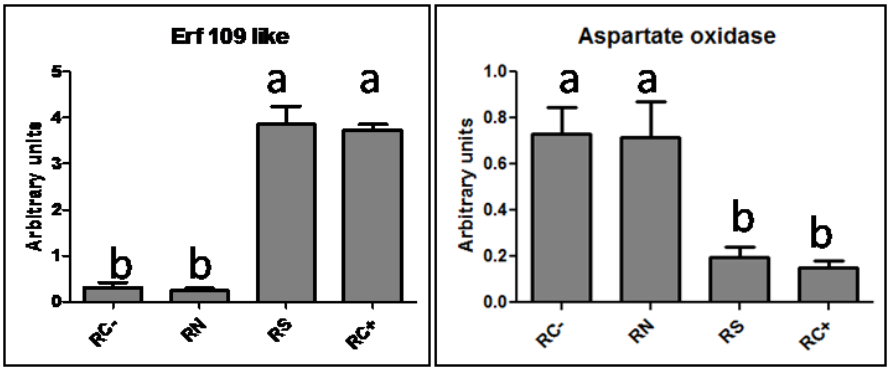


Fig.11. Expression of *Cs*ERF109 (a) and Aspartate oxidase(b) and was analyzed by RT-qPCR in cucumber split roots treated for 3 h with water (control) or 200 mM of salt. The *CsACT3* gene was used as reference.

3.2.5 Discussion

The present dataset provides clear evidence that the localised application of salt triggers an electrical signal that alters AP waveforms, unravelling the physiological relevance of such electrical phenomena with respect to systemic signalling in response to localised stress. As previously hypothesized by the changes in AP waveforms are unlikely to provide by itself a specific "defence-related" information, but it is more likely that APs, through the associated changes at the level of the plasma membranes and the proteins residing in the membrane matrix, could act as a fast forerunners to signal injury or threat.

Therefore, based also on the available data in the literature, the possible responses under localised stress as this, can be summarized as following: the addition of salt stress in part of the root system would

result in an immediate local membrane depolarization which, associated with the propagation of APs and probably Ca^{2+} waves, will propagate the signal systemically throughout the plants, with a propagation speed in the range of several hundred μm s^{-1}, depending on the plant's vascular connections (Choi *et al.* 2014). Locally, in the stressed roots, the strong membrane depolarization will then lead, in the following minutes and hours (depending on the species specific salt tolerance and the extent of the salt stress), in strong K^+ efflux mainly through K^+ channels and non-selective cation channels (Demidchik 2014) and will reduce the activity of the plasma membrane H^+-ATPase, which pumps protons and maintains electrochemical H^+ gradients (Sun *et al.* 2009).

Indeed, the ionic mechanism of moving electrical signals have been characterized by transient membrane depolarization/hyperpolarization leading to Ca^{2+}, Cl^- and K^+ ion fluxes as the changes in membrane potential always affect the electromechanical tension of the membrane lipid matrix, and thus membrane permeability and the conformational mobility of the protein systems residing in the membrane matrix (Davies 1993). The present results with vibrating probe technique and with PBFI fluorescent dye supports this hypothesis (e.g. see the observed increased K^+ uptake rate in the HR2). Interestingly, in animals, long-range signaling occurs through rapid ionic/membrane potential-driven signals throughout the nervous system; in an analogy between the plant and animal world, a review has recently brought forward the hypothesis that electrical signals in plants (i.e. electropotential waves that consist in the transmission of action and variation potentials) may be the vehicle for

the distribution of Ca^{2+} signals rapidly during inter-organ plant communication (van Bel *et al.* 2014). Interestingly during AP generation and propagation, Ca^{2+} enters the cytoplasm through voltage-gated Ca^{2+}channels of the plasma membrane and it is released from intracellular stores, rising the Ca^{2+} signal (Kudla *et al.* 2010), which supports the hypothesis of a linked electrical and calcium signal mechanism in plants. In a recent study it was demonstrated that localized application of salt stress at the root tip level elicits the spreading of a Ca^{2+} wave in the entire root system, that moves at a velocity of approximately 400 μm $s^{-1}$and triggers systemic molecular responses in the shoot (Choi *et al.* 2014). Hence, to determine whether the electrical signal might have been the vehicle for the distribution of Ca^{2+} signals during inter-organ plant communication, we estimated the propagation speed of our signal. Assuming that the signal travelled directly from the stressed root to the non-stressed root, and with an average root length of 10 cm, it was estimated that the electrical signal(s) that altered AP waveforms would have moved with a velocity of approximately 60 μm/s, which is an order of magnitude smaller than the propagation speed of the Ca^{2+} waves. On the other hand, if the signal were to travel to the shoot before arriving to the non-treated root, then the propagation speed of the electrical signal would increase to approximately 200 μm/s, thus in the same order of magnitude as the Ca^{2+} waves. Given that transmission of bioelectrical signals have been shown to takes place along the symplasmic route, especially within phloem cells, considered as the low resistance pathways for signal transmission (Fromm and Fei 1998; Masi *et al.* 2009), it is logical to expect that the propagation velocity would not

only depend on the distance considered but also would be affected by the vascular connections between organs, which also depend on plant anatomy (Callos and Medford 1994; Orians 2005). This would therefore explain the differences between the propagation speed in Arabidopsis and cucumber seedlings (Choi *et al.* 2014). Moreover, present results would suggest that this signal had to travel to the shoot and then to the roots, rather than travelling directly to the non-treated roots, as the measurements of stomata closure after 15 minutes of treatment suggests.

It is however important to note that present results suggest that the propagating signal moves first acropetally to the shoot and then subsequently basipetally to the non-treated roots, where it will trigger, first in the shoot and then in the roots, the systemic molecular responses (e.g. enhanced expression of K^+ channels or aquaporins (Choi *et al.* 2014), and then the physiological responses (e.g. stomatal closure observed in shoots, Fig. 8, or enhanced K^+ uptake in the HR2 of the root system in order to compensate K^+ efflux from HR1, the side of the root directly exposed to the salt, (Fig. 6 and 7). This view would also be supported by the observations that, under heterogeneous conditions triggered by salt addition, significant stomatal closure occurs within the first 15 minutes (Fig.8), thus substantially earlier than the observed changes in K^+ uptake rates in the roots.

The study of gene *GOLS1*, which plays a key role in raffinose biosynthesis and is mainly involved in relieving the osmotic stress induced by salt (Sun *et al.* 2013), confirmed that high salt concentrations induce osmotic stress in plants (Krasensky and Jonak

2012); indeed, the overexpression observed demonstrated its role in enhancing tolerance to high salinity and osmotic stresses. On the basis of our real-time qPCR analysis, we observed an upregulation of GOLS1 compared to the negative control in both sides of the split (Fig. 6*b*), indicating that the heterogeneous salinity in the rhizosphere altered plant responses.

Salt stress is one of the several factors that trigger the production of reactive oxygen species (ROS) and cause an increase of these compounds in plant cells (Apel and Hirt 2004). The generation of ROS as secondary messengers in the transduction pathway in plants under abiotic stress results in the expression of multiple stress-responsive genes or transcription factors (Huang *et al.* 2012). Among these transcription factors, ethylene response factor109 (ERF109,also known as RRTF1) is strongly activated under biotic stress and under salt treatment; the molecular mechanisms and the signaling pathway activated by this transcription factor still remains unclear to date (Matsuo *et al.* 2015). Present data demonstrate a strong induction of the ERF109 transcription factor only in the root side exposed to the salt (HR1). Moreover, Aspartate oxidase is downregulated under salt stress as expected (Winter *et al.* 2007), but only in the treated one. The data from ERF 109 and aspartate oxidase suggest that the ROS production is a tightly controlled mechanism and is only local and not systemic, meanwhile GOLS1 modulation seems to be not local, but systemic response.

References

Bazihizina, N, Barrett-Lennard, EG, Colmer, TD (2012a) Plant growth and physiology under heterogeneous salinity. *Plant and Soil***354**, 1-19.

Bazihizina, N, Barrett-Lennard, EG, Colmer, TD (2012b) Plant responses to heterogeneous salinity: growth of the halophyte Atriplex nummularia is determined by the root-weighted mean salinity of the root zone. *Journal of Experimental Botany***63**, 6347-6358.

Bazihizina, N, Colmer, TD, Barrett-Lennard, EG (2009) Response to non-uniform salinity in the root zone of the halophyte Atriplex nummularia: growth, photosynthesis, water relations and tissue ion concentrations. *Ann Bot***104**, 737-45.

Bertholon, N (1783) 'De L'Électricité Des Végétaux (etc.).' (Bernuset: Berthomieu, P, Conejero, G, Nublat, A, Brackenbury, WJ, Lambert, C, Savio, C, Uozumi, N, Oiki, S, Yamada, K,Cellier, F, Gosti, F, Simonneau, T, Essah, PA, Tester, M, Very, AA, Sentenac, H, Casse, F (2003)Functional analysis of AtHKT1 in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance.*Embo Journal***22**, 2004-2014.

Blatt, MR (2008) Ion Transport at the Plant Plasma Membrane. *eLS*

Bose, JC, 1926. The nervous mechanism of plants 1926. S,

Burdon-Sanderson, J, Klein, E, Foster, M, Brunton, TL (1873) 'Handbook for the physiological laboratory.' (J. & A. Churchill:

Callos, JD, Medford, JI (1994) Organ positions and pattern formation in the shoot apex. *The Plant Journal***6**, 1-7.

Choi, W-G, Toyota, M, Kim, S-H, Hilleary, R, Gilroy, S (2014) Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proceedings of the National Academy of Sciences***111**, 6497-6502.

Comstock, JP (2002) Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. *Journal of Experimental Botany***53**, 195-200.

Darwin, C (1875) Insectivorous plants. 1875. *Murray, London*

Datta, P, Palit, P (2004) Relationship between environmental factors and diurnal variation of bio. *Current Science***87**,

Davies, E (1993) 'Intercellular and intracellular signals and their transduction via the plasma membrane-cytoskeleton interface, Seminars in cell biology.' (Elsevier:

Davies, E (2004) New functions for electrical signals in plants. *New Phytologist***161**, 607-610.

Davies, E (2006) Electrical signals in plants: facts and hypotheses. In 'Plant electrophysiology.' pp. 407-422. (Springer:

Demidchik, V (2014) Mechanisms and physiological roles of K+ efflux from root cells. *Journal of Plant Physiology***171**, 696-707.

Dong, H, Kong, X, Luo, Z, Li, W, Xin, C (2010) Unequal salt distribution in the root zone increases growth and yield of cotton. *European Journal of Agronomy***33**, 285-292.

Dziubińska, H, Trębacz, K, Zawadzki, T (2001) Transmission route for action potentials and variation potentials in Helianthus annuus L. *Journal of Plant Physiology***158**, 1167-1172.

Felle, HH, Zimmermann, MR (2007) Systemic signalling in barley through action potentials. *Planta***226**, 203-214.

Ferrari, S, Galletti, R, Vairo, D, Cervone, F, De Lorenzo, G (2006) Antisense expression of the Arabidopsis thaliana AtPGIP1 gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to Botrytis cinerea. *Molecular Plant-Microbe Interactions***19**, 931-936.

Flowers, T, Troke, P, Yeo, A (1977) The mechanism of salt tolerance in halophytes. *Annual review of plant physiology***28**, 89-121.

Flowers, TJ, Colmer, TD (2008) Salinity tolerance in halophytes. *New Phytologist***179**, 945-963.

Fromm, J (2006) Long-distance electrical signaling and physiological functions in higher plants. In 'Plant Electrophysiology.' pp. 269-285. (Springer:

Fromm, J, Bauer, T (1994) Action potentials in maize sieve tubes change phloem translocation. *Journal of Experimental Botany***45**, 463-469.

Fromm, J, Fei, H (1998) Electrical signaling and gas exchange in maize plants of drying soil. *Plant Science***132**, 203-213.

Fromm, J, Lautner, S (2007) Electrical signals and their physiological significance in plants. *Plant, Cell & Environment***30**, 249-257.

Gil, PM, Gurovich, L, Schaffer, B, Alcayaga, J, Rey, S, Iturriaga, R (2008) Root to leaf electrical signaling in avocado in response to light and soil water content. *Journal of Plant Physiology***165**, 1070-1078.

Gowing, D, Davies, W, Jones, H (1990) A positive root-sourced signal as an indicator of soil drying in apple, Malus x domestica Borkh. *Journal of Experimental Botany***41**, 1535-1540.

Hedrich, R, Schroeder, JI (1989) The physiology of ion channels and electrogenic pumps in higher plants. *Annual Review of Plant Biology***40**, 539-569.

Knight, H, Knight, MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends in Plant Science***6**, 262-267.

Kong, X, Luo, Z, Dong, H, Eneji, AE, Li, W (2011) Effects of non-uniform root zone salinity on water use, Na+ recirculation, and Na+ and H+ flux in cotton. *Journal of Experimental Botany* err420.

Koressaar, T, Remm, M (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics***23**, 1289-1291.

Krasensky, J, Jonak, C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany***63**, 1593-1608.

Ksenzhek, OS, Volkov, AG (1998) 'Plant energetics.' (Academic Press)

Kudla, J, Batistič, O, Hashimoto, K (2010) Calcium signals: the lead currency of plant information processing. *The Plant Cell***22**, 541-563.

Lautner, S, Grams, TEE, Matyssek, R, Fromm, J (2005) Characteristics of electrical signals in poplar and responses in photosynthesis. *Plant Physiology***138**, 2200-2209.

Lu, C, Qiu, N, Wang, B, Zhang, J (2003) Salinity treatment shows no effects on photosystem II photochemistry, but increases the resistance of photosystem II to heat stress in halophyte Suaeda salsa. *Journal of Experimental Botany***54**, 851-860.

Lycoskoufis, I, Savvas, D, Mavrogianopoulos, G (2005) Growth, gas exchange, and nutrient status in pepper (Capsicum annuum L.) grown in recirculating nutrient solution as affected by salinity imposed to half of the root system. *Scientia Horticulturae***106**, 147-161.

Ma, S, Gong, Q, Bohnert, HJ (2006) Dissecting salt stress pathways. *Journal of Experimental Botany***57**, 1097-107.

Maathuis, FJ, Sanders, D (1997) Regulation of K+ absorption in plant root cells by external K+: interplay of different plasma membrane K+ transporters. *Journal of Experimental Botany***48**, 451-458.

Mancuso, S (1999) Hydraulic and electrical transmission of wound-induced signals in Vitis vinifera. *Functional Plant Biology***26**, 55-61.

Mancuso, S, Papeschi, G, Marras, AM (2000) A polarographic, oxygen-selective, vibrating-microelectrode system for the spatial and temporal characterisation of transmembrane oxygen fluxes in plants. *Planta***211**, 384-389.

Masi, E, Ciszak, M, Stefano, G, Renna, L, Azzarello, E, Pandolfi, C, Mugnai, S, Baluška, F, Arecchi, F, Mancuso, S (2009) Spatiotemporal dynamics of the electrical network activity in the root apex. *Proceedings of the National Academy of Sciences***106**, 4048-4053.

Matsuo, M, Johnson, JM, Hieno, A, Tokizawa, M, Nomoto, M, Tada, Y, Godfrey, R, Obokata, J, Sherameti, I, Yamamoto, YY, Bohmer, FD, Oelmuller, R (2015) High REDOX RESPONSIVE TRANSCRIPTION FACTOR1 Levels Result in Accumulation of Reactive Oxygen Species in Arabidopsis thaliana Shoots and Roots. *Molecular Plant***8**, 1253-1273.

Meiri, A, Plaut, Z (1985) Crop production and management under saline conditions. In 'Biosalinity in Action: Bioproduction with Saline Water.' pp. 253-271. (Springer).

Messedi, D, Labidi, N, Grignon, C, Abdelly, C (2004) Limits imposed by salt to the growth of the halophyte Sesuvium portulacastrum. *Journal of Plant Nutrition and Soil Science***167**, 720-725.

Orians, C (2005) Herbivores, vascular pathways, and systemic induction: facts and artifacts. *Journal of chemical ecology***31**, 2231-2242.

Oyarce, P, Gurovich, L (2011) Evidence for the transmission of information through electric potentials in injured avocado trees. *Journal of Plant Physiology***168**, 103-108.

Panta, S, Flowers, T, Lane, P, Doyle, R, Haros, G, Shabala, S (2014) Halophyte agriculture: Success stories. *Environmental and Experimental Botany***107**, 71-83

Pfaffl, MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research***29**,

Pyatygin, S, Opritov, V, Vodeneev, V (2008) Signaling role of action potential in higher plants. *Russian Journal of Plant Physiology***55**, 285-291.

Saab, IN, Sharp, RE (1989) Non-hydraulic signals from maize roots in drying soil: inhibition of leaf elongation but not stomatal conductance. *Planta***179**, 466-474.

Schachtman, DP, Goodger, JQ (2008) Chemical root to shoot signaling under drought. *Trends in Plant Science***13**, 281-287.

Shabala, L, Ross, T, Newman, I, McMeekin, T, Shabala, S (2001) Measurements of net fluxes and extracellular changes of H+, Ca2+, K+, and NH4+ in Escherichia coli using ion-selective microelectrodes. *J Microbiol Methods***46**, 119-29.

Shabala, S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. *Ann Bot***92**, 627-634.

Shabala, S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Ann Bot***112**, 1209-21.

Shabala, S, Cuin, TA (2008) Potassium transport and plant salt tolerance. *Physiol Plant***133**, 651-69.

Shabala, S, Pottosin, I (2014) Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. *Physiol Plant***151**, 257-279.

Shabala, SN, Newman, IA (1998) Osmotic sensitivity of Ca2+ and H+ transporters in corn roots: effect on fluxes and their oscillations in the elongation region. *J Membr Biol***161**, 45-54.

Shani, U, Waisel, Y, Eshel, A, Xue, S, Ziv, G (1993) Responses to salinity of grapevine plants with split root systems. *New Phytologist***124**, 695-701.

Sonneveld, C, De Kreij, C (1999) Response of cucumber (Cucumis sativus L.) to an unequal distribution of salts in the root environment. *Plant and Soil***209**, 47-56.

Spalding, E (2000) Ion channels and the transduction of light signals. *Plant, Cell & Environment***23**, 665-674.

Stahlberg, R, Cleland, RE, Van Volkenburgh, E (2006) Slow wave potentials—a propagating electrical signal unique to higher plants. In 'Communication in Plants.' pp. 291-308. (Springer).

Stahlberg, R, Cosgrove, DJ (1997) The propagation of slow wave potentials in pea epicotyls. *Plant Physiology***113**, 209-217.

Sten-Knudsen, O (2002) 'Biological membranes: theory of transport, potentials and electric impulses.' (Cambridge University Press).

Sun, J, Chen, S, Dai, S, Wang, R, Li, N, Shen, X, Zhou, X, Lu, C, Zheng, X, Hu, Z (2009) NaCl-induced alternations of cellular and tissue ion fluxes in roots of salt-resistant and salt-sensitive poplar species. *Plant Physiology***149**, 1141-1153.

Sun, Z, Qi, X, Wang, Z, Li, P, Wu, C, Zhang, H, Zhao, Y (2013) Overexpression of TsGOLS2, a galactinol synthase, in Arabidopsis thaliana enhances tolerance to high salinity and osmotic stresses. *Plant Physiology and Biochemistry***69**, 82-89.

Tabatabaie, S, Gregory, P, Ho, L, Hadley, P (2003) 'Split root system for the use of saline water in hydroponic tomato production, International Symposium on Managing Greenhouse Crops in Saline Environment 609.'

Trewavas, A (2003) Aspects of plant intelligence. *Ann Bot***92**, 1-20.

Tyerman, SD, Beilby, M, Whittington, J, Juswono, U, Newman, I, Shabala, S (2001) Oscillations in proton transport revealed from simultaneous measurements of net current and net proton fluxes from isolated root protoplasts: MIFE meets patch-clamp. *Functional Plant Biology***28**, 591-606.

Untergasser, A, Cutcutache, I, Koressaar, T, Ye, J, Faircloth, BC, Remm, M, Rozen, SG (2012) Primer3—new capabilities and interfaces. *Nucleic Acids Research***40**, e115.

van Bel, AJ, Ehlers, K (2003) Electrical signalling via plasmodesmata. *Plasmodesmata* 263-278.

van Bel, AJ, Furch, AC, Will, T, Buxa, SV, Musetti, R, Hafke, JB (2014) Spread the news: systemic dissemination and local impact of Ca2+ signals along the phloem pathway. *Journal of Experimental Botany* ert425.

Volkov, AG (2000) Green plants: electrochemical interfaces. *Journal of Electroanalytical Chemistry***483**, 150-156.

Volkov, AG (2006) Electrophysiology and phototropism. In 'Communication in Plants.' pp. 351-367. (Springer).

Volkov, AG, Carrell, H, Markin, VS (2009) Biologically closed electrical circuits in Venus flytrap. *Plant Physiology***149**, 1661-1667.

Volkov, AG, Pinnock, M-R, Lowe, DC, Ma'Resha, SG, Markin, VS (2011) Complete hunting cycle of Dionaea muscipula: consecutive steps and their electrical properties. *Journal of Plant Physiology***168**, 109-120.

Winter, D, Vinegar, B, Nahal, H, Ammar, R, Wilson, GV, Provart, NJ (2007) An "Electronic Fluorescent Pictograph" Browser for

Exploring and Analyzing Large-Scale Biological Data Sets. *PLoS ONE***2**, e718.

Zhu, Y, Ito, T (2000) Effects of nutrient stress by slit-root system on the growth and K, Ca, and Mg contents at different stages of hydroponically-grown tomato [Lycopersicon esculentum] seedlings. *Journal of the Japanese Society for Horticultural Science (Japan)*

Zhu, ZJ, Wei, GQ, Li, J, Qian, QQ, Yu, JQ (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (Cucumis sativus L.). *Plant Science***167**, 527-533.

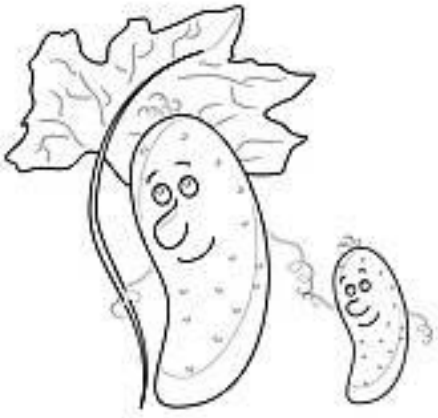
Zimmermann, D, Reuss, R, Westhoff, M, Geßner, P, Bauer, W, Bamberg, E, Bentrup, F-W, Zimmermann, U (2008) A novel, non-invasive, online-monitoring, versatile and easy plant-based probe for measuring leaf water status. *Journal of Experimental Botany***59**, 3157-3167.

Zimmermann, MR, Felle, HH (2009) Dissection of heat-induced systemic signals: superiority of ion fluxes to voltage changes in substomatal cavities. *Planta***229**, 539-547.

Zimmermann, U, Rüger, S, Shapira, O, Westhoff, M, Wegner, L, Reuss, R, Gessner, P, Zimmermann, G, Israeli, Y, Zhou, A (2010) Effects of environmental parameters and irrigation on the turgor pressure of banana plants measured using the non-invasive, online monitoring leaf patch clamp pressure probe. *Plant Biology***12**, 424-436.

Chapter 4

General conclusions



We can summarize that salinity stress is well reported to reduce plant growth and to affect plant development. The impact of salt stress on the growth of plant leads in altered plant morphology. The intense research activity performed during the last years has shed light on numerous details of salinity responses and mechanisms of adaptation at molecular level. However, many mechanisms should be still elucidated. For example, the regulation of gene expression and signaling cascades that regulates many Na^+ transporters remains unclear. Moreover, it remains to be assessed which of the sodium ions transport processes reviewed here could be employed to enhance plant performance under salt stress. Indeed both molecular breeding and advanced biotechnology methods should be helpful to develop crops with enhanced salt tolerance. In this context as illustrated in Fig. D, it is important to understand the underlying mechanisms that led to salt resistance and adaptation. Basically, molecular signaling components in plant adaptation and response to salt stress involved a network of Na^+ transporters, such as HKTs and NHXs, hormones (like ABA), transcription factors, MAPK pathways and ROS. Taken all together these are the major traits that influence plant resistance and adaptation to salt stress. In the next years the major goals will be to understand the cross-talk between different pathways that lead to resistance and adaptation. Moreover both stress tolerant and sensitive plants utilize the same signaling molecules, thus it will be important in the future works to dissect and discriminate signals from the signaling molecule.

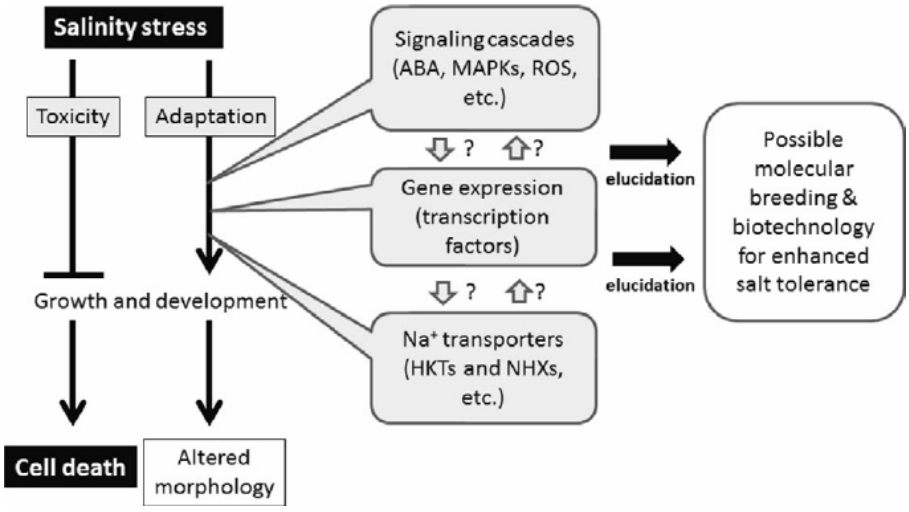


Fig. D. Ion transporters as possible targets of molecular breeding and biotechnological approaches for conferring the enhanced salinity tolerance in plant cells.

On the other hand, it is known that all living cells have evolved sophisticated tools to sense and respond quickly to various environmental stresses, and in particular sessile organisms such as plants often exposed to heterogeneous stimuli. In this context, stress signaling is pivotal for the maintenance of plant water status and all cellular functions and understanding systemic signaling in plants has long been recognized as a major scientific challenge (Zimmermann and Felle 2009). The results in the present study would indicate that

electrical signals carry the information following a localized stress that, then, not only affect shoot physiology, but also roots placed in a non-stressed environments. The ubiquity of the electrical signaling in plants would indicate that signal could have a wider and more general function in plant biology, and could be used by plants in long distance systemic signaling.

Summary

This work was divided in four chapters mainly dealt with the problem of salinity and plant physiology in particular in cucumber plants *(Cucumis sativus)*; as one of the most severe factors that limits the yield of filed, because most of crops employed in agriculture is salt sensitive.

In the first chapter an extensive and in-depth study on sensing and signaling mechanisms of salt stress and the tolerance strategies in plants was addressed.

In the second chapter, screening for salt tolerance in cucumber plants (*Cucumis sativus*) was conducted using several different physiological and genetic approaches. In the first phase six different cultivars of cucumber were screened for salt tolerance under 100Mm NaCl , but just two different cultivars (Parys, sensitive and Polan, tolerant), were selected based on their germination capability under salt stress, in order to continue the screening for salt tolerance as a second phase of the work by using several different physiological and genetic approaches . Thus, K^+ fluxes from roots were measured, as an immediate response to salinity, and the expression level of Inward Rectifying Potassium Channel (AKT1) was investigated as well. Not only that but also ROS production was also examined in both cultivars as an indicator for salt tolerance and the level of Respiratory Burst Oxidase Homolog F (RBOHF) gene, as well as a the induction of Ethylene Responsive Factor 109 (ERF109) transcription factor were investigated after salt treatment . Basing on the data obtained we

suggest that root ability to retain K^+, a higher level of RBOHF and a strong induction of ERF109, should all be considered important components for salt tolerance in *Cucumis sativus*.

In the third chapter, the work focused on studying plant signaling under heterogeneous salinity in the rizosphere ;starting from the idea that the distribution of salts in the root environment is unequal. and plant response to salt stress can differ greatly depending on environmental factors. So the aim of this study was to investigate plant signaling under heterogeneous salt conditions. For that a split root system was established and one half root apparatus (HR1) was treated with salt. The first measurements was conducted with a Multi Electrode Array (MEA) system in the non-stressed half roots (HR2) for the electrical signals as the initial responses of the plant to exterior stimulus and in this case (salt stress). We found that both duration and amplitude of the action potentials recorded increased, while in the control remains similar. In order to identify the nature of the signal traveling from one HR1 to HR2 we measured H^+ and K^+ fluxes in HR2 by using ion-selective microelectrodes with the vibrating probe technique., and interestingly we observed a net potassium influx after 40 minutes of the treatment while no change in proton flux was detected. To confirm this result a fluorescent dye potassium-binding benzofuranisophthalate (PBFI) was used and an increase in potassium concentration in HR2 was found compared with the control roots.

By corona sodium green (fluorescent dye) and confocal microscopy we confirmed that changes in electrical signals and fluxes are not associated with the simple apoplastic diffusion of sodium from HR1 to

HR2. To further investigate the transmission of signals among roots, the activation of salt stress marker genes were examined in the split roots system. The salt marker gene probed (CsGOLS1 like) was up-regulated in both portions of the root system after 3 and 4 h of the treatment with salt. A strong induction of Ethylene Responsive Factor 109 (ERF109) transcription factor was observed in the portion of root (HR1) after 3 hours of the salt treatment, meanwhile for the aspartate oxidase was found to be down regulated in the portion (HR1) after 3 hours of the treatment with salt.

The fourth chapter included the most important conclusions of this work