Study of reactive oxygen species-induced ion transport in different models by using electrophysiological approaches.

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A5 Human annexin 5
AKT, KAT *Arabidopsis* K^+ transporters
AtANN1 *Arabidopsis* annexin 1.
ann1 *Arabidopsis* annexin 1 loss of function
ANOVA Analysis of Variance statistical test

BCS Bathocuproinedisulfonic acid
BSM Basic salt medium
BTP bis-Tris propane

COPT1 *Arabidopsis* High Affinity Copper Transporter 1
C1^{oe} *Arabidopsis* COPT1 over-expressing plants
copt1 *Arabidopsis* COPT1 loss of function
CAX Calcium Exchanger 1
Ca^{2+}-ATPase Plasma membrane calcium pump
$[Ca^{2+}]_{cyt}$ Cytosolic calcium.

DMSO Dimethyl sulfoxide

GORK *Arabidopsis* gated outwardly- rectifying K^+ channel

HAK fungal-like high affinity K^+ transporter
HKT plant high-affinity potassium transporter
HP Holding potential
HR Hydroxyl radicals

$[K^+]_{cyt}$ Cytosolic potassium
KIR Inward-rectifying K^+ channel
KOR Outward-rectifying K^+ channel
KT Potassium transporter
KUP K^+ uptake transporter

MDA Malondialdehyde
MES 4-Morpholineethanesulfonic acid
MS Murashige and Skoog medium
½ MS half-strength Murashige and Skoog medium

$[Na^+]_{cyt}$ Cytosolic sodium
NADPH oxidase Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NHX Vacuolar Na^+/H^+ exchanger.

NSCC Non-Selective Cation Channel

ROS Reactive oxygen species

SOS1 Salt Overly Sensitive 1, Na1/H1 exchanger
SPIK *Arabidopsis* pollen inward- rectifying channel
SKOR *Arabidopsis* stellar potassium outward-rectifying channel
SPL7 *SQUAMOSA* Promoter Binding Protein–Like

PCD Programmed cell death
PLBs Planar Lipid Bilayers
PM Plasma membrane
PS Phosphatidylserine

TEA Tetraethylammonium chloride

$\mathbf{V_m}$ Plasma membrane potential

ZIP2 Zrt-, Irt-like Protein 2

Chapter 1

Ion transport in oxidative signaling/stress.

1.1 Reactive Oxygen Species (ROS) signaling.

Aerobic organisms use molecular oxygen (O_2) in mitochondrial respiration. In this process, carbon compounds are oxidized and their energy released and stored transiently in a compound, ATP, that will be used in maintenance and development. At the same time, respiration generates multiple carbon precursors required for biosynthesis in the cells.

As a consequence of O_2 partial reduction, different Reactive Oxygen Species (ROS) are formed in cells. Transition metals such as iron or copper (Cu) can also mediate ROS production by getting oxidized in the Fenton reaction and further get reduced in the net Haber-Weiss reactions. A schematic representation of the sequential ROS production is shown in the Fig 1.1.1.

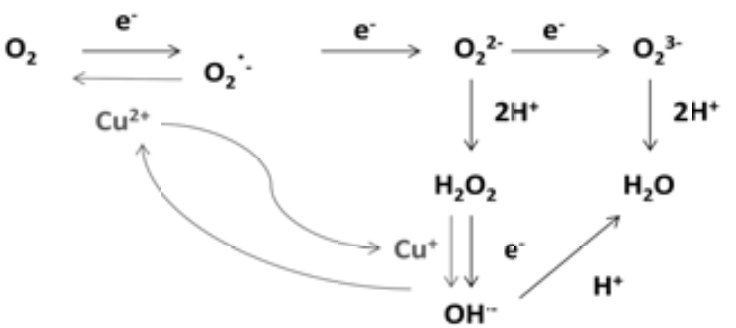

Figure 1.1.1 ROS production by multistep reduction of oxygen. (Adapted from Appel & Hirt, 2004; Gechev *et al.*,2006). Grey lines show the Haber-Weiss reactions.

ROS are produced in cells in response to a number of stimuli, including major abiotic and biotic stresses. These forms can be poorly reactive (non-radicals, such as H_2O_2 or O_3) or can react extremely quickly with other free radicals such as superoxide ($O_2^{\cdot -}$) or hydroxyl radicals ($OH\cdot$) (Halliwell & Gutteridge, 1999). Although ROS can control numerous processes, a control balance of ROS-producing and ROS-scavenging systems must be kept to ensure an accurate execution of signaling without provoking toxicity, due to their capacity of oxidizing proteins, lipids and DNA (Apell & Hirt, 2004).

Antioxidant defense includes enzymatic and non-enzymatic mechanisms. The first group includes the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione peroxidase. There are multiples isoforms of SOD and APX in the different subcellular compartments. The second group includes cellular redox buffers such as ascorbate, glutathione (GSH), tocopherol, flavonoids, alkaloids and carotenoids (Apell & Hirt, 2004).

Specificity of biological response to ROS varies depending, among other factors, on their chemical identity, plant zone tissue and the intensity of the ROS-induced signal. Moreover, ROS

can interact with other signaling molecules such as nitric oxide, lipid messengers or plant hormones (Reviewed by Gechev *et al.*, 2006).

In animal cells, an example of ROS-mediated process could be phagocyte activation and the generation of ROS for killing foreign organisms. Alterations in the ROS generation-scavenging system have been shown to generate oxidative-stress diseases such as heart failure, rheumatoid arthritis, or neurodegenerative diseases.

In plant cells, both photosynthetic and respiratory electron-transport chains, as well as the NADPH oxidases and peroxidases are involved in ROS generation (Apel & Hirt, 2004). ROS have been shown to regulate gene expression and signaling transduction pathways in plants. One of the main ways of plant-transmitted information in relation to environment fluctuations is the generation of superoxide bursts at the plasma membrane (Doke *et al.*, 1994). ROS can control numerous processes, such as root gravitropism, hypersensitive response to pathogens, stomatal closure and cell expansion and development (reviewed by Apel & Hirt, 2004; Laloi *et al.*, 2004; Mittler *et al*., 2004; Gechev *et al.*, 2006; de Pinto *et al*., 2012). During pathogen attack, Programmed Cell Death (PCD) is induced in order to isolate cells and therefore avoiding pathogen spread. ROS play a crucial role in this signaling network (Foyer & Noctor, 2005). In many cases, ROS production is genetically programmed and is induced during development. Generation of singlet oxygen induces controlled PCD in aleurone cells, leaf senescence, tracheary elements maduration or trichome development (Gechev *et al.*, 2006).

Numerous abiotic factors can perturb the net balance between ROS scavenging and production: salinity, heavy metals, UV light, ozone, paraquat, cold and osmotic shock, or aluminum toxicity (Murphy & Taiz, 1997; Apel & Hirt, 2004; Corrales *et al.,* 2008; Shabala & Cuin, 2008).

Copper has been shown to enhance antioxidant defenses in a widely range of species. Some examples are *Ulva compressa* (González *et al.*, 2010), *Zea mays* (Madejón *et al.*, 2010) or *Arabidopsis thaliana* (Drazkiewicz *et al.* 2004). The production of ROS by Cu^{+} ions by the Fenton reaction can occur in plant cells in presence of ascorbate or H_2O_2, (Fry, 1998), leading to the production of hydroxyl radicals (Hallowell & Gutteridge, 1999).

1.2 The role of ion channels in ROS-induced responses and Programmed Cell Death (PCD) in plant cells.

Different mechanisms have been proposed for ROS sensing in plant cells, including two-component histidine kinases, mitogen-activated protein kinases, and certain protein phosphatases and transcription factors (Apel & Hirt, 2004; Kwak *et al.*, 2006). In addition, ion channels could play also a role in this sensing. In animal cells, ion channels play a crucial role in ROS sensing and regulatory pathways (Lahiri *et al*., 2006), but their role in plant cells is still not well understood.

The interaction between ROS and $[Ca^{2+}]_{cyt}$ in plant cells was suggested not many years ago by Bowler & Fluhr (2000). Since then, it has been shown that an early event following the elevation in H_2O_2 levels is the alteration in calcium fluxes (Rentel & Knight., 2004). The activation of plasma membrane calcium influx by ROS in plant cells has been a "hot topic" during the last decade resulting in multiple publications (Pei *et al.*, 2000; Köhler *et al.*, 2003; Demidchik *et al.*, 2003, 2007, 2010; Foreman *et al.*, 2003). This induction is, moreover, spatial and dose –dependent, and varies with the type of ROS (Demidchik *et al.*, 2003, 2007).

Cation efflux in response to ROS has been less studied. However, K^+ efflux has been reported in the most of abiotic stresses that imply ROS generation, such as copper toxicity (de Vos et al., 1992) or salinity (Demidchik *et al*., 2003, 2010; Shabala 2009). First evidence of ROS-induced channel activation of potassium efflux was reported by Demidchik *et al*. (2003), and has been analyzed with detail few years ago suggesting that, as in animal cells, hydroxyl radicals-activated K^+ channels are involved in programmed cell death in plant cells (Demidchik *et al*., 2010).

1.3 Objectives

Taking into account this potential, but still not well characterized interaction between ROS signaling and ion channel activity in plants, the objectives of this doctoral thesis were to study the influence of oxidative stress on ion transport in different models, in order to contribute to a better knowledge of the mechanisms underlying the possible role of ROS ion transport regulation. For this purpose three different models were analyzed. The specific objectives of each of these models are outlined in the corresponding chapters:

- The role of potassium channels on salinity and oxidative stress are further studied in two halophytes in chapter 2.

- The differential effect of copper ions on calcium (Ca^{2+}) and potassium (K^+) homeostasis in two zones of Arabidopsis (*Arabidopsis thaliana* L.) seedlings differing in their ability of copper transport are discussed in chapter 3.

- Chapter 4 focuses on the calcium channels annexins, and it is subdivided in two sections. In the first section, the feasible implication of *AtANN1* on copper-induced pathways is analyzed. The implication of hydroxyl radicals in the calcium-activated current through human annexin V (AnnA5) is analyzed in the second section.

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Chapter 2

Linking oxidative stress with K^+ homeostasis: A case study on halophytes.

2.1.1 The importance of K^+/Na^+ ratio in salinity tolerance.

Under non-saline conditions, the cytosol of higher-plant cells contains from 100 to 200 mM K^+ and from 1 to 10 mM Na^+, an ionic environment in which many enzymes function optimally (Taiz & Zeiger 2002). Potassium (K^+) is an essential nutrient for plant cells, being the major osmotic component and regulator of enzymes. Decrease in cytosolic K^+ will therefore lead to alterations in metabolic and physiological functions in plant cells (Marschner, 2002). Given the low sodium (Na^+) concentrations and the negative values of membrane potential in plant cells (normally more negative than -100mV), rise in extracellular [Na^+] under saline conditions would cause a favorable electrochemical gradient for Na^+ entry.

It is well known that the ability of retaining a high K^+/Na^+ ratio is fundamental for salinity tolerance (Chen *et al*., 2005; Volkov & Amtmann, 2006; Amtmann, 2009). This ratio can be controlled by different ways:

- Keeping low levels of $[Na^+]_{cyt}$, that can be done by sodium efflux couple to protons (by the *SOS1* plasma membrane Na^+/H^+ exchanger, Blumwald, 2000; Zhu 2003) or by vacuole compartmentalization by the Na^+/H^+ antiporter *NHX* (Zhang & Blumwald, 2001).

- As salinity causes a decrease in $[K^+]_{cyt}$ (Carden *et al.*, 2003, Shabala *et al.*, 2006), under saline conditions, selective transport of K^+ over Na^+ seems to be another key process in salinity tolerance. Potassium uptake systems in plants are reviewed in the section 2.1.2 ant their implication on Na^+ transport in the section 2.1.3.

- A good control of preventing K^+ loss also seems to be another key process. In fact, salinity tolerance has been correlated with preventing K^+ loss in different wheat cultivars (Cuin *et al*., 2008).

Sodium transport mechanisms have been reviewed many times (Blumwald, 2000; Horie & Schroeder, 2004; Kronzucker & Britto, 2011) but the study of these mechanisms in halophytes, a key model for understanding salt tolerance, remains poorly understood (See section 2.1.3).

2.1.2 Potassium transport in plant cells. Non-selective cation channels, a heterogeneous group of low selectivity ion channels, are involved in many fundamental cell processes such as signaling and development but also in low affinity uptake of both divalent and monovalent cations, such as K^+ (Reviewed by Demidchik *et al.*, 2002; Demidchik & Tester, 2007).

At present, well characterized K^+ uptake transport systems in plasma membrane of plant cells includes three main families:

- HAK/KUP/KT family of K^+/H^+ symporters, related to the bacterial *KUP* or fungal *HAK* transporters (Rodriguez-Navarro, 2000). Members of this family can mediate both low and high affinity K^+ transport (Reviewed by Véry & Sentenac, 2003).

- HKT family includes members implicated in high K^+ affinity transport. The first member of this family to be characterized was the wheat transporter *TaHKT2;1* (previously named *TaHKT1*). Due of its transport kinetics and pH dependence, was first thought to mediate K^+:H^+ transport with stoichiometry 1:1 (Schachtman & Schroeder, 1994). At present, is its well known that members of this family can mediate Na^+ transport, and different coupled ions have been suggested (see below, in section 2.1.2).

- Shaker-like potassium channels, inhibited by tetraethylammonium (TEA^+) and divided in three groups based on their voltage-dependency (Reviewed by Véry & Sentenac, 2003).

    - Potassium Inward-Rectifying channels (KIR) mediate potassium uptake upon membrane hyperpolarization. Examples are *AKT1, KAT1, KAT2,* and *SPIK*.

    - Weekly inward-rectifying channels Potassium channels that mediate both uptake and release depending on K^+ electrochemical gradients. An example is *AKT2*.

    - Potassium Outward-Rectifying channels (KOR) mediate potassium release upon membrane depolarization. Two examples are *GORK* and *SKOR*.

  This family is the most likely implied in salt tolerance, as some of this members have transcriptional regulation by saline stress (Reviewed by Véry & Sentenac, 2003) and members of the family have been directly related to K^+ homeostasis under saline stress (Shabala & Cuin, 2008).

2.1.3 The role of potassium transport systems and Non-Selective Cation Channels in sodium transport.

HAK/KUP/KT transporters have shown to have a extremely high selectivity for K^+ than for Na^+, and the possibility of Na^+ mediated low affinity transport by members of this family has shown contradictory results (Reviewed by Kronzucker & Britto, 2011), thus is unlikely that substantial Na^+ transport under saline conditions occurs thought these transporters.

Salinity induces nutritional disorders and, specifically, K^+ deficiency. Both *HKT1* and *HKT2* transporters are up-regulated under K^+ deprivation (Wang *et al*., 1998; Horie *et al*., 2001, 2007, 2009; Garciadeblás *et al*., 2003; Yao *et al*., 2010). HKT members have been shown to transport Na^+ in multiple species either by heterologous expression systems or by direct measurements *in planta*, including Arabidopsis, eucalyptus, barley, rice and wheat (Rubio *et al*., 1995; Gassmann *et al*., 1996; Fairbairn *et al*., 2000; Uozumi *et al*., 2000; Horie *et al*., 2001, 2009; Laurie *et al*., 2002, Mäser *et al*., 2002; Garciadeblás *et al*., 2003; Jabnoune *et al*., 2009) and the halophyte *Mesembryanthemum crystallinum* (Su *et al*., 2003). However, the role of these transporters in salt tolerance is still controversial. Rice HKT transporters (in particular OsHKT2;1) have been shown to be down-regulated by external Na^+ (Horie *et al*., 2001, 2007). In contrast, reduction of wheat *TaHKT2;1*expression has been found to be coincident with less Na^+ influx (Laurie *et al*., 2002) , and mutations of the Arabidopsis *AtHKT1;1* led to a lower Na^+ tissue accumulation (Rus *et al*., 2001). However, Mäser *et al*. (2002) found that *AtHKT1;1* T-DNA insertion mutants had identical tissue Na^+ levels, and only reduced Na^+ content in roots. From this, its role was inferred to be internal Na^+ distribution, instead of primary uptake.

AKT1 has been shown to be capable of Na^+ transport (Santa-María et al., 1997; Amtmann & Sanders, 1999; Blumwald et al., 2000; Golldack et al., 2003), but its role under saline conditions is controversial, as transcriptional levels of *OsAKT1* are inhibited by Na^+ (Fuchs *et al*., 2005) and cytosolic Na^+ concentrations of 10mM inhibit its channel conductance (Qi & Spalding, 2004). By contrast, rice leaf protoplasts of a salinity sensitive cultivar showed a reduction in Na^+ content when K^+ channel blockers were applied (Kader & Lindberg, 2005), although these blockers had no effect on protoplasts of a salt tolerant cultivar. More recently, Nieves-Cordones *et al.* (2010) reported no differences on Na^+ tissue content in *Arabidopsis thaliana* between wild type and *akt1-2* mutant. These authors also suggested a role of *AtAKT1-2* transporter in K^+ efflux under saline conditions. (See section 2.1.4).

It is widely accepted that Na^+ influx under saline conditions is mainly driven by Non-Selective Cation Channels (NSCCs) (Reviewed by Amtmann & Beilby, 2010). Na^+ influx through these channels was characterized in Arabidopsis root protoplasts by Demidchik & Tester (2002), and revealed tetraethylammonium (TEA^+, a known channel blocker) and voltage insensitivity and Ca^{2+} sensitivity. Indeed, external Ca^{2+} has been shown to ameliorate salinity stress in many species (Cramer *et al*., 1987; Rengel, 1992). Different hypothesis have been suggested, including Ca^{2+} blockage of Na^+ uptake through NSCC (Demidchik & Tester 2002), prevention of K^+ leak from the cells (Shabala *et al*., 2003, 2005) and an enhanced Na^+ extrusion via SOS1 (Liu and Zhu 1998).

Wang *et al.* (2007), based on pharmacological studies, tissue Na^+ content and $^{22}Na^+$ influx measurements, showed that Na^+ entry in roots of the halophyte *Suaeda maritima* occurs though two pathways: one operating under low salinities (25mM), sensitive to Ba^{2+}, and the other under high salinities (150mM), sensitive to TEA^+. These authors suggested that HKT and AKT-like transport pathways might play a role in low and high salinities concentrations respectively As AKT channels are activated under membrane hyperpolarization, this would provide a good K^+/ Na^+ ratio by preventing K^+ leak through outward-rectifying channels and enhancing K^+ uptake through inward-rectifying channels (See section 2.1.2) Although electrophysiological evidence must be provided to identify channel identities, this suggests that different mechanisms could play a role in Na^+ transport in halophytes. Unfortunately, these mechanisms are still far away to be well understood. Two halophytes species studied in this chapter are further introduced in the section 2.1.6.

2.1.4 Potassium loss under saline conditions.

The importance of potassium leakage prevention under saline conditions has been shown in different species and is reviewed with detail by Shabala & Cuin, (2008). Briefly, GORK (KOR-type channel) and AKT (KIR-type channel) have been suggested to be the principal targets of salinity stress. GORK would mediate K^+ leak upon salinity depolarization, as the Arabidopsis *gork* mutant shows a more attenuated response to salinity addition compared to the wild type. Activation of H^+ATPase in salt tolerant species would repolarize membrane potential allowing K^+ entry into the cell through AKT channels, as hyperosmotic manitol treatment caused similar K^+ uptake in WT and *gork* roots, but had no impact on K^+ flux in *akt1* roots.

2.1.5 Link between salinity and oxidative stress.

Under saline conditions, the balance between ROS production and scavenging is broken, causing a rapid increase in ROS level (Apostol *et al*., 1989; Mittler, 2002; Apel & Hirt, 2004). Because of this, it has been suggested that ROS-scavenging activity is an important component of salinity tolerance (Zhu, 2003).

In animal cells, K^+/Na^+ ratio (fundamental in plant salinity tolerance) is the main parameter in PCD regulation (Bortner *et al*., 1997; Orlov *et al*., 1999).

K^+ efflux has been related to induce oxidative burst in Arabidopsis roots (Demidchik *et al*., 2003; Shabala *et al*., 2006) and hydroxyl radicals (HR) have been shown to activate specifically a population of non-selective K^+-efflux channels (encoded by the *GORK* gene) that are involved in activation of PCD nucleases and proteases (Demidchik *et al*, 2010). ROS-activated K^+ channels have been previously described in many animal systems (Kourie, 1998) and ROS-stimulated K^+ efflux has been observed in root cells of different plants (Demidchik *et al*., 2003, 2007, 2010; Shabala *et al*., 2006; Cuin & Shabala, 2007b). NaCl can stimulate HR production and this might lead to K^+ channel activation (Demidchik *et al*., 2010).

Accumulation of organic solutes might be necessary for balancing the osmotic potential of salt ions. Exogenous application of organic osmolytes (namely compatible solutes) has been shown to ameliorate Na^+ toxicity in barley roots (Cuin & Shabala, 2005, 2007b) and pea leaves (Shabala *et al.* 2007), and also mitigates K^+-induced efflux under saline and ROS stresses (Cuin & Shabala, 2005, 2007a,b). However, the effect of different polyamines has been shown to differ in different root zones, causing a reduction of K^+ leakage under saline stress only at the mature zone level of maize and Arabidopsis roots, but even increase it at the elongation zone level (Pandolfi *et al.,* 2010). Recently, Zepeda-Jazo *et al.*, (2011) have shown that some of these polyamines can induce HR-activated K^+ conductance an also have a differential effect on Ca^{2+} homeostasis: low concentrations stimulate Ca^{2+} pump (efflux) while higher concentrations stimulates net influx. The mitigating role of these solutes on salt toxicity must be then analyzed carefully in the different plant species.

2.1.6 Halophytes: A model for a better understanding of ionic mechanisms underlying salt tolerance.

Halophytes and glycophytes can use Na^+ and Cl^- ions compartmentalized in vacuoles as osmotic components (Blumwald *et al*., 2000).

The plant family of Chenopodiaceae is worldwide distributed specially in arid and saline environments. It contains 104 genera and more than 1400 species. In this family, Na^+ and Cl^- contribute up to 67% of total solute content (Albert *et al.,* 2000). Two of the members of this family are Quinoa and Atriplex.

Quinoa (*Chenopodium quinoa* Willd.) is a seed crop that has been cultivated in the Andean region for thousands of years (Jacobsen *et al*., 2003) due its high protein, mineral and vitamin content. This crop has been shown a remarkable tolerance to abiotic stress, such us frost, salinity and drought (Bosque Sanchez *et al.*, 2003; Jacobsen *et al.*, 2003, 2009; Hariadi *et al.*, 2011), as is able to form salt excreting structures (bladders) on leaf surfaces, which are important plant water retention mechanism (Bosque Sanchez *et al.*,2003). Optimal growth is achieved in plants grown in soil irrigated with NaCl solutions from 100 to 200 mM (Hariadi *et al*., 2011). Shoot sap K^+ progressively increased with saline treatments, showing the importance of K^+ retain under saline conditions (Hariadi *et al*., 2011).

Atriplex (*Atriplex lentiformis* (Torr.) S. Wats) has a valuable flavonoid content with antioxidant activity (Awaad *et al*., 2012). Although the main focus of previous studies derive from its ability to grow in dry areas of USA, Mexico and Australia, it can also grow under saline environments. In fact, *Atriplex* spp. was characterized by its high content of sodium chloride (Watt *et al*., 1962), but only few recent studies have focused on salt tolerance in this specie (Malcom *et al*., 2003; Jordan *et al.*, 2009; Soliz *et al.*, 2011) and it has been reported its ability to maintain higher K^+ content under saline conditions (Malcom *et al*., 2003).

Hypothesis tested.

As halophytes show a good control of both osmotic and toxic effects of Na^+, and therefore, prevent NaCl-induced ROS formation and their lethal effect on plant cells, and prevention of K^+ leakage has been suggested to be a key mechanism in both plant salt and oxidative stress tolerance, they may also have a better resistance under oxidative conditions by controlling K^+ loss. A range of techniques including growing experiments, ion flux estimation, viability assays, ion content determination and osmolality measurements were applied to test this hypothesis.

2.2 Material and methods

Plant material and growing conditions

Quinoa (*Chenopodium quinoa* Willd.) genotype 20 and Atriplex (*Atriplex lentiformis* L.) seeds were surface-sterilized with 1% commercial bleach for 15 min and washed three times with distilled water.

For root ion flux estimations, seeds were surface-sterilized with commercial bleach. Afterwards, seeds were placed on floating polypropylene nets in sterilized chambers wrapped with aluminum foil. The chambers contained Basal Salt Medium (BSM) with, in mM: 0.2 KCl and 0.1 $CaCl_2$, pH 5.7 adjusted with KOH, with continuous aeration. Germination took place in darkness (1 day for Quinoa and 10 days for Atriplex) and the seedlings were grown under a 16/8 h photoperiod for 4 more days after germination.

For growing experiments, seeds were germinated in BSM plates jellified with 1.2% phytagel (Duchefa, Netherlands) with the same light conditions as above. Uniform seedlings were then transferred to 1.2% phytagel containing plates with the indicated amounts of NaCl, $CuSO_4$, H_2O_2 and DMSO, and grown at 27ºC under a 16/8h photoperiod (light/darkness) and 100 μmol m^{-2} s^{-1} PAR irradiance.

Quinoa adult plants for K^+ and Na^+ determinations and sap osmolality measurements were grown under glasshouse-controlled conditions and irrigated with increasing NaCl solutions (50, 100, 150, 200 and 300 mM) 7 weeks more.

Ion flux estimation

4-day-old roots were placed on Petri dishes and softly fixed with silicone tubs. Measuring chambers contained BSM at the same concentrations used for germination (see above), unbuffered for NaCl and H_2O_2 additions and buffered with TRIS/MES pH 5.6 for Cu/asc addition. Roots were left under these conditions at least 40 minutes prior to measurements.

Net K^+ and H^+ fluxes were measured non-invasively using a microelectrode ion flux measuring (MIFE) technique (UTas Innovation Ltd, Hobart, Australia). Specific details on microelectrode fabrication and calibration are given in Shabala S & Shabala L (2002), and the principles of the MIFE measurements are comprehensively described in Shabala, 2006. Electrodes were equilibrated in an appropriate set of standards (0.2–1 mM for K+ and Na^+; pH 5.3–7.8 for H^+). Electrodes with a response of <50 mV per decade and correlation <0.999 were discarded.

Microelectrodes were mounted on a multimanipulator providing three-dimensional positioning. Net ion fluxes were measured in roots at the elongation (300μm from the root tip) and mature (10 mm from the tip) zones. Electrodes were positioned 30 μm above the surface. During

measurement, electrodes were moved in a square-wave manner between 50 μm at a frequency of 0.1 Hz by a computerized stepper motor as described in Shabala, 2006.

Flame photometry

Young leaves (2nd and 3rd floors from the tip) from Quinoa plants grown at different salinity levels for 7 weeks were frozen at -18ºC. Close to the time to the measurements, samples were thawed and hand squeeze to extract the sap as described in Cuin *et al.* (2009). Samples were diluted by a factor of 100 and its K^+ and Na^+ contents were determined by flame photometry (Corning 410C, Essex, UK).

Sap osmolality

An undiluted aliquot of the samples described above was used for osmolality measurement ussing a vapour pressure osmometer (Vapro; Wescor Inc., Logan, UT, USA)

Stainings

Fresh roots tips were harvested after the different times of salt/oxidative treatments (10', 1h and 4h), washed with phosphate buffer and double-stained using fluorescein diacetate (FDA) and propidium iodide (PI), according to Koyama *et al*. (1994), modified from Jones & Senft (1985). Intact cells exhibit green fluorescence due to FDA, and PI provides red fluorescence of nuclei in damaged cells, due the low penetrability in intact membranes (Hamilton *et al.*, 1980).

For the Evans blue assay, root tips were stained with an aqueous Evans blue solution for 15 min, and then washed 3 times with distilled water for 10 min each, according Baker & Mock (1994).

2.3 Results

Elongation zone shows higher sensitivity to both saline and oxidative stress. Atriplex has higher sensitivity to HR.

As prevention of K^+ leakage under saline conditions is a crucial in salinity tolerance, and saline stress have been shown to stimulate HR production, the effect of 100mM NaCl and 1mM copper/ascorbate (hydroxyl radical generating mixture, Halliwell & Gutteridge, 1999) addition on K^+ efflux was analyzed in Quinoa and Atriplex seedlings at two root levels: mature and elongation zone. NaCl and hydroxyl radical (HR)-induced K+ efflux was higher at the elongation zone level in both species (Fig. 2.3.1), and considerably higher with HR-generation (2-fold at the mature zone and from 2 to 5-fold at the elongation zone when compared to salt addition).

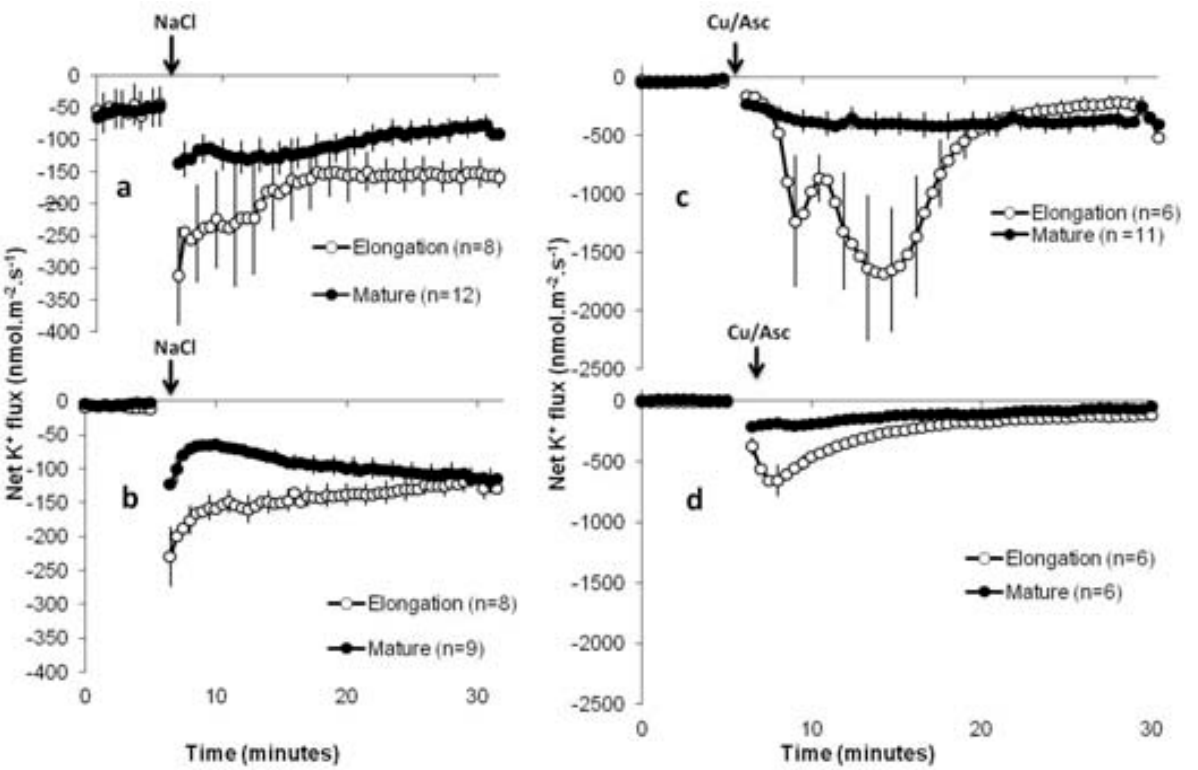


Figure 2.3.1 K^+ efflux in response to saline and oxidative stress. Atriplex (a.c) and Quinoa (b,d) K+ net efflux following to 100mM NaCl (a,b) or 1mM Cu/asc (c,d) addition (indicated by arrows) at mature and elongation zone levels. Mean±SE is shown. For simplifying the figure, SE is shown every 3 points. n =6-12 seedlings.

H_2O_2 stimulates a dose-dependent K^+ efflux in Atriplex roots, highly sensitive at the elongation zone level.

The effect of different hydrogen peroxide (H_2O_2) concentrations on K+ flux was analyzed in Atriplex seedlings (Fig. 2.3.2). Low (1-10 μM) H_2O_2 evoked a net K^+ efflux at the elongation zone levels, while higher concentrations (1-10mM[1]) were necessary for a response at the mature zone level (Figure 2.3.2).

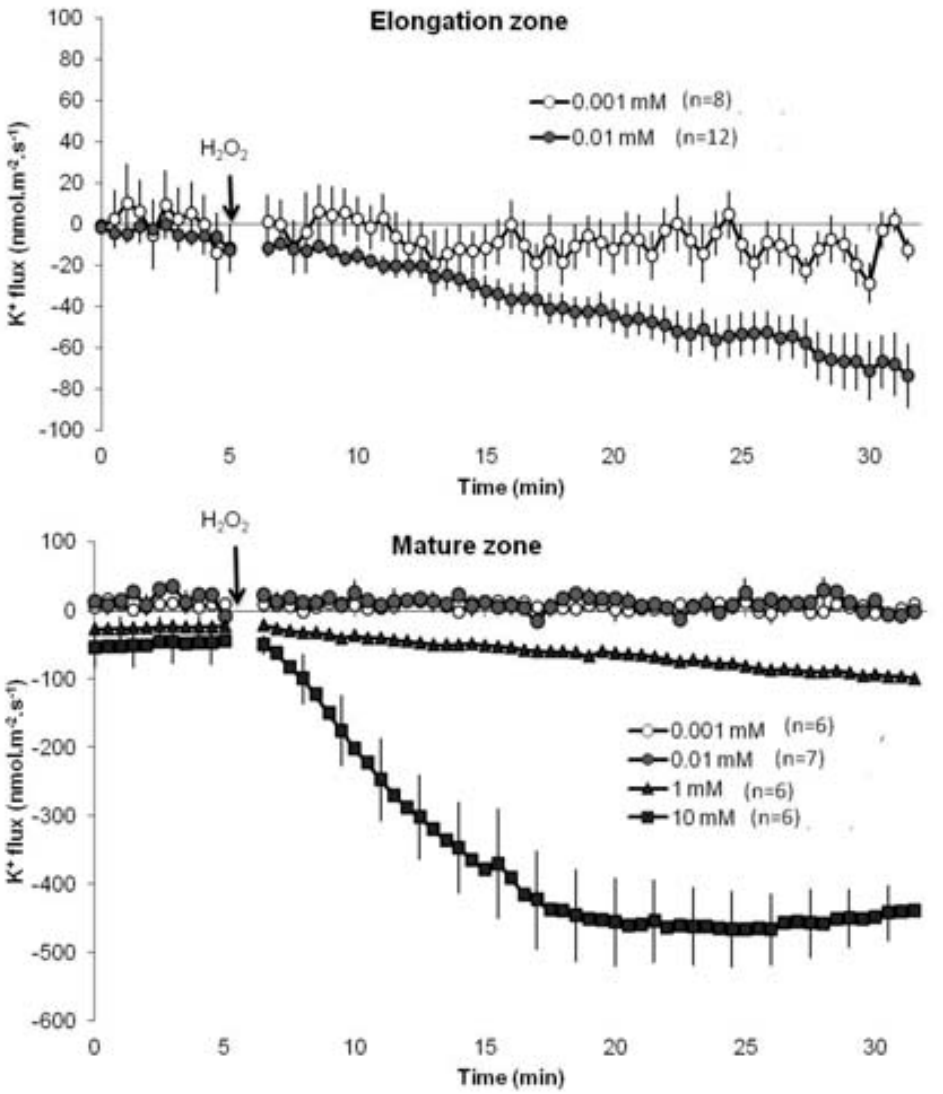


Figure 2.3.2 K^+ efflux in response to H_2O_2 in Atriplex roots. K^+ net flux at the elongation (up panel) and mature (lower panel) zones of Atriplex roots upon H_2O_2 addition (indicated by arrows). Mean±SE is shown. For simplifying the figure, SE is shown every 3 points. n =6-12 seedlings.

[1] High H_2O_2 concentrations (1 and 10mM) results were obtained by S. Shabala & J. Bose, in collaboration with the present study

Low H_2O_2 stimulates K^+ efflux at the elongation zone of Quinoa roots, and inhibits root growth.

Exogenous application of 10 μM H_2O_2 also evoked K^+ efflux at the elongation zone of Quinoa seedlings (Figure 2.3.3a). H_2O_2 also caused inhibition of root growth in a dose dependent manner. Interestingly, seedlings showed inhibition of root growth from 10μM, the same level of H_2O_2 that had K^+ efflux response at the elongation zone level. (Fig. 2.3.3b)

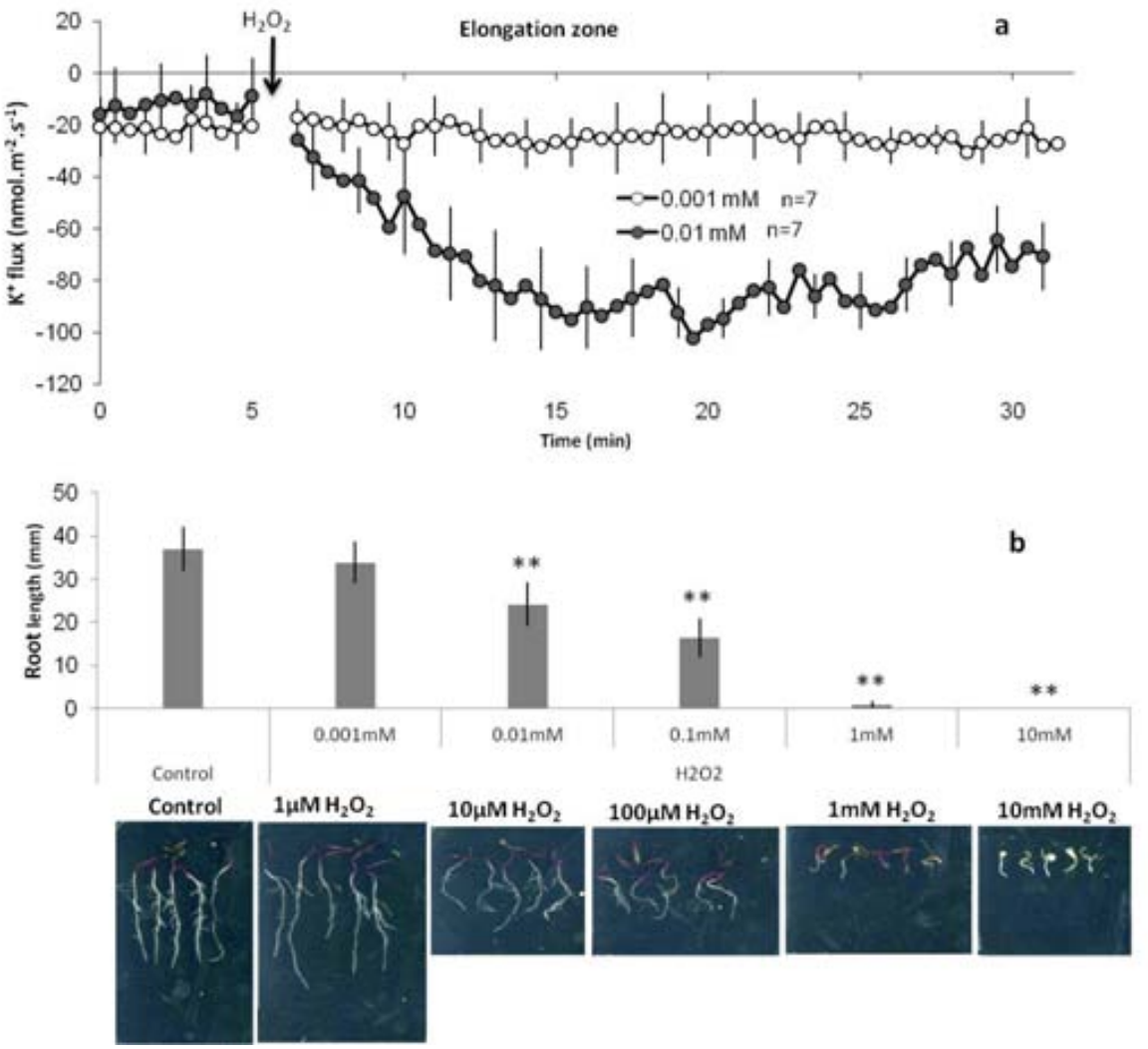


Figure 2.3.3 Quinoa responses to H_2O_2 (a) Effect of low H_2O_2 addition at the elongation zone level of Quinoa roots **(b)** (Up) Average of relative root length for 5-day old quinoa seedlings transferred from BSM medium with 1.2% phytagel to plates with the indicated amounts of salt, copper or DMSO and grown for 4 days. Mean±SD is shown. (n=5) Asterisks indicate statistically significant differences with respect to the control, ANOVA test **(P <0.01). (Down) Phenotype of the seedlings grown for 1 week on plates with the indicated amounts of H_2O_2.

Salt addition stimulates net H^+ efflux while H_2O_2 causes decrease in net H^+ efflux in a dose-dependent manner at mature zone levels of Quinoa roots[2].

The effect of NaCl and high concentrations of H_2O_2 (1 and 10mM) on H^+ net flux was tested at the mature zone level of Quinoa roots. NaCl caused initial net H^+ efflux, which was maintained after 1h of salt treatment (Fig. 2.3.4). H_2O_2 caused initially net H^+ influx in a dose-dependent manner. While in treatments with 1mM H_2O_2 the final value after 1h was similar to the NaCl treatment, treatment with 10mM caused a sustained net H^+ influx.

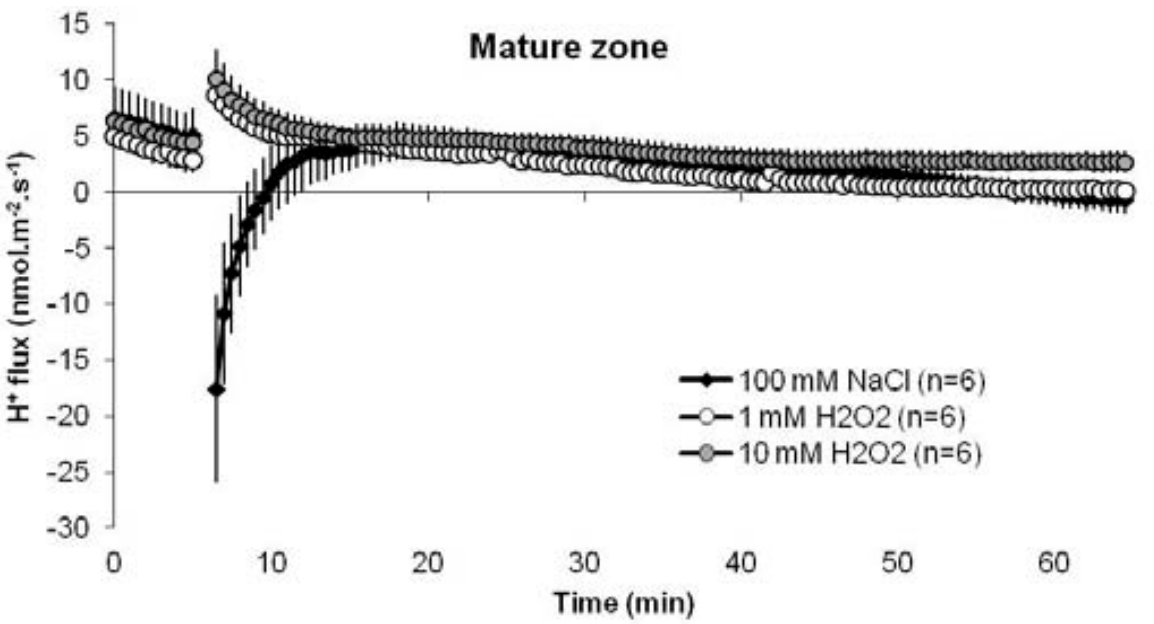


Figure 2.3.4 H^+ efflux in response to NaCl and H_2O_2 in Quinoa roots. K^+ net flux at mature (panel) zone level upon NaCl or H_2O_2 addition (indicated by arrows). Mean±SE is shown. For simplifying the figure, SE is shown every 3 points. n =6-12 seedlings.

[2] Results were obtained by S. Shabala & J. Bose, in collaboration with the present study

Oxidative stress causes rapid membrane disruptions in both species while salinity does not affect to membrane integrity in medium term expositions.

After different times of saline and oxidative treatments, root tips were excised and washed and membrane permeability disruptions were analyzed by the double staining with fluorescein diacetate and propidium iodide (Fig. 2.3.5 and 2.3.6). Both species resulted saline-tolerant in medium term expositions (24h), but oxidative sensitive. Only ten minutes after HR stress addition, disruptions on membrane integrity were detected in both species, causing high damage after one hour. Similar results than with HR addition were found for H_2O_2 stress in Atriplex roots (Fig. 2.3.5), although Quinoa showed greater tolerance to H_2O_2 stress (Fig. 2.3.6). In Atriplex, both 1 and 10mM H_2O_2 caused permebility disruptions after 1h, while in Quinoa the lower treatment with 1mM did not cause permeability disruptions until 4h. Saline treatment did not cause any permeability disruptions in the first 4 hours at any of the halophytes but some membrane disruptions were detected after 24h of saline treatment in Quinoa, absent in Atriplex roots.

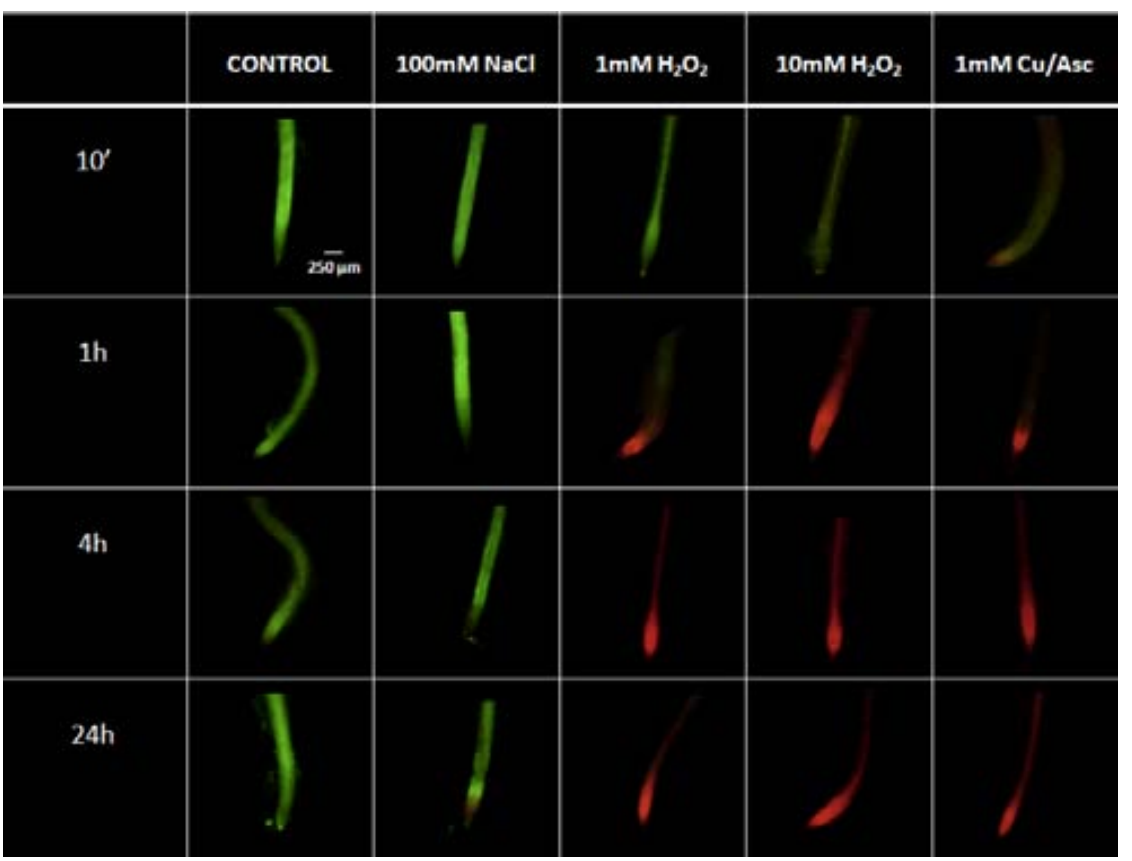


Figure 2.3.5 Vital staining of Atriplex roots after different times of saline and oxidative treatments. A representative photograph for each treatment is shown. n =6 seedlings.

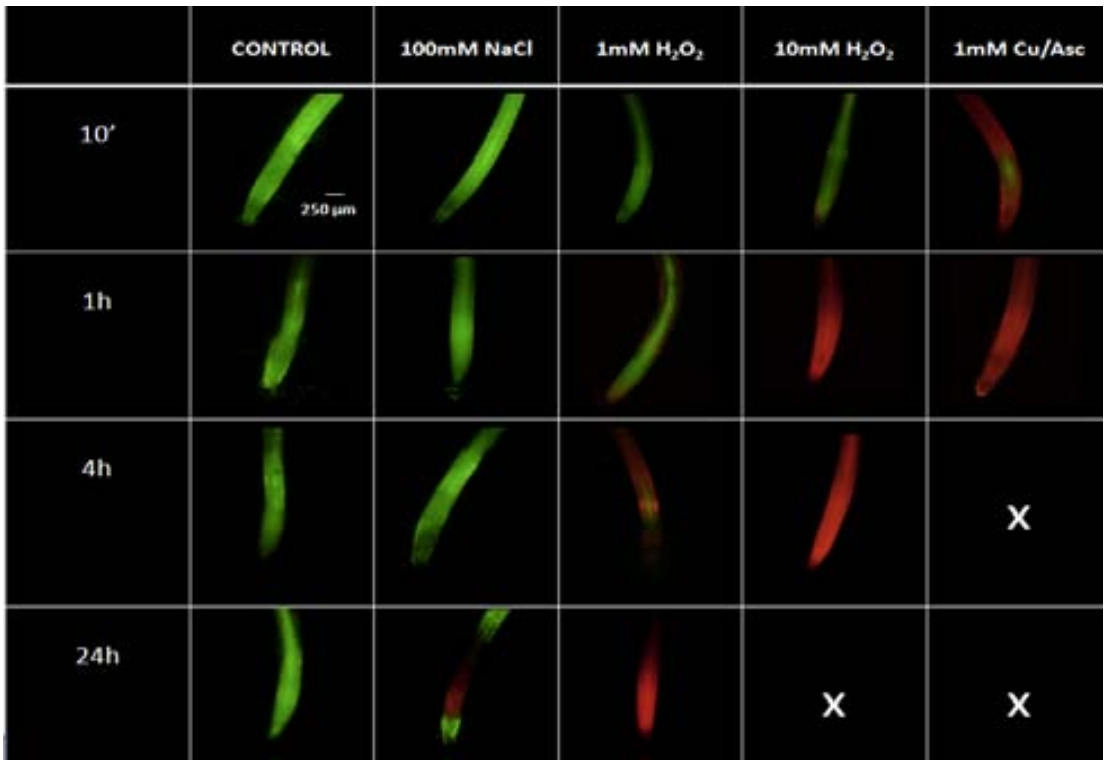


Figure 2.3.6 Vital staining of Quinoa roots after different times of saline and oxidative treatments. A representative photograph for each treatment is shown. n =6 seedlings.

Evans blue assay for cell viability revealed that damaged cells in root tips of 24h plants exposed to salt were minly restricted to the epidermical layers (Fig. 2.3.7).

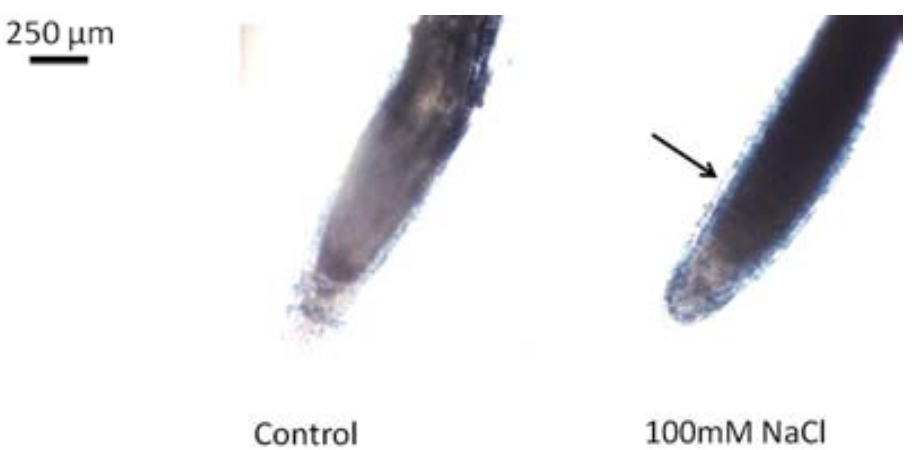


Figure 2.3.7 Evans blue staining of Quinoa roots after 24h of saline treatment. A representative is shown. n =6. Arrow shows the localization of non-viable cells.

Medium and long term saline treatments effect.

Analysis of K^+ steady state net flux after 48h under saline conditions revealed that the K^+ efflux detected in short term additions in Quinoa and Atriplex roots can be ameliorated and be reverted to values close to zero (Fig. 2.3.8).

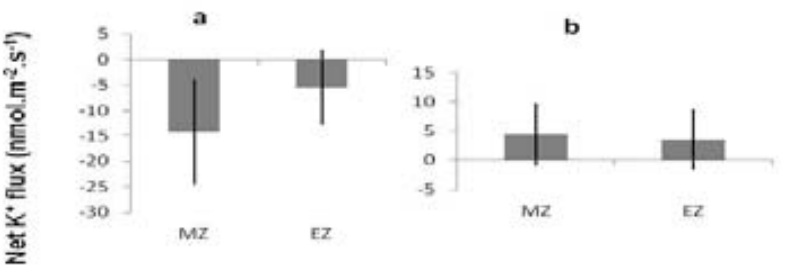
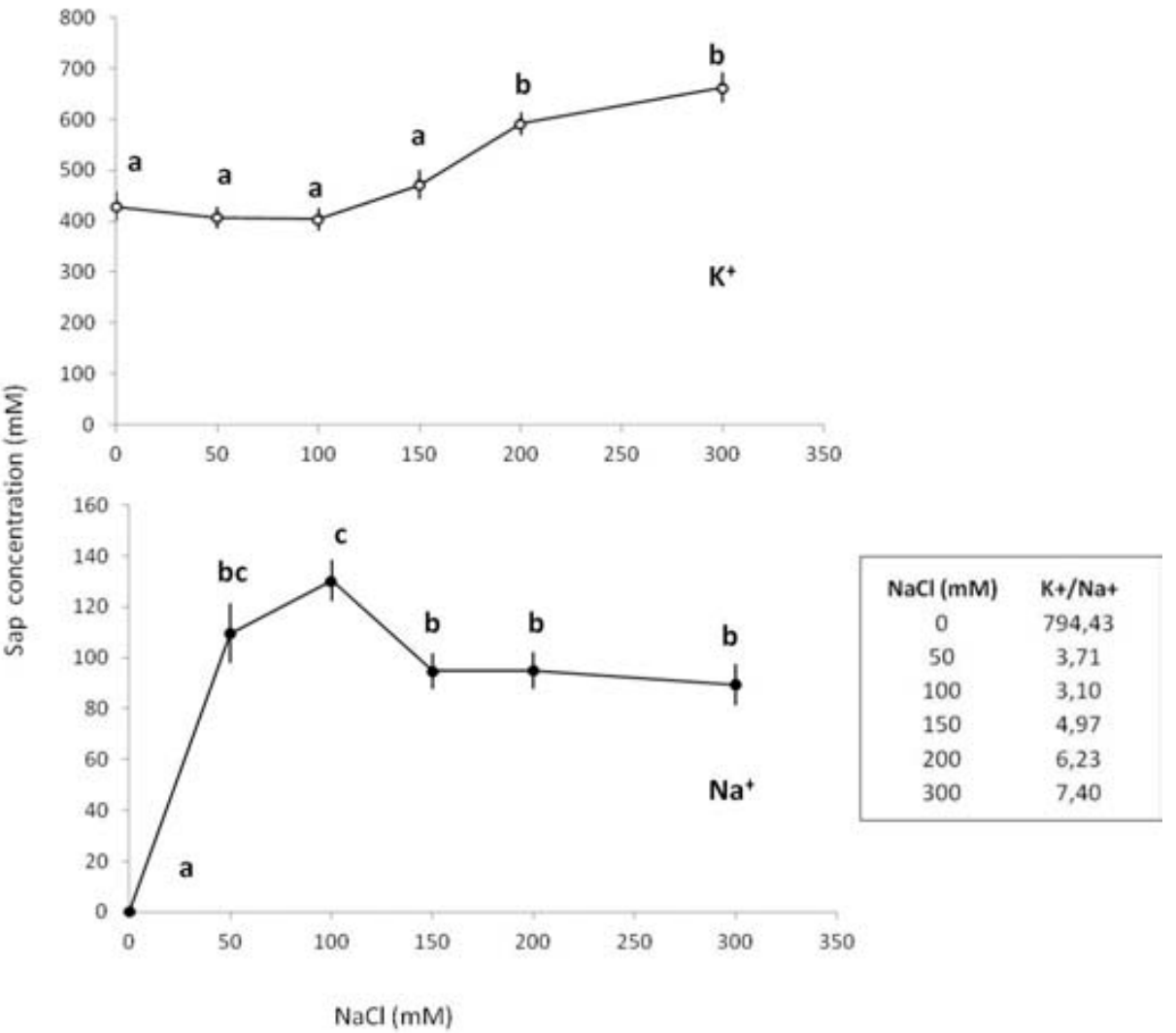


Figure 2.3.8 Atriplex (a) and Quinoa (b) steady state K+ flux at both mature and elongation zones after 48h of saline treatment with 100mM NaCl (n=4-9)

The effect of long term expositions to saline treatments was tested in Quinoa plants grown for several weeks in glasshouse controlled conditions and irrigation with different concentrations of NaCl (from 0 to 300mM, in steps of 50mM). Na^+ and K^+ contents of plants irrigated 7 weeks with different saline solutions were analyzed by flame photometry only in young leaves, as old leaves began to present senescence symptoms (Fig. 2.3.9). As expected, all salinity treatments caused Na+ accumulation in Quinoa leaves. Na^+ content showed a maximum at 100mM NaCl, while higher salt treatments caused a decrease in the maximum Na^+ content and a higher K^+ accumulation. Analysis of total osmolality in young leaves (n=10) and the total contribution of Na^+ and K^+ are shown in table 2.I.

Table 2.I Relative contribution of sodium and potassium ions to the total osmolality in Quinoa salinized leaves.

Treatment (NaCl)	K^++Na^+ (mOsm)	Osmolality (mOsm)	% Measured
Control (0)	429.5±40.6	565.8±65.0	75,9
50 mM	516.3±47.9	815.4±90.3	63,3
100 mM	534.2±42.6	916.5±50.2	58,3
150 mM	566.3±51.2	947.2±36.4	59,8
200 mM	686.8±42.1	1013.7±56.0	67,8
300 mM	751.8±53.01	1057±22.8	71,1



NaCl (mM)	K+/Na+
0	794,43
50	3,71
100	3,10
150	4,97
200	6,23
300	7,40

Figure 2.3.9 Flame photometry determination of K^+ (top panel) and Na^+ (bottom panel) content in young leaves of 7 weeks grown Quinoa plants irrigated with different salt concentrations. Mean±SE is shown. (n=6) Different letters mean statistical differences between treatments (P<0.01), ANOVA test. Inset shows K^+ to Na^+ ratio in the different treatments

2.4 Discussion

As previously mentioned, Na^+ ions enter the cell though NSCC (Maathuis & Amtmann, 1999; Tyerman & Skerret, 1999) Following Na^+ entry, it is expected a consequent depolarization of the cells, as has been shown in salinized barley seedlings (Shabala *et al*., 2007), and Arabidopsis leaves and roots (Shabala *et al*., 2006; Demidchik *et al*., 2010). In addition, NaCl-induced plasma membrane depolarization will cause activation of depolarization- activated Ca^{2+} channels (DACC), leading to an increase in cytosolic free Ca^{2+} and a consequent stimulation of NADPH oxidase and forward ROS generation in a positive feedback (Demidchik & Maathuis, 2007).

The initial K^+ loss under saline treatment (Fig 2.3.1) however, did not cause membrane permeability disruptions in any of both species (Figs. 2.3.5 to 2.3.7), and would be understood as a mechanism for ameliorating Na^+ induced depolarization. Na^+ will be transported to shoots for osmotic adjustment (Fig. 2.3.9, Table 2.I). Both halophytes can revert K^+ efflux to values close to zero in few days (Fig. 2.3.8), and even increase sap K^+ content in longer treatments (Fig. 2.3.9). Contribution of both ions (Na^+ and K^+) to the total osmolality in Quinoa (table 2.I) was similar between control and salinized plants. Last, Quinoa roots are able to increase K^+/Na^+ ratio under increasing saline treatments (see inset in Fig. 2.3.9). Overall, these results show the importance of retaining K^+ in salt tolerance.

First studies on ROS modulation of ion channels in Arabidopsis roots failed to show H_2O_2-induced K^+ conductance (Demidchik *et al*., 2003). In 2007, Demidchik *et al.* characterized the H_2O_2 dose-dependency on Ca^{2+} fluxes in Arabidopsis roots: H_2O_2 can stimulate Ca^{2+} inward current through hyperpolarized-activated channels (HACCs), but is still unknown if the effect of H_2O_2 on this channels occurs at the intracellular or intracellular side. Several studies have been shown H_2O_2 to activate K^+ fluxes, but no studies have been shown K^+ dose-dependency at such low H_2O_2 concentrations. 10μM of H_2O_2 is enough to stimulate K^+ efflux at the elongation zone in both species (Fig. 2.3.2 and 2.3.3), and inhibits root growth in Quinoa seedlings (Fig. 2.3.3). Higher H_2O_2 expositions caused K^+ leakage in a dose-dependent manner in Atriplex roots (Fig. 2.3.2) and cell permeability disruptions in both species, being quicker in Atriplex (Figs. 2.3.5 and 2.3.6). At present, no K^+ channel population has been characterized for the H_2O_2 effect on roots, and it has been suggested that the effect is caused by its further conversion to hydroxyl radicals by cell wall transition metals. In addition to the higher sensitivity to H_2O_2 detected in Atriplex, HR induced K^+ efflux at the elongation zone was also markedly higher in this specie (Fig. 2.3.1). HR caused quick cell permeability disruptions in both species (Figs. 2.3.5 and 2.3.6), suggesting that both halophytes and specially Atriplex, are oxidative-stress sensitive.

Differential activation of the plasma membrane H+-ATPase might underlie on the differential sensitivity to salt and oxidative stresses in halophytes.

Sustained depolarization is probably the best marker of cell damage and death (Bortner *et al*., 2001). Indeed, ameliorative Ca^{2+} effect on NaCl toxicity has been attributed to a less dramatic depolarization in Arabidopsis roots (Shabala *et al*., 2006).

Oxidative stress has been shown to cause membrane depolarization in different studies. Exogenous applied hydroxyl radicals (HR) cause depolarization in barley (Cuin & Shabala 2007) and Arabidopsis (Demidchik *et al*., 2010). And this effect is irreversible after 30-45min exposure in Arabidopsis thaliana root protoplasts (Demidchik *et al*., 2010). This explains the quick cell permeability disruptions in both species assessed by vital staining in both species under HR treatment (Figs. 2.3.5 and 2.3.6). According to this, exogenous application of 1/10mM H_2O_2 induces net H^+ influx (otherwise, depolarization) in roots of Quinoa (Fig. 2.3.4).

In contrast, previous work on the halophyte *Chenopodium quinoa* showed an increasing H^+ net efflux under increasing salinity treatments (Hariadi *et al*., 2011). Exogenous application of 100mM NaCl also induced net H^+ efflux (hyperpolarization) in roots of the present genotype of Quinoa (Fig. 2.3.4). NaCl-induced increase in plasma membrane H^+-ATPase activity has been reported in many halophytic species (Yao *et al*., 1992; Vera-Estrella *et al*., 1999; Beilby & Shepherd, 2001; Sibole *et al*., 2005) as it energizes SOS1 H^+/Na^+ antiport. Maughan *et al*. (2009) cloned two *SOS1* loci homologues in Quinoa, but they have not been characterized at present.

The activation of the H^+-ATPase pump might, in addition with the initial K^+ efflux, be mechanisms to ameliorate Na^+ depolarization, while Na^+ ions would be transported to the leaves and used for osmotic adjustment (table 2.I, Fig.2.3.9).

Model proposed under salt and oxidative stress in halophytes.

Under saline treatments (black lines in Fig. 2.4.1), Na^+ ions (1) will enter the cell through NSCC. The depolarization caused by its entry will be ameliorated by three ways (2): Activating PM H^+-ATPase, by K^+ loss through GORK-like channels (Reviewed by Shabala & Cuin, 2008) and last, by long transport of Na^+ to the leaves (3) for osmotic adjustment. (4) Decrease in $[Na^+]_{cyt}$ and K^+ influx probably mediated by AKT-like channels (Shabala & Cuin, 2008) will keep a (5) high K^+/Na^+ ratio.

Under oxidative conditions (grey lines in Fig 2.4.1), HR and H_2O_2 conversion to HR by cell wall transition metals (Fry, 1998) (1) will activate a Ca^{2+} entry through NSCCs (Demidchik *et al*., 2003) and K^+ leakage through GORK-like channels (Demidchik *et al*., 2010). (2) $[Ca^{2+}]_{cyt}$ increase will activate ROS production by activation of the NADPHoxidase in a positive feedback (Foreman *et*

al., 2003). (3) Decrease in $[K^+]_{cyt}$ will (4) activate PCD caspases and nucleases and (5) initiate PCD (Shabala 2009; Demidchik *et al.*,2010).

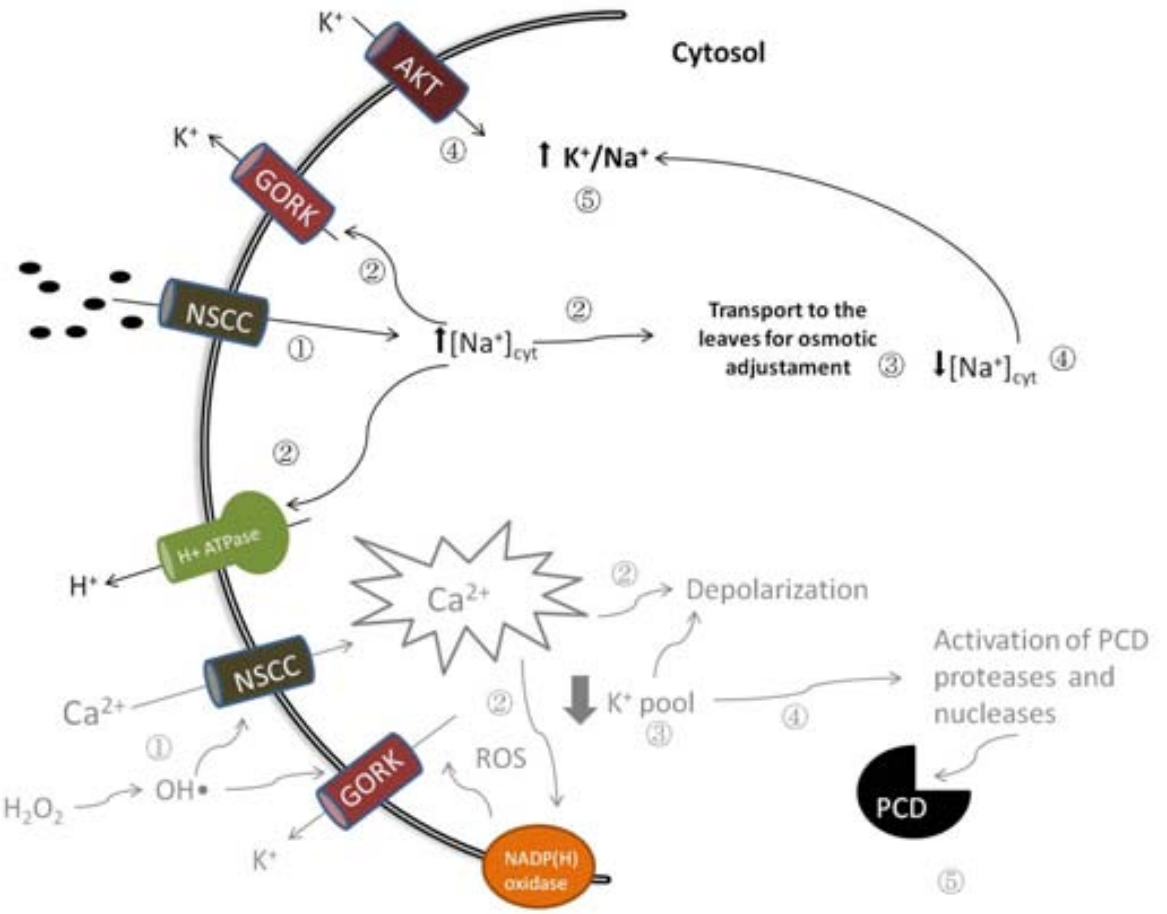


Figure 2.4.1 Tentative model proposed in halophytes under salt (black lines) and oxidative (grey lines) stresses. See text for explanations. Abbreviations used: NSCC: Non-Selective Cation Channel; H^+-ATPase: Plasma membrane proton pump; NADP(H) oxidase (Nicotinamide Adenine Dinucleotide Phosphate-Oxidase); GORK: gated outwardly-rectifying K^+ channel; AKT: Potassium transporter; PCD: Programmed Cell Death

Conclusions and future work

In this study, a good control of membrane depolarization and K^+ loss by repolarization of the V_m by the plasma membrane H^+-ATPase is the most likely mechanism underlying salinity tolerance. This effect is likely to be salinity-mediated, as depolarization on ROS-treated roots lead to depolarization, K^+ loss and activation of nucleases and proteases that will initiate PCD. K^+ loss, previously correlated with salinity tolerance, must be understood in a different manner in the case of halophytes. In halophytes, K^+ leak helps to keep V_m at hyperpolarized values, preventing the effect of sustained membrane depolarization: Ca^{2+} increase that would activate further ROS formation by the plasma membrane NADPH oxidase, and finally will lead to PCD.

Salinity and ROS stresses, in conclusion, seem to activate differential ionic mechanisms in halophytes.

Further research will include membrane potential measurements under both oxidative and saline conditions, K^+ transport identity by pharmacological experiments, Ca^{2+} responses and specific H^+-ATPase regulation under saline conditions.

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Chapter 3

Copper-induced oxidative stress in K^+ and Ca^{2+} homeostasis.

3.1.1 Copper uptake mechanisms in roots.

Copper (Cu) is an essential micronutrient in higher plants. Copper participates as a catalytic cofactor in many fundamental processes such as electron transport, hormone signaling, cell wall metabolism, pollen formation and fertilization, nodulation and nitrogen fixation, and carbohydrate metabolism (Marschner, 2005). In addition, copper is present as a metal component of the prosthetic group in superoxide dismutases (CuZnSOD) and, thus, plays a central role in detoxification of the superoxide anion free radicals (Shabala, 2009a). On the other hand, being a transient (and, hence, highly reactive) metal Cu ions can lead to the generation of harmful reactive oxygen species (ROS) via Fenton and Haber-Weiss reactions thus causing oxidative damage to cells (Halliwell & Gutteridge, 1984). Copper toxicity is well-reported and, despite some Cu-tolerant species may accumulate as many as 1000 μg g^{-1} DW of copper, leaf concentrations above 30 μg g^{-1} DW are considered to be toxic for most crop species (Marschner, 2005). This duality of copper has resulted in the development of a complex homeostatic network for copper acquisition and use in aerobic organisms.

While major focus of previous studies were on copper metabolism and its role in photosynthesis (see above), little is known about fundamental mechanisms underlying copper transport in plant root cells. The CRT family of trimeric transporters, called COPT1 in plants, mediates copper transport towards the cytosol in eukaryotic cells (Kampfenkel *et al.*, 1995). It was suggested that copper transport may require the ion reduction from Cu^{2+} to Cu^+ by a reductase (Sancenón *et al.*, 2004, Garcia-Molina *et al*., 2011). Cu^+ is then transferred towards the cytosol in a process that requires conserved sequence motifs (reviewed by Peñarrubia *et al*., 2010). Sancenón *et al*. (2003) identified six members of the family COPT of high affinity copper transporters in Arabidopsis (COPT1-6). Some members of the COPT1 family are induced under copper deficiency via interaction with the SPL7 transcription factor (reviewed by Peñarrubia *et al.*, 2010), and the expression of COPT1 and COPT2 is downregulated by Cu (Sancenón *et al.*, 2003). Four of the members of the COPT family can complement yeast *crt1/crt3* defective growth (COPT 1-3 and COPT5), but only two of them have been characterized in detail. COPT5 it is located at the tonoplast and it has been shown to mediate copper transport under severe copper deficiency (Garcia-Molina *et al*., 2011).

The first member of the COPT family that was functionally characterized was COPT1. This COPT member is expressed at the plasma membrane level in root tips under copper deficiency and is responsible of high affinity copper uptake in Arabidopsis (Sancenón *et al*., 2004, Andrés-Colas *et al*., 2010). COPT1 antisense plants (C1AS) have reduced copper uptake and show defective root elongation (Sancenón *et al.*, 2004). The effect of overexpressing COPT1 ($C1^{oe}$) results not only in higher copper accumulation by the seedlings, but also in deregulated development (Andrés-Colás *et al*., 2010). Some aspects of COPT1 expression, copper accumulation and the associate phenotypes grown on Murashige and Skoog medium (MS) and MS supplemented with Cu are detailed in Table 3.3.I.

In addition to COPT, members of the ZIP (Zrt, Irt-like Protein) family (such as ZIP2 or ZIP4) could mediate copper transport in plant cells, as they complement yeast Cu and Zn transport mutants

(Wintz *et al*., 2003; Burkhead *et al*., 2009). In addition, ZIP2 transcript levels are up-deregulated in response to copper deficiency (Yamasaki *et al*., 2009).

Table 3.3.I. COPT1 expression, copper uptake and associate phenotype of Arabidopsis seedlings differing in copper-transporting activity.

		Phenotype	COPT1 expression	Cooper uptake/ accumulation
Seedlings grown on MS medium	**Col-0**	Root reduction after 3w grown with 100µM BCS (c)	Heart stage embryos, root tips, pollen grains, trichomes, stomata guard cells (a)	
	COPT1 overexpression ($C1^{oe}$)	Root length similar to the wild type (b)	2.5 fold increase (b)	moderate increase in seedling Cu content (b)
	COPT1-antisense (C1AS)	roots three to six times longer than controls when grown on the sucrose-depleted medium (a)	Transcript levels of *CCH* and *COPT2* (upregulated in response to decreasing copper) with 4 fold increase (a)	
Seedlings grown on MS medium supplemented with copper	**Col-0**	Root growth unaffected with concentrations up to 20µM (b), and reduced with 50µM (b)		Cu uptake is prevented when intracellular Cu levels rise (Wu et al., 2009).
	COPT1 overexpression ($C1^{oe}$)	Root length dramatically reduced with 10µM. Lateral roots. (b)	3.5 fold increase (b)	Moderate increase in seedling Cu content (b). Increase and modification of Cu uptake pattern (b)
	COPT1-antisense (C1AS)	Roots reverts o WT length with 30µM copper addition (a)		Both reduced 40-60% (a)

(a) Sancenón *et al*., 2004, (b) Andrés-Colás *et al*., 2010 (c) Garcia-Molina *et al*., 2011 (d) Wu *et al*., 2009

3.1.2 Link between copper and oxidative stress.

As mentioned before, Cu is a transition metal and, as such, can mediate ROS production. In the presence of either ascorbate or hydrogen peroxide (H_2O_2), copper can produce highly toxic hydroxyl radicals ($OH^{\bullet}$) via Fenton and Haber-Weiss reactions (Fry, 1998). $OH^{\bullet}$ is a prime cause of oxidative damage to proteins and nucleic acids as well as lipid peroxidation during stress, in addition it is involved in oxidative stress signaling and programmed cell death (Demidchik, 2010). The *in vivo* half-life of $OH^{\bullet}$ is approximately 1 ns, which allows $OH^{\bullet}$ diffusion over only very short distances (<1 nm) (Halliwell & Gutteridge, 1999). Because of this, $OH^{\bullet}$ generation was normally associated with two major sites: cell walls and chloroplasts. In the former, $OH^{\bullet}$ production was shown to be dependent on the activity of NADPH oxidase RbohC that forms H_2O_2 which is later catalysed by transition metals of the cell wall (Foreman *et al.,* 2003). Here, ascorbic acid is likely to serve as a reductant, because its concentration in the cell wall is very high (1-20 mM) (Fry, 2002). In chloroplasts, $OH^{\bullet}$ is generated by Fenton-like reaction of H_2O_2 with free transition metals present in stroma (Šnyrychová *et al.,* 2006; Pospíšil *et al.,* 2004; Pospíšil, 2009). Copper is a constituent of plastocyanin and thus is also crucial for the electron transport between PSII and PSI, and majority of total copper present in plants are located in chloroplasts (Marschner, 2005).

Generation of $OH^{\bullet}$ has significant implication to cell metabolism. In addition to causing lipid peroxidation (Apel & Hirt, 2004), free oxygen radicals have been proven to regulate Ca^{2+} and K^+ plasma membrane (PM) channels in Arabidopsis root cells (Demidchik *et al.*, 2003; 2007; 2010). Hydroxyl-induced activation of K^+ efflux channels resulted in massive K^+ loss from the cell, leading to programmed cell death via stimulation of K^+-dependent cell death proteases and endonucleases (Demidchik *et al.,* 2010). Interestingly, ROS-sensitivity of root plasma membrane channels showed significant tissue-specificity (Demidchik *et al.*, 2007), with elongation zone tissues being much more sensitive to exogenously applied H_2O_2 and Cu^{2+}/ascorbate ($OH^{\bullet}$-generating mixture, Halliwell & Gutteridge, 1999). This difference was explained by the existence of the different population of ROS-sensitive channels in different root zones. But is this the only reason?

Hypothesis tested.

An alternative explanation for the above differential sensitivity of plasma membrane K^+ and Ca^{2+} permeable channels to ROS could be that (1) in plant roots $OH^{\bullet}$ may be formed not only in the cell wall but also in the cytosol, and that (2) cytosolic Cu concentration may vary between root apex and mature zone tissues.

This hypothesis was tested in this study by electrophysiological and imaging assessment of Arabidopsis seedlings differing in their ability of copper transport. Wild type (Col-0), COPT1 T-DNA insertion mutant (copt1) and COPT1 over-expressing plants ($C1^{oe}$) were compared in order to see whether copper can stimulate $OH^{\bullet}$ production and *in vivo* $OH^{\bullet}$-activation of ion channels in Arabidopsis root epidermis.

3.2 Material and methods.

Plant material and growing conditions

Arabidopsis (*A.thaliana* L.) seeds ecotype Columbia-0 wild type (Col-0), COPT1 T-DNA insertional mutant (copt1) and COPT1-overexpressing seeds ($C1^{oe}$) were surface-sterilized using commercial bleach and placed on Petri dishes with half strength Murashige & Skoog (½ MS) medium with 1% (w/v) sucrose 1.2% (w/v) phytagel, pH 5.7 adjusted with KOH. Petri dishes were placed for 48h in darkness and 4°C to synchronize germination. Seeds were grown vertically in a growing chamber (16h light-8h darkness, 25°C, 78µmol m^{-2} s^{-1} irradiance).

Microelectrode ion flux measurements

Seven to 11 days-old intact seedlings were removed from the dish by cutting a piece of agar without affecting roots and placing it in a small Petri dish. Seedling was immobilized by gently placing a glass on the top of agar block, letting apical 5-6 mm of the root uncovered. The bath media was .1mM $CaCl_2$, 0.2mM KCl, pH 5.7, unbuffered. The seedling was placed on an inverted microscope (NIKON Eclipse TE2000-E). Electrode tips were positioned at the selected distance from the root surface at 100x magnification and measured 45 min after immobilization; the process was visualized and recorded by a digital camera (NIKON digital sight DS-U2) and the software NIS-Elements F 2.30. Electrodes were fabricated using PULL-100 (WPI Europe, Hertfordshire, UK) puller and silanized with dichlorodimethylsilane (catalogue number 40136, Fluka, Buchs, Switzerland). Electrode tips were filled with commercial ionophore cocktails (60031 for K^+ and 21048 for Ca^{2+}; both from Fluka), and calibrated in a range of standard solutions (see Shabala & Shabala (2002) for details). Electrodes with a low response (less than 50mV for K^+ and 25mV for Ca^{2+}) or a correlation under 0.999 were discarded. Net ion fluxes were measured by the system developed at the Autonomous University of Barcelona (see http://sine.ni.com/cs/app/doc/p/id/cs-12681 for details). In brief, the movement of the electrodes was controlled by a hydraulic manipulator connected to a stepping motor, with a high precision of displacement (1µm). The electrode movement was fully computerized using the software NI Motion (National Instruments Spain) and a 2-axis card (NI PCI 7330); this allowed a full control of the movement in both axis X/Y. Electric signal was amplified by an electrometer (FD223a, WPI) and send to a 16bits data acquisition/conversion card (A/D NI PCI 6220). This acquisition system allows changes in movement, time, frequency and number of samples prior the beginning of the experiments.

Cell viability assays

Seedlings were harvested after 2h of control and copper treatments, washed with a phosphate buffer and double-stained using fluorescein diacetate (FDA) and propidium iodide (PI), according to Koyama *et al*. (1994) as modified by Jones & Senft (1985). Intact cells exhibit green fluorescence due to FDA, and PI provides red fluorescence of nuclei in damaged cells, as has very low penetrability in intact membranes (Hamilton *et al.*, 1980).

For the Evans blue assay, seedlings were pre-incubated for 1h with the different channel blockers and stained with an aqueous Evans blue solution for 15 min, and then washed 3 times with distilled water for 10 min each, according Baker & Mock (1994).

Peroxide detection

Two hours after treatments, roots were washed in 10mM Tris-HCl buffer and incubated for 30min at 37° with 25µM 2',7'Dichlorofluorescein diacetate (DCF-DA), according the protocol by Sandalio *et al.* (2008).

3.3 Results.

Short-term copper exposure causes permeability disruptions in C1^{oe} seedlings

The effect of short-term exposure (2 hours) to 10µM copper was analyzed by the double staining with fluorescein diacetate and propidium iodide (Fig. 3.3.1) Col-0 and copt1 showed intact membrane permeability (green in the figure), while in C1^{oe} seedlings damage was localized at the root tip (red). The damage at the root tip of C1^{oe} seedlings was prevented by addition of 10 µM of bathocuproinedisulfonic acid (BCS), a known copper chelator (Sandmann & Böger, 1983; Märschner, 2005).

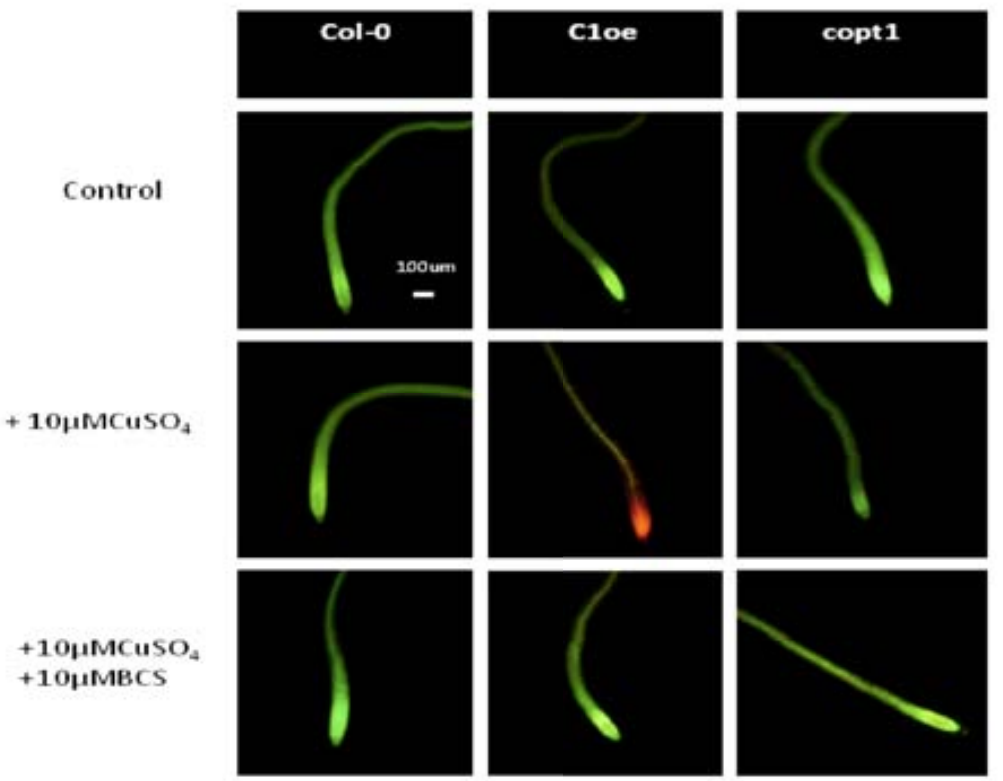

Figure 3.3.1 Copper-induced damage to the root apex in C1^{oe} seedlings. Intact seedlings were double stained with fluorescein diacetate (intact cells, green) and propidium iodide (damaged cells, red) after 2h with the different treatments. A representative image for each treatment is shown (n=6).

Copper stimulates dose-dependent net Ca^{2+} influx in the meristem but not mature root zone

Calcium fluxes were measured from the meristematic (50-100 µm from the apex) and mature (~ 1 mm) zone regions of Arabidopsis roots using ion-selective microelectrodes. 15 to 20 minutes after 10µM $CuSO_4$ addition, a transient peak of Ca^{2+} influx was detected at the meristematic zones of wild type (76.5% of tested roots, n=17) and $C1^{oe}$ (64.3% of tested roots, n=14) seedlings, but in none of 10 copt1 seedlings (Fig. 3.3.2a). The amplitude of net calcium influx peak in $C1^{oe}$ seedlings was an order of magnitude higher compared with wild type (49.6 ± 14 and 6.6 ± 1.4 $nmolm^{-2}s^{-1}$ respectively, significant at $p<0.01$; Fig.3.3.2b) Net calcium influx was proportional to copper concentration applied to plant roots (Fig.3.3.2c).

Only slight Ca^{2+} influx was detected in the mature zone of $C1^{oe}$ seedlings (Fig. 3.3.2b); this was not significant at $P < 0.05$ ($n = 9$). Also, no apparent changes in net Ca^{2+} flux kinetics was seen around the mature region of either Col-0 or copt1 roots (n=10 for Col-0, and n=11 for copt1; neither is significant at $P < 0.05$).

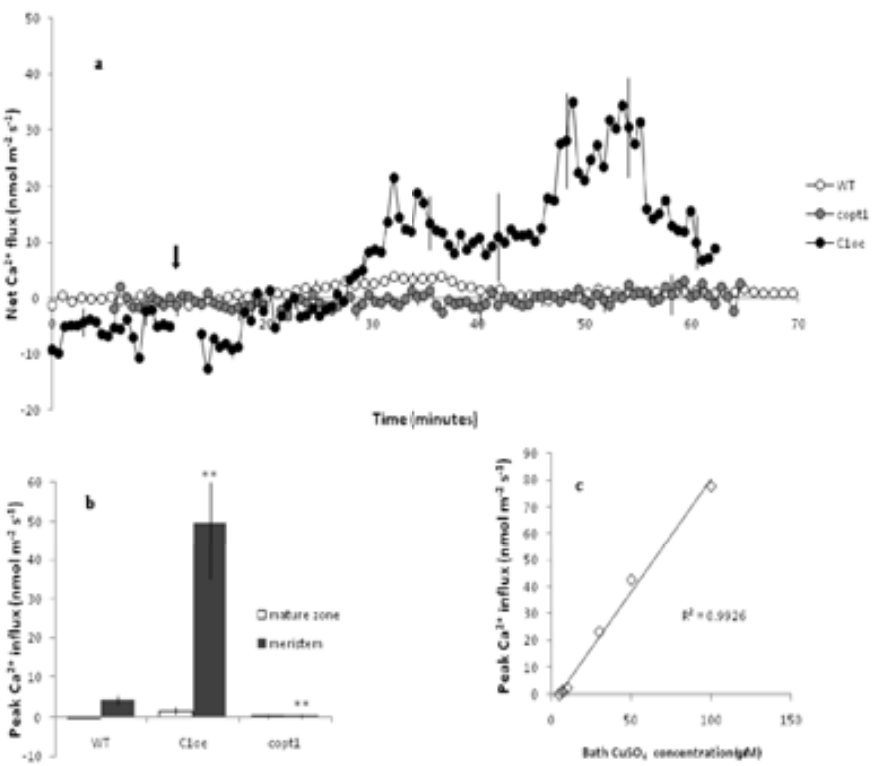

Figure 3.3.2 Copper induces Ca^{2+} influx at meristem of WT and $C1^{oe}$ seedlings. (a) Transient flux kinetics upon addition of 10µM $CuSO_4$ addition (indicated by an arrow). Error bars are SEM. **(b)** Peak calcium influx measured in mature and elongation zones in response upon 10µM $CuSO_4$ addition (n= 6 to 13). Asterisks indicate statistically significant differences with respect to the wild type, ANOVA test (**P <0.01). **(c)** Dose-dependency of Ca^{2+} flux response.

Ca^{2+} net flux is de-regulated in $C1^{oe}$ seedlings grown under neutral photoperiod

$C1^{oe}$ seedlings have been shown to have de-regulated copper transport under neutral photoperiod that affects growth (Andrés-Colás *et al.*, 2010) but the mechanisms of this process remain elusive. One possibility is that calcium could be a good intermediate in the putative pathway between copper and circadian clock components. Importantly, basal net Ca^{2+} flux beforecopper addition was lower in $C1^{oe}$ seedlings when compared to the wild type (Fig 2a). Accordingly, basal Ca^{2+} fluxes were analyzed at the meristematic level on 7-days old seedlings grown under neutral photoperiod at Zeitgeber times 0 (T_0) and 12 (T_{12}) (Fig 3.3.3). At T_0, differences in basal Ca^{2+} flux between Col-0 and $C1^{oe}$ seedlings had statistical significance at $p<0.01$. No statistically significant difference in Ca^{2+} flux levels was detected for T_{12} grown plants, consistent with the above hypothesis of Ca^{2+} involvement in de-regulated copper transport under neutral photoperiod.

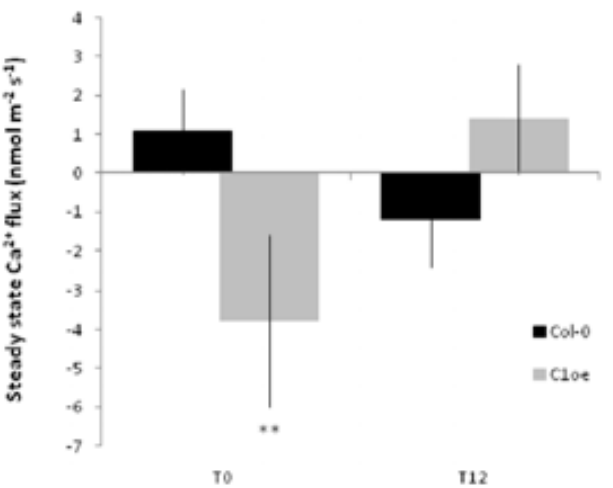

Figure 3.3.3 Calcium basal fluxes are de-regulated in $C1^{oe}$ seedlings. Basal net calcium flux was measured in seedlings grown under neutral photoperiod at Zeitgeber times 0 and 12. Mean ± SE (n = 5-8 seedlings). Asterisks indicate statistically significant differences with respect to the wild type (**P <0.01, ANOVA test).

Pharmacology of copper-induced Ca^{2+} influx

The effect of different blockers on calcium net flux was investigated. Seedlings were pre-incubated with 10mM TEA^+ (potassium channel blocker), 30µM verapamil and 50µM Gd^{3+} (two known blockers of non-selective cation (NSCC) channels). In wild type plants, TEA^+ had no effect on calcium flux, while both gadolinium and verapamil caused significant blockage of the calcium flux response (Fig 3.3.4a). Gadolinium completely suppressed calcium flux (from 6.6 ± 1.4 to 0.6 ± 0.4 nmol m^{-2} s^{-1}, n=4; significant at $p<0.01$), while verapamil caused approximately 50% reduction in copper-induced Ca^{2+} influx (n=4, significant at $p<0.05$). Application of above concentration of Gd^{3+} and verapamil to $C1^{oe}$ seedlings resulted in reduction in net Ca^{2+} influx to the level identical to wild type (n=4, Fig. 3.3.4b).

As COPT-silenced seedlings revert their phenotype when treated with 30µM copper (Sancenón *et al*., 2004), the effect of this concentration was tested at the meristematic zone of copt1 seedlings. Under these conditions, Ca^{2+} influx response, absent when copt1 roots were treated by 10µM of copper, becomes evident. In this case, peak Ca^{2+} influx had a magnitude comparable with those for wild type treated with 30µM copper (as reported in Fig. 3.3.3). Pharmacology experiments revealed that the calcium influx was blocked in a similar way to that observed in wild type seedlings (Fig. 3.3.4c).

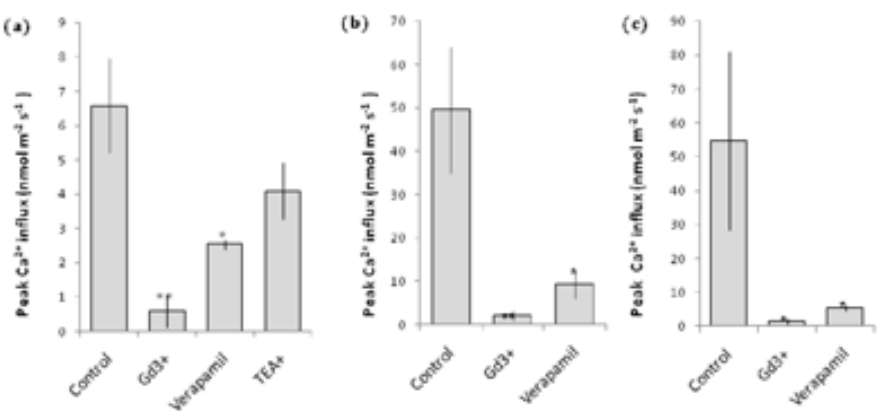

Figure 3.3.4 Pharmacology of copper-induced Ca^{2+} flux responses. (a) Gd^{3+} and verapamil block net calcium influx at the meristem of wild type seedlings. Seedlings where pre-treated with (in mM) 0.05 mM Gd^{3+}, 0.02 mM verapamil or 10 mM TEA 1h prior 10µM $CuSO_4$ addition. Mean ± SE (n= 4 to 8). **(b), (c)** effect of Gd^{3+} and verapamil on net Ca^{2+} influx at the meristem of $C1^{oe}$ (**b**) and copt1 (**c**) seedlings. Seedlings where pre-treated with 0.05 mM Gd^{3+} or 0.02 mM verapamil 1h prior to 10µM (for $C1^{oe}$) or 30µM (for copt1) $CuSO_4$ addition. Mean ± SE (n=4). Asterisks indicate statistically significant differences with respect to the wild type (*P<0.05**; P <0.01, ANOVA test).

Copper induces K^+ efflux in $C1^{oe}$ seedlings

Addition of 10μM of copper to Arabidopsis roots has also resulted in net K^+ efflux from the meristematic region. This efflux was relatively small in wild type seedlings (-13.1 ± 9, n=4) but five-fold higher in C1oe seedlings (-75.1 ± 31, n=5; difference significant at $p < 0.05$) (Fig 3.3.5). The K^+ efflux at the meristematic zone of $C1^{oe}$ was suppressed by pre-incubation with Gd^{3+} (n=4), but not with TEA^+ (n=5) (see inset in Fig. 3.3.5).

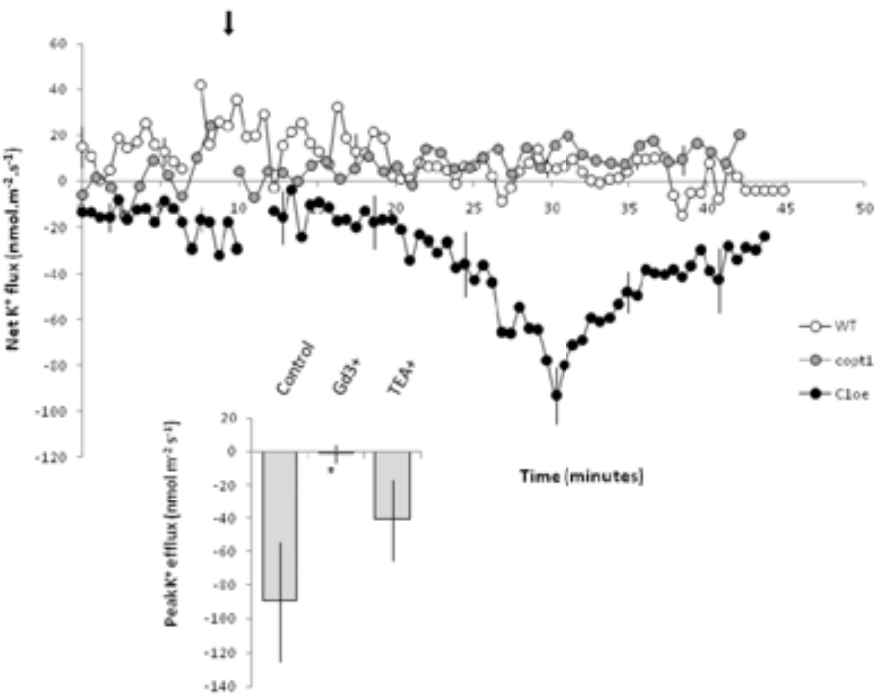

Figure 3.3.5 Copper induces K^+ efflux at meristem of $C1^{oe}$ seedlings. Intact seedlings were placed in a chamber with 0.1 mM $CaCl_2$ and 0.2mM KCl, pH 5.7. 10μM $CuSO_4$ was added at around 10 min (as indicated by an arrow). Mean ± SE (n = 5 to 8). **Inset -** Gd^{3+} blocks potassium net efflux at the meristem of $C1^{oe}$ seedlings. Seedlings where pre-treated with 0.05 mM Gd^{3+} or 10 mM TEA^+ 1h prior 10μM $CuSO_4$ addition. Mean ± SE (n=4). Asterisks indicate statistically significant differences with respect to the control (*P<0.05, ANOVA test).

Gd^{3+} prevents root tip damage caused by toxic copper exposure

Effect of different ion channel blockers in preventing root damage under toxic concentrations of copper was tested using wild type seedlings by Evans blue assay (Baker and Mock, 1994). Two hours of exposition to 50 µM $CuSO_4$ caused substantial damage to root apex (dark blue staining in Fig 3.3.6). Verapamil reverted root tip damage in ~30% of roots, while Gd^{3+} completely prevented the toxic effect of copper. No beneficial effect of TEA^+ pretreatment was found. No root damage was observed in the presence of 10 µM of BCS.

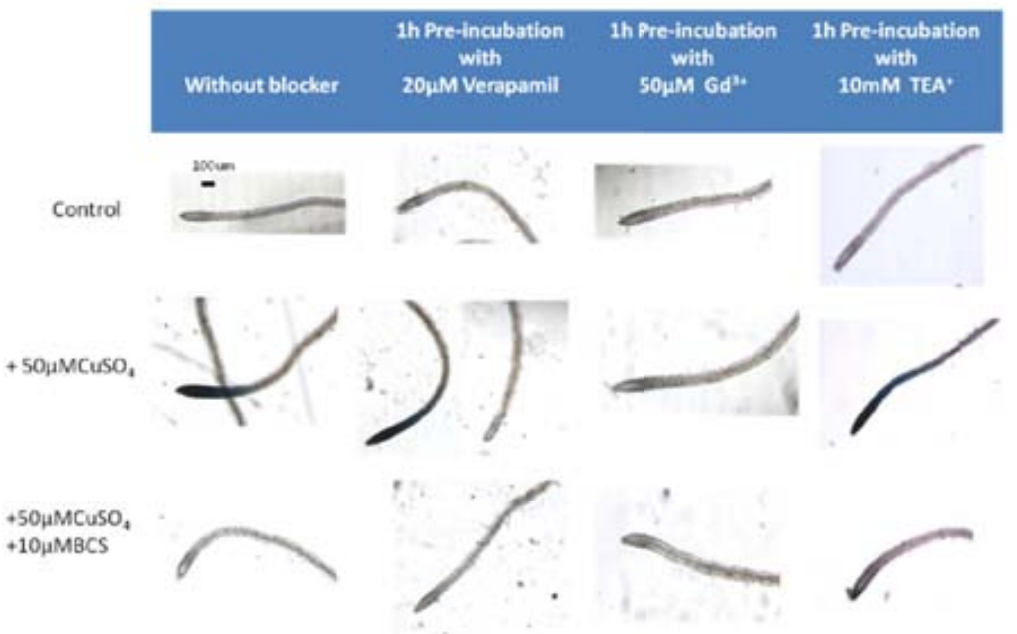

Figure 3.3.6 Gd^{3+} prevents root tip damage caused by copper toxicity in wild type seedlings. Seedlings where pre-treated with inhibitors (0.02 mM verapamil, 0.05 mM Gd^{3+} or 10 mM TEA^+) 1h prior to root exposure to 50µM $CuSO_4$ for 2h. A representative image for each treatment is shown (n=8-12).

Basal peroxide levels decrease in copper-treated C1^{oe} root meristem

In order to asses of whether copper is able to react with peroxide and produce OH·, roots were washed and incubated with 2',7'Dichlorofluorescein diacetate (DCF-DA) after 2h of the different treatments (Fig. 3.3.7a). Average basal peroxide accumulation was analyzed in the section 0-100 µm from the root apex in plants with copper treatments (Fig.3.3.7b). Peroxide accumulation in this section was statistically higher at the meristem of copt1 seedlings respect to the control and C1^{oe} seedlings ($p<0.01$, n=6, Fig. 7c). C1^{oe} had lower peroxide accumulation than wild type seedlings (significance at $p<0.05$, n=6, Fig 3.3.7c).

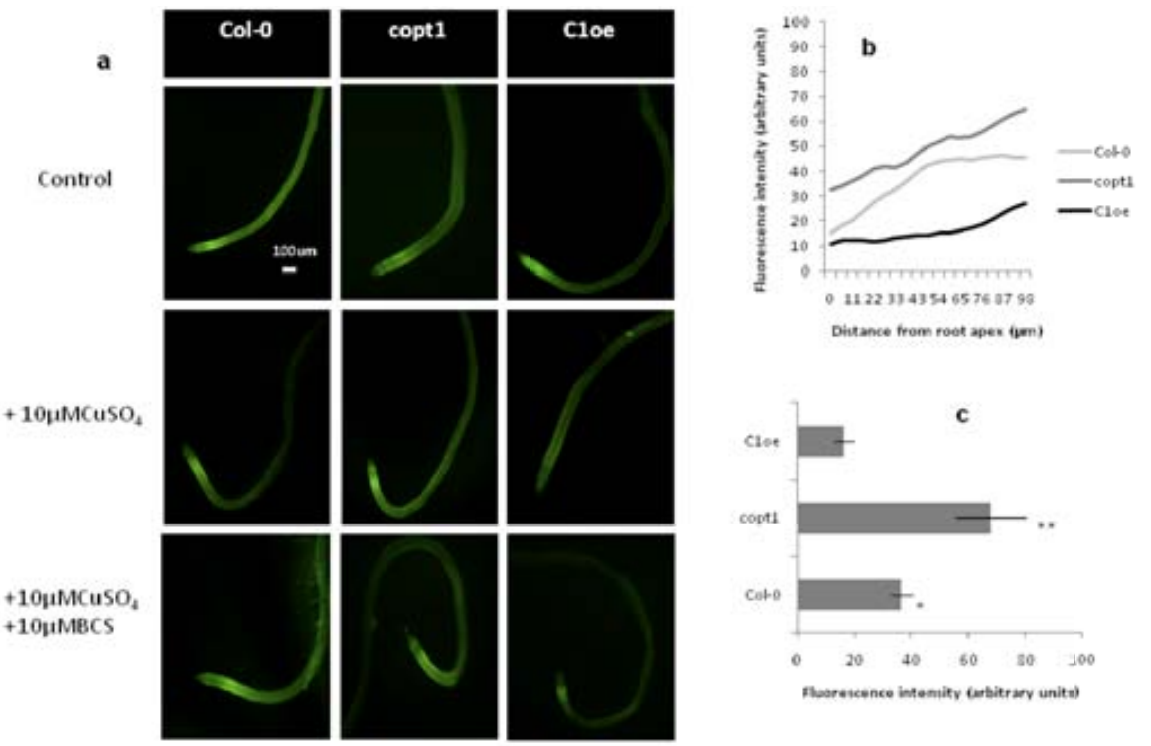

Figure 3.3.7 Copper treatment causes decrease in basal peroxide accumulation at the meristem of C1^{oe}. (a) A representative image for each treatment is shown (n=6). **(b)** Fluorescence intensity profile at the root tip of the seedlings under copper treatments shown in (a). **(c)** Average fluorescence intensity at the root tip (0 to 100 µm) of the seedlings with copper treatment (Mean±SE, n=6) Asterisks mark significant differences with respect the control (**P<0.01; *P<0.05, ANOVA test).

3.4 Discussion.

Hydroxyl radical-sensitive plasma membrane ion channels are activated by from the cytosolic side *in planta*

All previous studies reporting *in planta* $OH^{\bullet}$ effects of plasma membrane ion channels activity assumed that $OH^{\bullet}$ generation occurs in the apoplastic space, as a result of interaction between transient metals (such as Cu) and H_2O_2 in the presence of ascorbate located in the cell walls (Demidchik *et al*, 2003, 2007). In this study we provide strong evidence that the similar process occurs also in the cell cytosol, and that $OH^{\bullet}$ produced in the latter compartment may have significant impact on plasma membrane transport activity and intracellular homeostasis in intact plants. Several lines of evidence support this claim.

First, the damage caused by application of low (10 μM) concentration of copper was observed only in C1oe seedlings, with 2.5-fold expression of COPT1 (Andrés-Colás *et al.*, 2010) and is absent in wild type and copt1 seedlings (Fig 3.3.1). Localization of the damage at the root tip in wild type seedlings (Fig 3.3.6) is consistent with previously reported COPT1 expression (Sancenón *et al.*, 2004) and the reversibility of damage with the copper chelator bathocuproinedisulfonic acid (BCS) confirms that cell permeability disruptions are copper-induced.

Second, significant difference between Cu-induced net Ca^{2+} and K^+ fluxes was found between most $C1^{oe}$ and wild type roots (Fig 3.3.2 and 3.3.5). This difference was observed only in root meristem but not mature zone, and was fully consistent with expression patterns of COPT1 transporters (Sancenón *et al.*, 2004). Thus, differential sensitivity of Ca^{2+} and K^+ permeable plasma membrane ion channels to ROS reported in previous studies (Demidchik *et al*, 2002, 2003, 2007) may be explained not only by differential sensitivity/expression of $OH^{\bullet}$-activated channels in different root tissues but also by the fact that cytosolic Cu concentrations in the root apex (and, hence, its potency to generate $OH^{\bullet}$ in the cytosol) are much higher compared with mature region, due to preferential expression of COPT1 transporters in this tissue.

Third, basal peroxide (H_2O_2) detection reveals a decrease at the meristematic zone of $C1^{oe}$ under copper treatment (Fig. 3.3.7a-c), being consistent with this cytosolic $OH^{\bullet}$ generation from copper reaction with H_2O_2. The decrease in peroxide at the mature zone of C1oe and copper-treated wild type seedlings could be due to a lignifications process, as has been reported previously in Arabidopsis roots under copper treatments (Lequeux *et al.*, 2010).

Fourth, all previous studies on hydroxyl-activated ion channels have used non-physiologically high concentration of $OH^{\bullet}$-generating Cu/ascorbate mix (Demidchik *et al.*, 2002, 2010; Zepeda-Jazo *et al.*, 2011). In this study, fluxes were measured in response to 100 times lower copper addition, 10μM, and in absence of ascorbate. Both Ca^{2+} and K^+ transient peaks occurred 20 to 25 min after copper addition, further suggesting that the effect of copper on these channels was indirect and occurred from the cytosolic but not apoplastic side.

Physiological implications and underlying mechanisms

During root growth, the meristem is the zone that first contacts with a new soil environment. A higher sensitivity to $OH^{\bullet}$ in this root zone may provide an efficient mechanism for signaling changes in the chemical properties in the rhizosphere. $OH^{\bullet}$-induced Ca^{2+} signaling appears to be essential in this process. Ca^{2+} flux responses were absent in copt1 mutant treated with 10 μM Cu (Fig. 3.3.2) but reverted when plants were treated with higher (30μM) copper (Fig. 3.3.4c). This is consistent with reports that COPT-silenced seedlings revert their phenotype when treated with higher Cu concentrations (Sancenón *et al*., 2004), suggesting a causal relationship between Cu transport and Ca^{2+} flux changes. The most likely explanation may be that, at lower Cu concentrations, COPT1 is the only transporter mediating Cu uptake into the cell, while at higher concentrations Cu uptake may be carried by some other (unspecific) transporter such a ZIP2. Regardless of whether copper is transported through COPT1 or through another transporter, the effect on activating calcium influx is similar. A moderate $OH^{\bullet}$ generation would generate a transient Ca^{2+} influx necessary for root tip growth, as $C1^{oe}$ seedlings have been shown to have deregulated growing (Andrés-Colás *et al*., 2010), that could be explained by a deregulated basal Ca^{2+}influx (Fig. 3.3.3).

Another possible role of Ca^{2+} flux may be in regulation of circadian clock. Membrane transporters represent an intrinsic part of both circadian (Njus *et al.,* 1974) and ultradian (Shabala, 2003; Shabala et al., 2006) oscillators. In this work, net Ca^{2+} flux is de-regulated in C1oe seedlings grown under neutral photoperiod (Fig. 3.3.3). Both CCA1 and LHY mRNA levels, which are nuclear components of the Arabidopsis circadian clock (Mizoguchi *et al.*, 2002, 2005; Ito *et al.*, 2007; Niwa *et al.*, 2007) have a peak of expression at Zeitgeber time 0 h (Mizoguchi *et al.*, 2002). Previous work on COPT1 (Andrés-Colás *et al.*, 2010) has shown that in wild type both mRNA levels are reduced when seedlings where grown with 10μM of copper and that LHY oscillation phase is delayed by Cu. In addition, $C1^{oe}$ seedlings grown on MS showed higher Cu content and lower fresh weigh when compared to wild type seedlings. It has been shown that extracellular pH, ROS and Ca^{2+} have oscillations and modulate tip growth of Arabidopsis root hairs (Monshausen *et al*., 2007).

Neither Ca^{2+} influx nor K^+ efflux were affected by TEA^+ (Figs 3.3.4 and 3.3.5) suggesting that K^+-selective channels were not part of responses to Cu treatment. At the same time, both gadolinium and verapamil, two known NSCC blockers, were highly efficient (Fig 4-6), implicating NSCC as a downstream target of Cu signaling mechanism. The specific identity of these channel(s) should be revealed in patch-clamp experiments. The NSCC-mediated $OH^{\bullet}$-activated Ca_{in} analyzed by Demidchik *et al*. (2003) showed a similar pharmacological profile (insensitivity to TEA^+ and sensitivity to lanthanides and to verapamil). Permeability sequence of these channels suggested that this $OH^{\bullet}$-activated conductance was mediated by a different population of NSCCs.

It was shown in direct patch-clamp experiments that outward-rectifying depolarization activated K^+ permeable GORK channels are also activated by hydroxyl radicals (Demidchik *et al*., 2010). Both $OH^{\bullet}$-activated K_{out} currents, and net K^+ fluxes in the mature root zone of Arabidopsis were sensitive to TEA^+ (Demidchik *et al.*, 2003; 2010; Cuin and Shabala, 2007). In all these reports, high (1 mM) concentration of Cu/ascorbate mix was used. In our study, TEA^+ did not have effect on

K^+ efflux when plants were treated with much lower (10 μM) Cu concentrations. Also rather different was K^+ flux kinetics. While in all referred above papers the peak K^+ efflux was achieved within 5-7 min of 1 mM Cu/A application, maximum K^+ efflux in our work was observed 25 to 30 min after 10 μM Cu treatment. This difference may be explained assuming that GORK channels have $OH^•$ binding site on external side (and, hence, activated from the apoplast) while NSCC channels have their $OH^•$ binding sites from the cytosol and, hence, take longer to be activated. Indeed, animal hERG channels, which are closely related to plant GORK channels (BLAST analysis; NCBI), have extracellular transition-metal-binding centers that generate HRs in the presence of H_2O_2 and are responsible for channel opening (Yu et al., 1997; Yu, 2003). It remains to be answered if similar metal-binding centers do exist in NSCC channels, and of whether they are located on the inner site of the plasma membrane.

Copper homeostasis model under deprivation and toxic conditions

Based on above data, the following model describing copper homeostasis and hydroxyl radical generation/signaling can be put forward (Fig. 3.4.1). Under limiting copper conditions, ① the expression of COPT1 is induced. This high affinity transporter is located at the PM of root tips and ② mediates copper transport into the cytosol (Sancenón *et al*., 2004, Andrés-Colás *et al*., 2010). Once in the cytosol, copper will be transported by metal chaperones to major intracellular compartments for essential functions in the cells. Part of the copper would generate ③ a moderate $OH^•$ increase, that activates ④ a moderate Ca^{2+} influx necessary for root growth. Once this signaling is over, elevated cytosolic calcium would be reverted by the orchestrated action of ⑤ CAX1 at the tonoplast and Ca^{2+}-ATPase at the PM. At the same time, the increase in $[Cu]_{cyt}$ ⑥ regulates COPT1 expression via negative feedback (Sancenón *et al.*, 2004) (illustrated in Fig 3.4.1 as black lines).

If excessive copper supply follows copper deprivation ② COPT1 and perhaps other unspecific transporters as ZIP2 would mediate copper entry towards the cytosol (grey lines in Fig. 3.4.1). The resultant increase in cytosolic Cu pool would result in ③ a massive generation of $OH^•$, due to Cu interaction with cytosolic H_2O_2. This will result in ④ activation of $OH^•$-sensitive Ca^{2+} and K^+-permeable NSCC. The effect will be two fold. First, such activation may further increase net Ca^{2+} uptake into cytosol via ⑤ positive feedback regulation of NADP(H)oxidase (Lecourieux *et al*., 2002). Second, $OH^•$-induced K^+ efflux will result ⑥ in the rapid decrease in $[K^+]_{cyt}$ pool, ⑦ activating caspase-like proteases and leading to PCD (Shabala, 2009b; Demidchik *et al*, 2010). This will explain the rapid loss of viability in $C1^{oe}$ plants as indicated by viability staining experiments (Fig. 3.3.1).

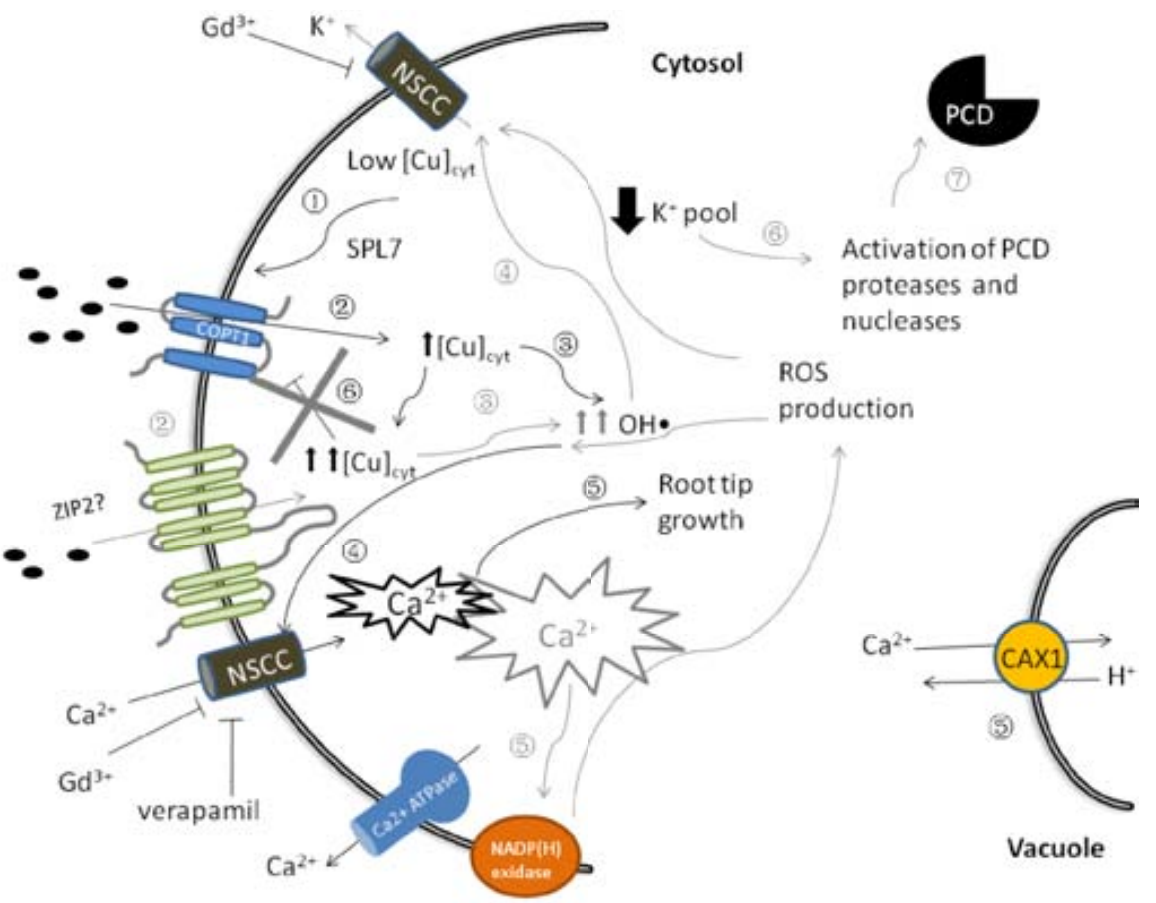

Figure 3.4.1 The tentative model depicting hydroxyl radical production and activation of plasma membrane Ca^{2+} and K^+ permeable channels in Arabidopsis copper transport mutants. See text for explanations. Black lines relate to signaling under copper sufficient condition; grey lines refer to copper toxicity. Abbreviations used: COPT1: High Affinity Copper Transporter 1; ZIP2: Zrt-, Irt-like Protein 2; NSCC: Non-Selective Cation Channel, CAX1: Calcium Exchanger 1; NADP(H) oxidase (Nicotinamide Adenine Dinucleotide Phosphate-Oxidase); SPL7: *SQUAMOSA* Promoter Binding Protein–Like7; Ca^{2+}-ATPase: Plasma membrane calcium pump; PCD: Programmed Cell Death

Conclusions and future work

Copper-induced generation of hydroxyl radicals (HR) occurs not only at the extracellular side, but also in the cytosol by reaction with H_2O_2 in the Fenton and Haber-Weiss reactions, as basal peroxide decreased with copper treatments and gain and loss of function mutants in copper transport have been shown to have differential HR-channel activation.

Copper has been shown to regulate circadian components, and overexpressing-copper transport mutants have been shown impaired growth. As basal calcium net flux was impaired in these mutants, copper transport is, therefore, likely to be a key process in HR-generation and growth under both favorable and toxic copper environments.

Patch clamp experiments must be further conducted in order to reveal channel identities.

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Chapter 4

Oxidative stress- mediated activation of annexins.

4.1.1 Annexins: Part of the Ca^{2+}-signaling network.

Annexins are widespread through the Prokaryote and Eukaryote domains (Morgan & Fernández, 1997; Braun *et al.*, 1998; Hofmann, 2004; Bouzenzana *et al.*, 2006). At neutral pH, annexins can bind to negatively charged phospholipids such as phosphatidylserine (PS), phosphatidylglycerol and phosphatidylinositol (Reviewed by Lahoavist at al, 2011).

Most annexin functions are linked to their ability to interact with membranes in a Ca^{2+}-regulated and reversible manner, and, as a component of the Ca^{2+}-signaling network, annexins participate in many fundamental processes, such as regulation of membrane domains and membrane-cytoskeleton linkages, vesicle transport, or ion flux across the membranes. (Reviewed by Creutz *et al.,* 1992; Kourie & Wood, 2000; Gerke & Moss, 2002; Gerke *et al.*, 2005; Grewal & Enrich, 2009; Monastyrskaya & Babiychuk, 2009). Unlike conventional channels, annexins are soluble phospholipidbinding proteins, and they can associate or directly insert into membranes (Kourie & Wood, 2000; Gerke & Moss, 2002; Gorecka *et al.*, 2007; Mortimer *et al.*, 2008; Laohavisit *et al.*, 2009; Laohavisit & Davies, 2009).

Animal annexins have been intensely studied as one of its members, annexin A5, is implicated in apoptotic events (Koopman *et al*., 1994). This member is amongst the annexins that have extracellular presence in addition to intracellular localization (Reviewed by van Genderen *et al.*, 2008), and its binding to PS at the plasma membrane (PM) face has been frequently used as an animal cell death marker (Boersma *et al.*, 2005). Multiple studies have focused on A5 gene, protein structure and its ability to bind to phospholipid membranes in a Ca^{2+}-dependent manner (Huber *et al,*, 1990a,b; Brissom & Mosser, 1991; Sun *et al.,* 1993; Burguer *et al.*, 1994; Demange *et al.*, 1994; Fernández *et al,*, 1994; Goossens *et al.*, 1995; de Oliveira Santos *et al.*, 2001) and its role in development (Giambanco *et al.,*, 1993; Arcuri *et al.*, 2002). Several electrophysiological studies have focused on A5 transport properties and voltage dependency (Andree *et al.*, 1990; Rojas *et al*., 1990; Burguer *et al.*, 1994; Demange *et al.*, 1994; Arispe *et al.*, 1996; Hofmann *et al.,* 1997; Han *et al.*, 1998; Neumann *et al.*, 2000). Based on these studies, it can be concluded that A5 binds preferentially to acidic phospholipids, that its adsorption to membranes is highly calcium, pH and voltage-dependent, and that can mediate different ion transport (K^+, Na^+, Mg^{2+} in addition to Ca2+) and form different channel types (Totally o partially crossing PM) depending on the protein concentration.

Plant annexins, in contrast, have been much lesser studied, although they are abundant proteins and are widespread in the Plant Kingdom (Reviewed by Lahoavisit *et al.*, 2011). As in animal cells, annexins are components of the Ca^{2+}-signaling network (Lahoavisit *et al.*, 2009, reviewed by Lahoavisit *et al.*, 2010, 2011).

In Arabidopsis, annexin family has eight members, expressed in all plant tissues (Clark *et al.,* 2001; Cantero *et al.*, 2006) and their expression patterns were shown to change under several abiotic factors, indicating a role of this family in stress responses (Cantero *et al.*, 2006), that has been

recently sustained by the participation of *AtANN1* and *AtANN4* in salt and drought stresses (Huh *et al.*, 2010).

4.1.2 Are annexins regulated by ROS?

ROS can have a harmful effect on membranes as they cause lipid peroxidation (Halliwell and Gutteridge, 1984; Apel & Hirt, 2004). One of the by-products of this phenomena is malondialdehyde (MDA), frequently measured as an indicator of lipid peroxidation in both animal and plant cells (i.e Rodríguez-Serrano *et al.*, 2006; Andrés-Colás *et al*., 2010; García *et al.,* 2011).

Together with forming Ca^{2+} channels in non-oxidized conditions, annexin A5 can also mediate peroxide-induced Ca^{2+} influx across the PM of chicken DT40 cells (Kubista *et al.*. 1999; Kourie & Wood, 2000; Gerke & Moss, 2002), and this annexin has been shown to bind preferentially to MDA-modified lipids (Balasubramanian *et al.*, 2001).

On the other hand, production of ROS is consequence of multiple abiotic factors that also have been shown to increase plant annexin abundance and recruitment (Bianchi *et al.*, 2002; Repetto *et al.*, 2003; Lee *et al.*, 2004; Cantero *et al*., 2006; Vandeputte *et al.*, 2007; Konopka-Postupolska *et al.*, 2009; Huh *et al.*, 2010). Plant annexins also have been found in pollen tubes and root hairs, points at which ROS play important roles in signaling and development. (Reviewed by Mortimer *et al.,* 2008). This suggests that annexins may play a role linking both ROS and Ca^{2+} signaling.

ROS have been shown to regulate plant ion channels (Demidchik *et al*, 2003, 2007, 2010) and at present, there is strong evidence that supports that hydroxyl radicals activate *AtANN1*, and that this channel provides a molecular link between reactive oxygen species and Ca^{2+} signalling in plants (Lahoavisit *et al.*, unpublished results).

Hypothesis tested.

In this chapter the research was focused in to main hypothesis.

- In section A, some preliminary studies were conduced in order to asses the role of copper-induced cytosolic hydroxyl radical (HR) generation (see chapter 3) in *Arabidopsis thaliana* annexin 1 loss of function *ann1*, due that this mutant has been shown to have impaired Ca^{2+} response to HR (Lahoavisit *et al.*, unpublished results).

- Section B focus in the possible activation of human annexin A5 by HR, as this annexin has been shown to have a role in apoptotic events in which ROS generation is implied.

4.2 Material and methods

Section A. Effect of copper deficiency- toxicity on *ANN1* loss of function growth, weight and chlorophyll content.

Seeds of Arabidopsis (*Arabidopsis thaliana* L.) Col-0 and the T-DNA loss of function *atann1* were surface-sterilized with commercial bleach and grown vertically on half-strength Murashige and Skoog (½ MS) medium (Duchefa, Netherlands) with 0.8% (w/v) bactoagar, pH 5.7 adjusted with KOH, in absence of sucrose, After 48h in darkness and 4°C to synchronize germination, seeds were grown vertically in a growing chamber (16h light, 8h darkness, 25°C, 78μmol $m^{-2}s^{-1}$ irradiance).

After 4 days, uniform seedlings were transferred to plates supplemented with different copper concentrations (in form of $CuSO_4$ salt), absence of either copper or bathocuproinedisulfonic acid, a copper chelator (BCS, 146625 SIGMA), and presence of BCS in different concentrations.

After 10 days of growing in the different conditions, the following parameters were analyzed: root length (with the software Image-Pro Plus 6.0), and fresh weight.

Section B. Study of HR-activation of annexin A5 using planar lipid bilayers (PLBs).

Bilayers were formed by the painted lipid technique. A schematic representation from Neumann *et al.* (2000) is shown in Fig. 4.2.1.

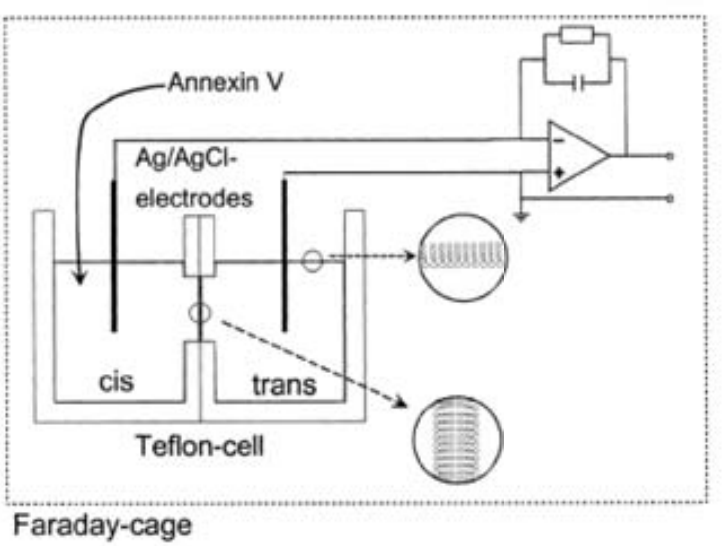

Figure 4.2.1 A schematic representation of the experimental set-up for the painted lipid bilayer technique. *Cis* emulates intracellular side while *trans,* extracellular side. In all the experiments, annexin was added to the *cis*-side (From Neumann *et al.*, 2000)

Lipids, obtained from Avanti Polar Lipids (Alabama, USA), included 1-palmitoyl 2-oleoyl phosphatidylethanolamine (POPE) 1-palmitoyl 2-oleoyl phosphatidylserine (POPS) and cholesterol.

Bilayers were composed of a mixture of lipids (10mg ml-1, with ratio POPE:Cholesterol:POPS 5:3:2), evaporating previously the chloroform and resuspending the mixture in n-decane (Sigma).

Bilayers were formed across a 200µm diameter hole spreading the lipid mixture across the septum between *cis* and *trans*. The *cis* compartment was connected to the headstage of an Axopatch 200B amplifier, while the *trans* compartment was connected to the ground by an Ag/AgCl electrode, in both cases the connection was done using salt-bridges (3M KCl, 1% agar).

The software Clampex 8.0 was used to measure the final resistance (from 2 to 5 GΩ) and capacitance (from 150 to 200 pF) of the painted bilayers. An example of a stable bilayer in the interface of the software is represented in Fig 4.2.2.

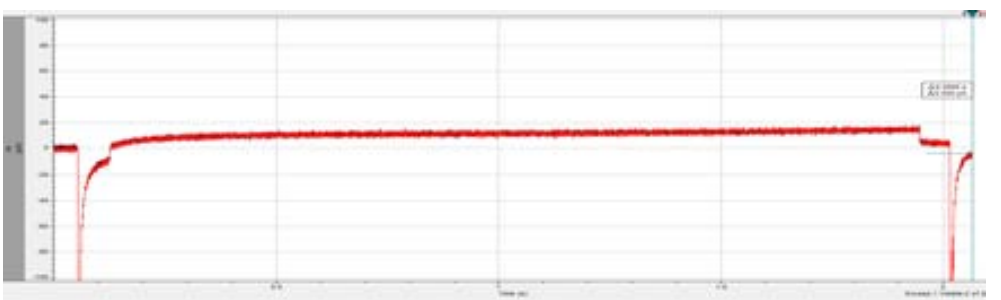

Figure 4.2.2 Clampex 8.0 program interface showing a stable bilayer of composition POPE:Cholesterol:POPS 5:3:2 across a 200µm diameter hole.

Following the formation of a stable bilayer, 1µl of A5 protein (SIGMA, A9460) was added to the *cis* side in a final concentration of 70 nM. The bilayer was held at negative value (From -90 to -150mV and the *cis* side was stirred from 3 to 5 minutes to aid protein insertion.

Symmetrical *cis-trans* conditions were used (50mM $CaCl_2$) at two different pH values, 7.4 and 6.3 (buffered with MES/BTP). All the solutions used in the experiments were previously filtered using a 0.22 µm filter.

Different step-voltage protocols were also tested in order to get the optimal experimental conditions.

4.3 Results

Section A. Effect of copper deficiency- toxicity on *ANN1* loss of function growth, weigh and chlorophyll content.

After 4 days of germination on ½ MS, uniform seedlings were placed in ½ MS plates supplemented with copper (5, 10, 25 or 50 μM), BCS (100μM) or absence of both (0 Cu 0 BCS). Initial growth was smaller in *ann1*, significant at P<0.01, ANOVA test (Fig. 4.3.1).

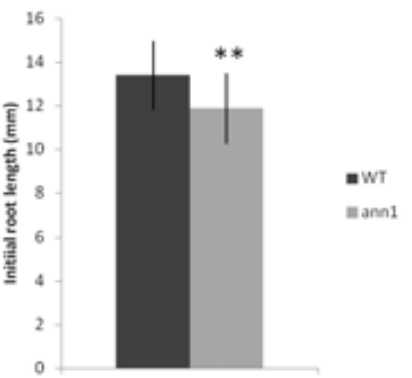

Figure 4.3.1 Average root length for 4d old seedlings grown on ½ MS medium. Mean ± Standard deviation is shown (n= 120). Asterisks indicate statistically significant differences with respect to the wild type, ANOVA test (**P <0.01)

Seedlings were transferred to the different treatments and root length was again measured after 6 days (Fig. 4.3.2a). The phenotype of the seedlings after 3 weeks grown under the different treatments is shown in Fig 4.3.2b. The statistical differences between wild type and *ann1*, detected for all treatments (P<0.01, ANOVA test), were suppressed at the highest copper treatment tested, 50 μM.

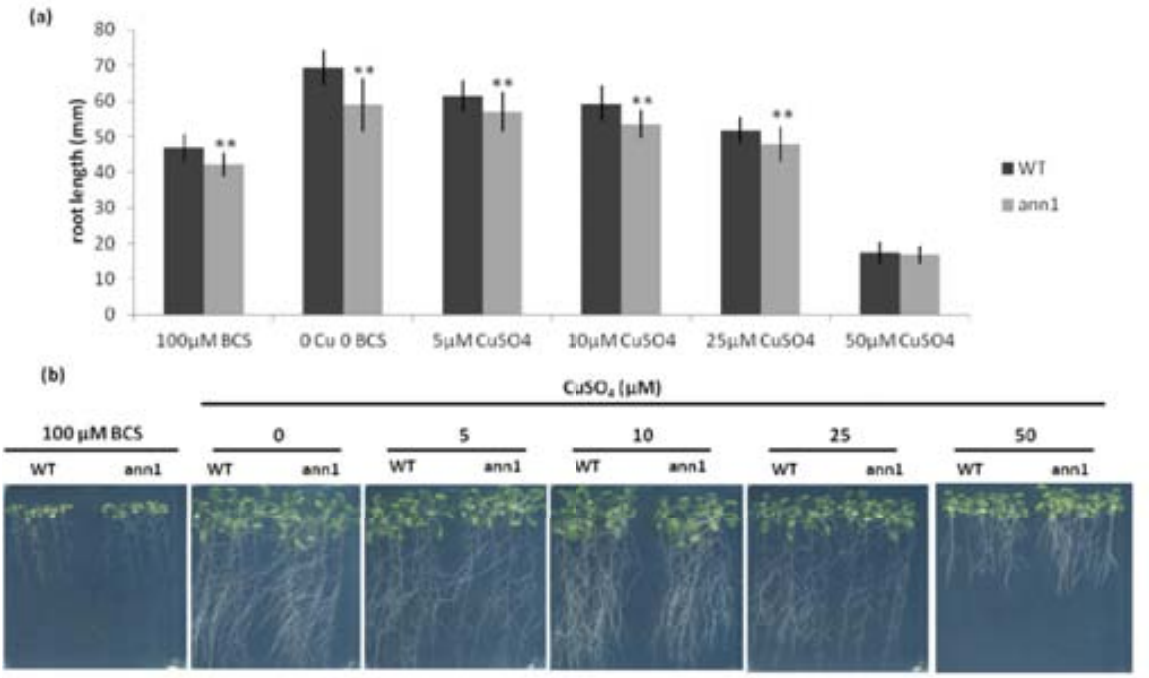

Figure 4.3.2 (a) Average root length for 4d old seedlings transferred from ½MS medium to Cu or BCS supplemented plates and grown for 6 days. Mean ± Standard deviation of four independent experiments is shown (n=20). Asterisks indicate statistically significant differences with respect to the wild type, ANOVA test (**P <0.01) **(b)** Phenotype of the seedlings grown for 3 weeks on the ½MS medium with the indicated amounts of BCS or $CuSO_4$.

Fresh weight (Fig. 4.3.3) of *ann1* seedlings was lower under nontoxic copper treatments (from deficiency to 5µM), and progressively increased with 10 and 25µM copper treatments. Last, was similar to the wild type under high copper toxicity (50µM).

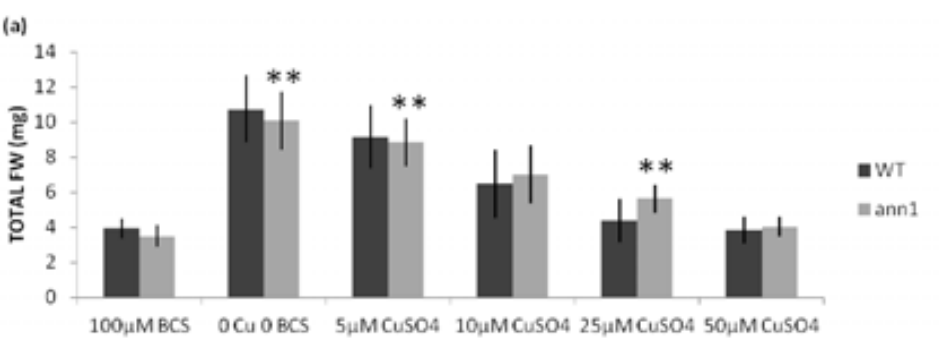

Figure 4.3.3 Total fresh weight of 4d old seedlings transferred from ½MS medium to Cu or BCS supplemented plates and grown for 10 days. Mean ± Standard deviation of four independent experiments is shown (n= 14-20).

Section B. Study of HR-activation of annexin A5 using planar lipid bilayers (PLBs).

Initially, pH in both *cis*-trans was set to 7.4 (MES/BTP), as activity of A5 has been found previously under these conditions (Rojas *et al.,* 1994, Hofmann *et al.,* 1997; Neumann *et al.*, 2000), and symmetrical conditions were used (50mM CaCl2, 3mM MES, 3mM BTP pH 7.4). Final A5 protein concentration in *cis* was 70nM. The bilayer was held at -90mV. No conductance appeared after 1.5 hours.

As annexins have shown high pH-dependency, pH was changed to more acidic values. For the rest of attempts, symmetrical *cis-trans* conditions were kept (50mM $CaCl_2$, pH 6.3 MES/BTP). Holding potential was changed in order to achieve an optimal protein insertion, and also different step-voltage protocols were tested for getting optimal experimental conditions. The holding potential was kept at -90mV in the first two experiments at the new pH conditions (Fig. 2.3.4 and 2.3.5), and at -120mV for the rest of the experiments, due to a quicker integration of the protein observed at more negative values (Fig. 2.3.6).

In all the attempts (five), no protein insertion was observed under non-oxidised conditions after 40 to 60 minutes (Fig. 4.3.4 to 4.3.6).

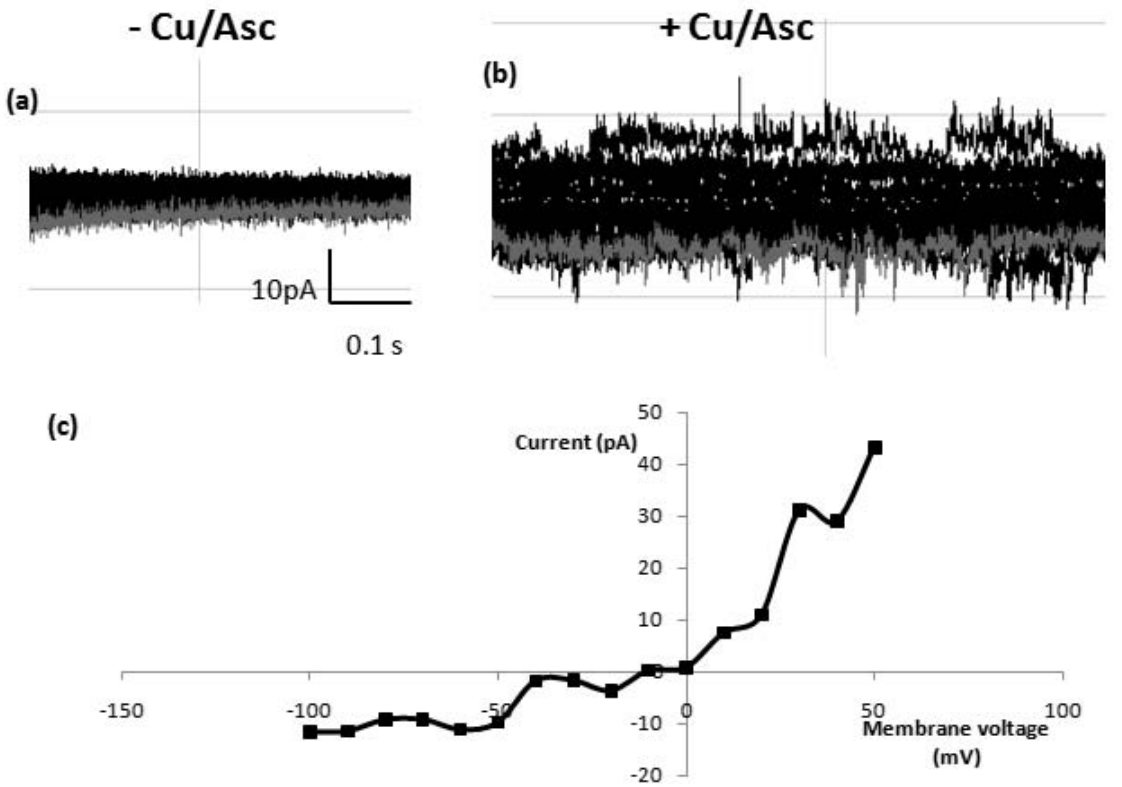

Figure 4.3.4 Hydroxyl radical-activated current by annexin V (A5). Experimental conditions: Symmetrical *cis-trans* 50mM $CaCl_2$, pH 6.3. Holding potential was -90mV. **(a)** After 45', no conductance was observed in the absence of hydroxyl radical production. **(b)** A5-induced current was observed 22' after Cu/asc addition. Voltage changed in steps of 10mV, from -100 to +100mV. **(c)** I-V relationship constructed from the voltage step protocol.

Following the addition of 1mM Cu/Asc (OH·-generating mixture, Halliwell & Gutteridge, 1999), conductance was observed in all cases, requiring from 20 to 40 minutes when the holding potential was -90mV (See fig, 4.3.4 and 4.3.5) and 5 to 10 minutes when it changed to -120m or -150mV (Figure 4.3.6). At this more negative holding potential, conductance was seen in the four attempts, but only in two of them a complete step-voltage protocol was possible, due to the high integration of the protein in the bilayer, causing its rupture (data not shown).

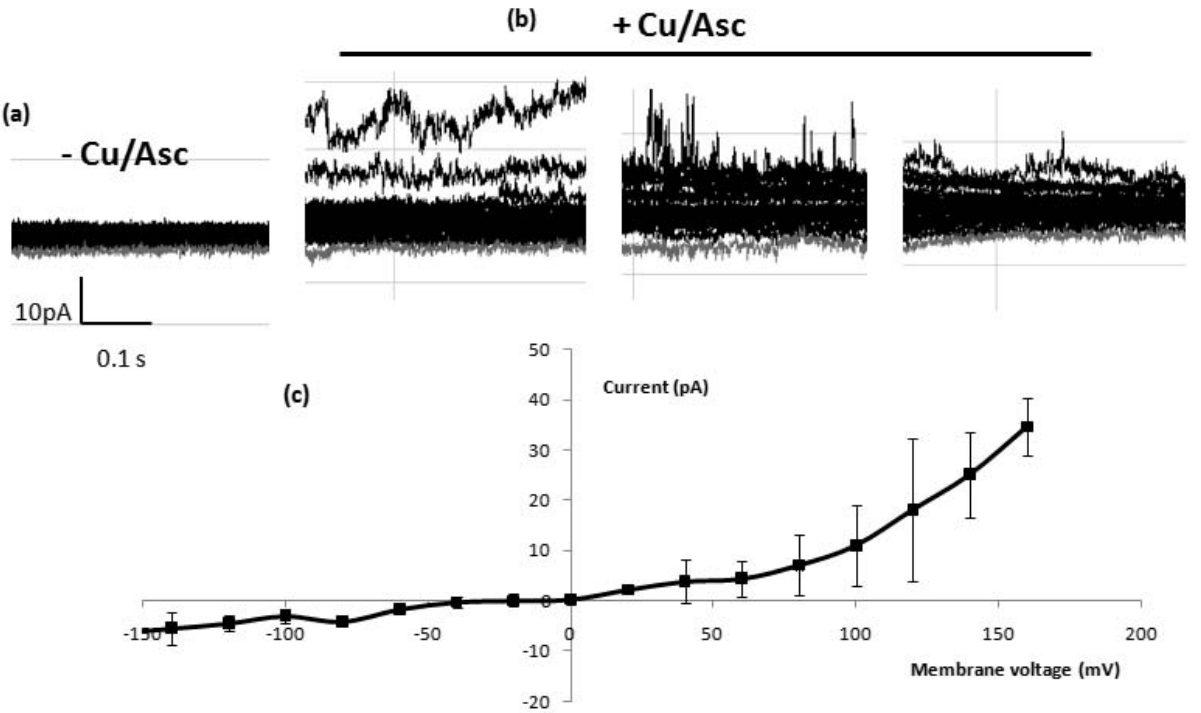

Figure 4.3.5 Hydroxyl radical-activated current by A5. Experimental conditions: Symmetrical *cis-trans* 50mM $CaCl_2$, pH 6.3. Holding potential (HP) -90mV. **(a)** After 1h, no conductance was observed in the absence of hydroxyl radical production. A5-induced current was not observed after 45' of Cu/asc addition and A5 was taken to a final concentration of 140nM. HP was kept at -90mV. **(b)** Change in HP to more negative values showed A5-mediated conductance. Voltage changed in steps of 20mV, from -200 to +200mV. **(c)** I-V relationship constructed from the voltage step protocols.

Gadolinium (50 μM) was tested , causing a partial inhibition in the conductance promoted by the A5 protein (Fig.4.3.6).

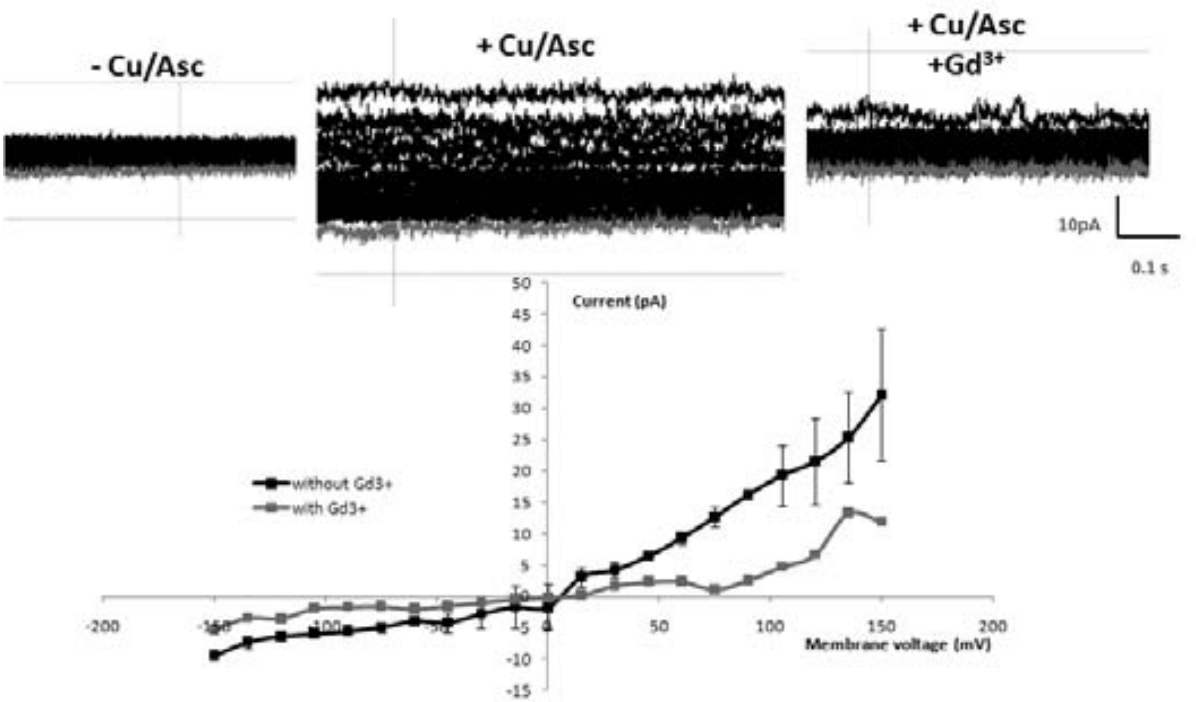

Figure 4.3.6 Hydroxyl radical-activated current generated by A5. Experimental conditions: Symmetrical *cis-trans* 50mM $CaCl_2$, pH 6.3. Holding potential (HP) -120mV. **(Up panels)** After 40', no conductance was observed in the absence of hydroxyl radicals production. (left) A5-induced current was observed after 10' of Cu/asc addition (middle panel) Addition of gadolinium to the *trans* chamber caused inhibition of the current (right). **(Down)** I-V relationship constructed from gadolinium panel and from two consecutive step protocols is shown in absence of the blocker.

4.4 Discussion

Section A. Effect of copper deficiency- toxicity on *ANN1* loss of function growth, weigh and chlorophyll content.

There is clear evidence for *AtANN1* activation by hydroxyl radicals. The loss of function *ann1* has impaired root cell growth and does not have ability to elevate cytosolic free Ca^{2+} in response to hydroxyl radicals. Root epidermal protoplast $[Ca^{2+}]_{cyt}$, measured with *apoaequorin* revealed that in *ann1*, more than 20% of the signal was lost. In addition, *AtANN1* Ca^{2+}-mediated conductance has been shown to be suppressed by the calcium channel blocker gadolinium (Lahoavisit *et al.*, unpublished results). Copper has been shown to produce hydroxyl radical *in vivo* in Arabidopsis roots that regulates K^+ and Ca^{2+} channels, and that Ca^{2+} conductance is blocked by two Ca^{2+} channel blockers, verapamil and gadolinium (see chapter 3).

Seedlings of Col-0 and *ann1*showed a reduction of root length and biomass under both deficiency (BCS) and excess of copper conditions, according to previous results (Lequeux *et* al., 2010; Garcia-Molina *et al.* 2011).

The preliminary results presented here point to a role for *AtANN1* in copper toxicity, supported by the fact that both impaired root growth and fresh weight in *ann1* are suppressed at highly toxic copper concentrations. If Ca^{2+} conductance generated by hydroxyl radicals is impaired, the positive feedback through NADPHoxidase would then generate moderate ROS accumulation and the consequent K^+ leakage should be considerably lower. This moderate hydroxyl radical generation would be necessary for root growth (Foreman *et al.,* 2003).

Section B. Study of HR-activation of annexin A5 using planar lipid bilayers (PLBs).

No spontaneous A5 protein insertion into the bilayer in non-oxidized conditions at different pH values (7.4 and 6.3) was achieved. However, insertion of the protein was successful in all the attempts after adding the OH·-generating mixture (1mM Cu/Asc). The insertion of A5 into the bilayers showed a high holding voltage-dependency on A5 protein insertion into the bilayer. A holding potential of -90mV not always guaranties the insertion, while changing the potential to more negative values (-120mV) causes a quick insertion in about minutes.

Cellular distribution of annexins has been shown to be regulated by oxidative stress (Sacre *et al.*, 2002). Human annexin 5 is a Ca^{2+}-dependent-phospholipid binding protein that binds to cell surface in early stages of apoptosis (Koopman *et al*., 1994). In human, oxidative stress is involved in many diseases, like heart failure (Maack *et al.,* 2009) and neurodegenerative diseases (Allsop *et al.*, 2008). Annexin A5 has been reported to have a function in the peroxide-induced Ca^{2+}-influx pathway (Kubista *et al.*, 1999) and might be involved in the regulation of early apoptotic events during cardiac pathological situations (Monceau *et al.*, 2006). This HR-activated conductance of annexin A5 could be relevant in the function of the protein in some of these human oxidative stress-involved diseases.

Conclusions and future work

The loss of function *ann1* has been shown impaired hydroxyl activated-Ca^{2+}, and preliminary experiments showed here revealed that its response to external copper varies with respect to the wild-type. This mutant has impaired growth that reverts to the wild type when toxic copper conditions were applied. This corroborates the idea that Ca^{2+}-dependent feedback ROS formation by NADPH oxidase is a key mechanism during growth.

In humans, hydroxyl radical are implicated in many oxidative diseases, such as heart failure or neurodegenerative diseases. A role for annexin V (A5) in apoptosis it is also clear. The Ca^{2+}-activated conductance of A5 showed here by hydroxyl radicals might explain the function of this protein in some of these oxidative induced-diseases.

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Conclusions

- K^+ leakage, a parameter that normally correlates with salinity and oxidative stress tolerance, must be understood in a different way in halophytes, as a mechanism for preventing the lethal effect of membrane depolarization due to Na^+ entry in the cells.
- Salinity and ROS stresses are likely to activate differential ionic mechanisms in halophytes.

- Copper transport into cytosol is a key factor contributing to intracellular hydroxyl radicals generation and a consequent regulation of plasma membrane $OH^{\bullet}$-sensitive Ca^{2+} and K^+ channels in Arabidopsis roots.

- Copper transport is likely to be a key process in HR-generation and growth under both favorable and toxic copper environments.

- The loss of function *ann1* that has impaired hydroxyl activated-Ca^{2+}conductance and, also lower copper toxicity response. This corroborates the supported idea that Ca^{2+}-dependent feedback ROS formation by NADPH oxidase is a key mechanism during growth.

- The Ca^{2+}-activated conductance of A5 by hydroxyl radicals shown here might explain the function of this protein in some of the oxidative induced-diseases.