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Role of ion channels in programmed cell death induced by hyperosmotic stresses in plant cells

Emanuela Monetti

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PhD Thesis

Emanuela Monetti

ROLE OF ION CHANNELS IN PROGRAMMED CELL DEATH INDUCED BY HYPEROSMOTIC STRESSES IN PLANT CELLS

PhD defense on November 17th 2014

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The present work was done in the Laboratoire Interdisciplinaire des Énergies de Demain (LIED-UMR 8236) of Université Paris Diderot-Paris 7 and in the LINV-DiSPAA (Department of Agri-Food and Environmental Science) of University of Florence

Abstract

The work presented in the present thesis relates to the role of ion channels in response to (ionic and non-ionic) hyperosmotic stresses and their interactions with signaling events leading to PCD in plant. Early cell responses such as cytosolic calcium increase and ROS production classically involved in PCD process, seems not to be involved in hyperosmotic-induced cell death in BY2 tobacco and *A. thaliana* cultured cells. When BY2 tobacco cells were subjected to hyperosmotic stress, an early influx of sodium through non-selective cation channels participates in the development of PCD through mitochondrial dysfunction and NADPH-oxidase-dependent $O_2^{\cdot-}$ generation. On the contrary, non-ionic hyperosmotic stress resulted in an early decrease in anion currents. To further investigate the role of anion channels in non-ionic hyperosmotic stress further experiments were conducted by using *A.thaliana* cells of the anion channel mutant SLAC1. Results showed that the delayed activation of SLAC1 channels was involved in the non-ionic hyperosmotic stress induced pathway leading to cell death. Interestingly, the early anion channel activity decrease could participate to signalisation or osmotic adjustment allowing cell adaptation and survival, when a second set of events, namely superoxide anion ($O_2^{\cdot-}$) generation by NADPH-oxidase and anion channel activation could participate in PCD development of a part of the cell population. In addition, the potential role of small peptides belonging to the FMRFamide-like peptide (FLP) family described in metazoan in osmoregulation in *A. thaliana* was investigated. By using synthetic peptides, based on FLPs homolog genes existing in *A. thaliana*, it was possible to demonstrate that these putative FLPs are involved in hyperosmotic stress response. Overall, the present work shed light on the importance and the complexity of ion channels regulation in the signaling pathways and the processes leading to PCD.

Résumé

Le travaux présenté dans cette thèse concerne le rôle des canaux ioniques de la membrane plasmique en réponse à des stress salins et non salins ainsi qu'aux interactions possibles avec d'autres événements de signalisation conduisant à la mort cellulaire programmée (PCD). Nous avons montré que les réponses cellulaires précoces:

tels que l'augmentation du calcium cytosolique et la production de ROS, classiquement impliqués lors de la PCD, ne semblaient pas être impliqués dans la mort cellulaire induite par les stress hyperosmotiques chez les cellules en culture de tabacco BY2 ou d'*A. thaliana*. Nous avons montré que, dans les cas de stress salin chez les cellules de BY2 un influx précoce de sodium à travers des canaux cationiques non spécifiques participe au développement de la PCD en entraînant un dysfonctionnement mitochondrial et la production de $O_2^{\bullet -}$ par des NADPH oxydases. Dans le cas de stress hyperosmotique non-ionique, nous avons observé une diminution précoce de l'intensité des courants anioniques. Afin de poursuivre l'étude du rôle des canaux anioniques lors du stress hyperosmotique non salin, nous avons utilisé des cellules *A.thaliana* nous permettant de travailler avec le mutant de canal anionique SLAC1. Nous avons constaté que l'activation retardée des canaux SLAC1 participait au développement de la PCD induite par un stress hyperosmotique non salin. La réduction précoce de l'activité des canaux anioniques pourrait participer à la signalisation ou l'ajustement osmotique permettant l'adaptation et la survie cellulaire alors que des événements retardés, à savoir la production d'anion superoxyde ($O_2^{\bullet -}$) par les NADPH-oxydases et l'activation des canaux anioniques pourraient participer au développement de la PCD d'une partie de la population cellulaire. Nous avons aussi étudié le rôle potentiel des petits peptides appartenant à la famille des peptides FMRamide décrite chez les métazoaires à l'osmorégulation chez des cellules d'*A. thaliana*. Des gènes susceptibles de coder de tels peptides sont en effet présent dans le génome d'*A. thaliana*. En utilisant des peptides synthétiques, nous avons montré que ces FLPs putatifs pourraient participer aux réponses induites lors de stress hyperosmotique chez les plantes. Ce travail illustre la complexité et l'importance de la régulation des canaux ioniques dans les voies de signalisation et les processus conduisant à la PCD.

Riassunto

Il lavoro presentato in questa tesi riguarda il ruolo dei canali ionici nella membrana plasmatica in risposta a stress di tipo iperosmotico (salino e non salino) e il loro coinvolgimento nelle vie di segnalazione che portano alla PCD in pianta. Le risposte precoci (es. aumento di calcio citosolico e produzione di ROS) a stress di tipo iperosmotico, coinvolte negli eventi che portano alla PCD, non sembrano essere

implicate nel modello da noi studiato (cellule di tabacco BY2 e di *Arabidopsis*). Abbiamo dimostrato che nel caso di stress ionico l'influsso di sodio attraverso i canali NSCC (Non Selective Cation Channels) partecipa allo sviluppo della PCD inducendo depolarizzazione mitocondriale e produzione di O_2^- da parte dell'enzima NADPH ossidasi. Nel caso di stress non ionico abbiamo riscontrato una precoce diminuzione delle correnti anioniche. Per studiare meglio il ruolo dei canali anionici abbiamo usato cellule di *A.thaliana* che ci permettono di lavorare su mutanti. Abbiamo dimostrato che i canali SLAC1 risultano coinvolti negli eventi che portano alla PCD in seguito a stress di tipo non ionico. La diminuzione di correnti anioniche potrebbe partecipare alla regolazione osmotica che permette l'adattamento cellulare. Un'attivazione delle correnti anioniche e generazione di O_2^- da parte dell'enzima NADPH-ossidasi potrebbe partecipare allo sviluppo della PCD di una parte della popolazione cellulare. Abbiamo inoltre indagato il potenziale ruolo di piccoli peptidi appartenenti alla famiglia FMRF amide-like peptide (FLP) nella regolazione di stress osmotico in colture cellulari di *A.thaliana*. Utilizzando peptidi sintetici, abbiamo dimostrato che questi peptidi potrebbero partecipare alla regolazione di stress osmotico nelle piante. Questo lavoro mette in luce l'importanza e la complessità della regolazione dei canali ionici nelle vie di segnalazione degli eventi che portano alla PCD.

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Chapter 1

Introduction

1 Hyperomotic stress

In nature, plants are subjected to different biotic and abiotic stresses. Among abiotic stresses, drought and salinity are the primary causes of crop loss worldwide. Global warming, due to climate change, will enhance these environmental stresses that may severely affect crop productivity (Peters *et al.*, 2013). Drought, defined as soil and/or atmospheric water deficit, reduces plants water potential and turgor. Among drought effects are loss of water, which is involved in stomatal closure and limitation of gas exchange that results in reduced plant growth and productivity and could lead to death. It is expected an increase of drought frequency mainly due to climate change, as a result of decreasing regional precipitations and increasing evaporation driven by global warming (Seneviratne *et al.*, 2012; Sheffield *et al.*, 2012). Since 1970 significant increases in drought extent and severity have already been estimated for Africa, southern Europe, east and south Asia and eastern Australia (Lobell and Gourdj, 2012; Sheffield and Wood, 2008). Water deficit and aridity conditions lead also to salinization of the soil. Salinity has also been identified as one of the major threats because of its degrading effects on landscapes (Ghassemi *et al.*, 1995). Increased salinization of arable lands is expected, resulting in 50% land loss by the year 2050 (Wang *et al.*, 2003).

Drought and salinity have osmotic, ionic and nutritional constraint effects on plants. These effects lead to growth retardation, metabolic disturbances and oxidative stress. Plants may tolerate and adapt to these stressors with different mechanisms including changed leaf architecture, osmotic adjustment, ion exclusion and compartmentalization and a more efficient reactive oxygen species (ROS) scavenging systems. However, depending on the strength and duration of these stresses and on the plant's genetic features, it can be a matter of life or death since there is always the so-called "stability limit", which when exceeded leads the organism to death. In Figure 1 is shown the main abiotic stress that affects plants in nature.

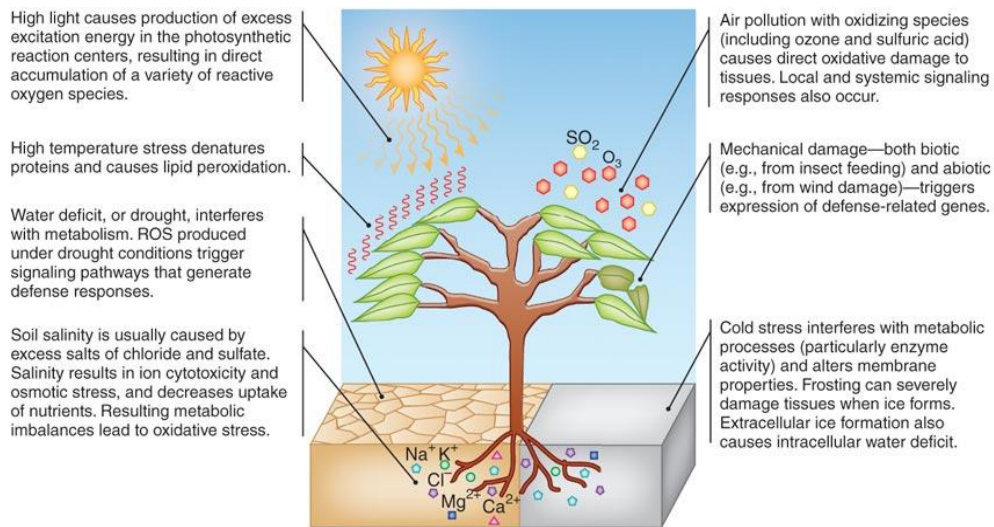


Figure 1: Overview of abiotic stresses that affect plants (Vickers *et al.*, 2009)

1.2 General plant responses to osmotic stresses

Plants have evolved sophisticated systems to detect and respond to osmotic stresses (Hirayama and Shinozaki, 2010). The general plant osmotic stresses response include a complex signaling pathway, that involves: (i) the reception of the stress, through the osmosensors that are probably located at the plasma membrane level; (ii) the signal transduction (i.e. osmosignaling) to the nucleus where (iii) the last step of controlled expression of specific genes occurs. The molecular basis of osmosensing is not fully understood in plants and little is known about the receptors that detect osmotic stress, much information is available for numerous transcription factors acting in the regulation of gene expression. The signals transductions, amplify and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression or ion-channel activity.

1.2.1 Osmosensor

Osmosensing is pivotal for maintaining a normal water status and all cellular functions in plants. All living cells developed sophisticated tools to sense and respond quickly to osmotic stresses. Osmosensors are proteins whose primary role is to monitor fluctuations in external osmolarity and start an activation of signalling pathways for osmo-adaptation. (Osakabe *et al.*, 2011; Yamaguchi-Shinozaki and Shinozaki, 2006). In general, an osmosensor can be defined as a component that is altered by osmotic stresses. The molecular basis of osmosensing in plants is not fully understood and little is known about the receptors that detect osmotic stress. Signals emanate from several osmosensors are involved in a combinational way that allows highly specific outcomes despite the lack of exclusively specific osmosensors (Kültz, 2012). It has been hypothesized that osmosensing involve the sensing of cell volume, shape, membrane tension or macromolecular crowding by osmosensor proteins (Burg *et al.*, 2007; Kumar *et al.*, 2013; Schliess *et al.*, 2007). In animal cells slight osmolality can be sensed by calcium sensing receptors and transient receptor potential (TRP), in particular TRPV4 in vertebrates (Liedtke *et al.*, 2000; Nilius *et al.*, 2004; Strotmann *et al.*, 2000; Wissenbach *et al.*, 2000). Although is not very clear whether TRP is involved in volume regulation, it was found that it mediates the Ca^{2+} increase which subsequently stimulates the Regulatory Volume Decrease (RVD) with the activation of K^+ channels (Numata *et al.*, 2007). Interestingly, a recent study made a parallelism between plant and animal kingdom after finding a Calcium permeable Stress-gated cation Channel 1 (AtCSC1) in *A. thaliana* (Hou *et al.*, 2014). The analysis of whole-cell two-electrode voltage clamp (TEVC) on *Xenopus* oocytes expressing this gene, led to the conclusion that this protein is a ion channel, which is permeable to various cations, including Ca^{2+} , K^+ and Na^+ . Interestingly, the C-terminal of AtCSC1 comprising domain of unknown function 221 (DUF221) belongs to transmembrane channel-like (TMC) proteins that are cation channel components. Since CSCs are a family of cation channels permeable to calcium triggered by physical signals such as hyperosmotic stress, and these properties are reminiscent of TRPs in animals, these channels are candidates for involvement in osmo- or mechano-sensitive calcium signaling processes in plants (Hou *et al.*, 2014). Receptor-like kinases (RLKs), which form a large gene family in plants, contain Ser/Thr kinase as a cytosolic domain while having structural elements similar to animal

receptor tyrosine kinases (RTKs) (Osakabe *et al.*, 2013; Shiu and Bleecker, 2001). The RLKs are involved in water stress responses, and this receptor like kinase (RLK) family is composed by more than 600 members, with the leucine rich-repeat (LRR)-RLKs forming the largest subgroup (Gish and Clark, 2011; Osakabe *et al.*, 2011). Several RLKs localized in the plasma membrane are known to be involved in the early steps of osmotic-stress signaling in a variety of plant species (Osakabe *et al.*, 2013). Several studies have been performed to explore the involvement of RLK gene in environmental stresses (Chae *et al.*, 2009; Lehti-Shiu *et al.*, 2009). RLKs involvement in sensing environmental cues and abiotic stresses have been reported for various plant species, such as *Arabidopsis* (Bai *et al.*, 2009; Osakabe *et al.*, 2005; Osakabe *et al.*, 2010; Tanaka *et al.*, 2012), rice (*Oryza sativa*) (Ouyang *et al.*, 2010), *Medicago truncatula* (De Lorenzo *et al.*, 2009), and soja (Yang *et al.*, 2010). In particular in plants there are some RLKs such as RPK1, CYSTEINE-RICH RLK (CRK36), PROLINE-RICH-EXTENSIN-LIKE RLK4 (PERK4), and GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1), that studies have been reported to control water stress signalling directly in *Arabidopsis* (Bai *et al.*, 2009; Osakabe *et al.*, 2005; Osakabe *et al.*, 2010; Osakabe *et al.*, 2013). Interestingly, de Lorenzo *et al.* (2009) identified a salt stress-inducible LRR-RLK gene (SRLK) in *M. truncatula*, and root growth inhibition by high salinity stress was reduced in SRLK RNA interference (RNAi) transgenic *Medicago* roots. Epidermal cell-specific expression of the SRLK gene was observed in roots under salt stress, and SRLK was shown to control the expression level of several salt-responsive genes. These findings suggest that SRLK activates the signalling pathway involved in the adaptive response of *Medicago* roots to salt stress (De Lorenzo *et al.*, 2009; Osakabe *et al.*, 2013). It tempting to suppose that plants as well as vertebrate cells, have evolved a sophisticated mechanism at the plasma membrane to sense osmotic changes. Clearly, it is not surprising that plants have evolved mechanisms to sense osmotic forces at the plasma membrane, as these are the first cell component to come in contact with the stress. In animal cells, because they lack a cell wall, as well as the membrane itself cannot provide a mechanical barrier, the actin cytoskeleton has been proposed to control shape after osmotic challenge in animal cells (Papakonstanti *et al.*, 2001). In different cell lines, a rapid organization of the actin filaments of cytoskeleton has been observed as a result of osmotic stress. This type of organization

varies between different cell types, but generally leads to an increase of the F-actin (Pedersen *et al.*, 1999). Recent studies have demonstrated that microtubule-associated (MT) to the cytoskeleton are regulated by osmotic shrinkage. Nevertheless, the mechanisms that link the sensing of the perturbation of the volume to the rearrangement of the cytoskeleton have not been completely elucidated. In plants there is a strong and transient response of microtubules to hyperosmotic stress: microtubules first disappear, but soon they are replaced by massive bundles, called macrotubules (Komis *et al.*, 2002). The formation of macrotubules can be suppressed by oryzalin, which at the same time blocks osmoadaptation, demonstrating that this microtubule response is not a product of adaptation but represents an essential event. A pharmacological study on this phenomenon revealed that inhibitors of phospholipase D, such as *n*-butanol or *N*-acetyethanolamine suppress both macrotubule formation and osmotic adaptation (Komis *et al.*, 2006). In vertebrate cells, the integrins have an important role in osmotic sensing. Different studies had shown (Komis *et al.*, 2002) that integrins were activated as a result of disruption of the cell volume, and has been proposed that they serve as the volume sensors after swelling or shrinkage. It was demonstrated an involvement of integrins in the regulation of osmosis and in the movement of ions across cell membranes, in particular they control the flow of calcium, potassium and chlorine, either directly (Hoffmann and Pedersen, 2011) or indirectly (Hoffmann and Pedersen, 2011; Shakibaei and Mobasheri, 2003). It is difficult to transfer these mechanisms to plants, since they have not yet identified canonical integrins in plant cells (Baluška *et al.*, 2003) and the situation in plant cells differs fundamentally. In plants, the shape is maintained thanks the cell wall, where elongate load-bearing elements (cellulose microfibrils) are embedded in an amorphous matrix (hemicelluloses, pectins, proteins). In these cells, the interphasic plant cytoskeleton is not directly required to support cell shape and therefore it is free to adopt additional functions. In plants, cortical microtubules are connected with the extracellular matrix through transmembrane proteins that have not been identified so far, but it seems that they share some analogies with animal integrins (Baluška *et al.*, 2003). This link seems to stabilize cortical microtubules. For instance, removal of the cell wall makes microtubules more cold-sensitive in tobacco cells (Akashi *et al.*, 1990). Moreover, cobtorin, a compound identified from a screen that specifically perturbs the parallelity of microtubules and

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microfibrils (Yoneda *et al.*, 2007) has meanwhile been found to target the cell wall pectins.

1.3 Signalisation in response to osmotic stress

Reactive Oxygen Species (ROS), Calcium (Ca^{2+}), Mitogen activated protein kinases (MAPK) and ion channels are recognized to be important in plant signalisation responses to osmotic stress.

1.3.1 Reactive Oxygen Species (ROS) signalling

The ROS signalling network controls a wide range of biological processes such as growth, development and responses to biotic and/or abiotic stimuli (Mittler *et al.*, 2011). ROS are produced as a normal cellular process in all aerobic organisms and include free radicals such as superoxide anion (O_2^-) hydroxyl radical ($\cdot\text{OH}$), as well as non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$). ROS are produced by reduction of molecular oxygen (O_2) by means of the electron transport activities of chloroplasts, mitochondria, and plasma membranes (Foyer *et al.*, 1995; Heyno *et al.*, 2011). Also NADPH oxidases respiratory burst oxidase homologues (RBOHs), is involved in ROS production (Suzuki *et al.*, 2011; Torres and Dangl, 2005). During osmotic stress, drought and salinity, ROS production increases (Serrato *et al.*, 2004). During water deficit there is an accumulation of abscissic acid (ABA) in leaves, which minimize water loss by stimulating stomatal guard cell closure (Li *et al.*, 2000). The CO_2 available for photosynthesis is reduced and it can misdirect electrons in the photosystem and lead to the formation of ROS. Unlike biotic stress, where the so called oxidative burst is frequently involved in the events that lead to programmed cell death (PCD), the role of ROS production during osmotic stress needs to be demonstrated. Under abiotic stress ROS seem to be involved in a dual process depending on their cellular concentrations. At low levels ROS have functions as components of a pathway that lead to stress defense/acclimation responses (Vranova *et al.*, 2002). When reaching a level of phytotoxicity ROS become detrimental and participate in the events triggering to cell death (Mittler, 2002).

1.3.1.1 Plant ROS generation

1.3.1.1.1 Singlet Oxygen (1O_2)

Singlet oxygen (1O_2) is a highly reactive short-lived product (half-life, approximately 200 ns) with a strong oxidizing potential (Apel and Hirt, 2004; Vellosillo *et al.*, 2010). 1O_2 is produced constitutively by a photochemical excitation of the chlorophyll of photosystem II with oxygen (Krieger-Liszkay *et al.*, 2008). In Figure 2 is shown the generation of 1O_2 by excited chlorophyll (Fischer *et al.*, 2013). 1O_2 can be also produced by phytochemicals during plant-pathogen interaction, and by peroxidases (POX) (Kanofsky, 2000; Kawano *et al.*, 1998).

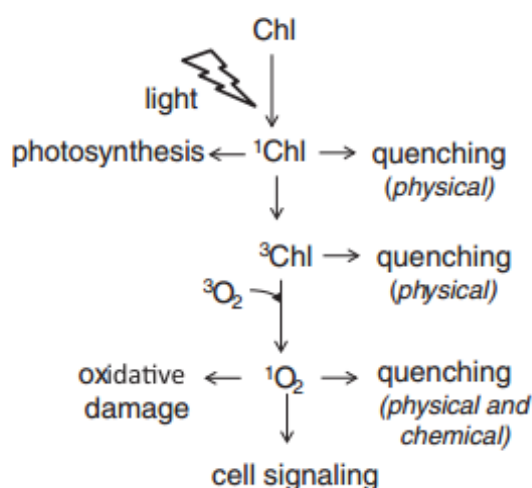
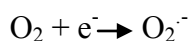


Figure 2: 1O_2 generation by excited chlorophyll (from Fischer *et al.*, 2013)

Peroxidases (POXs) could also be involved in 1O_2 generation (Kanofsky *et al.*, 1988; Kawano *et al.*, 1998). Peroxidases are involved in a wide range of physiological processes, such as lignification, suberization, auxin catabolism, wound healing and defense mechanisms against pathogen infection (Kawano, 2003). The plant POXs in the apoplastic space are often involved in ROS generation. Peroxidases are considered essential to catalyze the generation of aromatic oxyl radicals from several aromatic compounds (Kawano, 2003; Takahama and Yoshitama, 1998) and this POX-dependent production is often associated to ROS generation (Kawano, 2003).

1.3.1.1.2 Superoxide anion ($O_2^{\cdot-}$)

The first reaction of molecular oxygen is a reduction to form the superoxide anion ($O_2^{\cdot-}$).



$O_2^{\cdot-}$ is the product of the reduction of oxygen by one electron. An enzymatic sources include NAD(P)H oxidases located on the cell plasma membrane of peroxisomes. Dismutation of $O_2^{\cdot-}$ produces hydrogen peroxide (H_2O_2), which may be reduced to water (H_2O) or partially reduced to hydroxyl radical (OH^{\cdot}).

The partial reduction of O_2 may generate different Reactive Oxygen Species (ROS). Moreover, ROS may be produced through transition metal-mediated pathways in the Fenton reaction or in the net Haber-Weiss reactions. A schematic representation of the sequential ROS production is shown in Figure 3

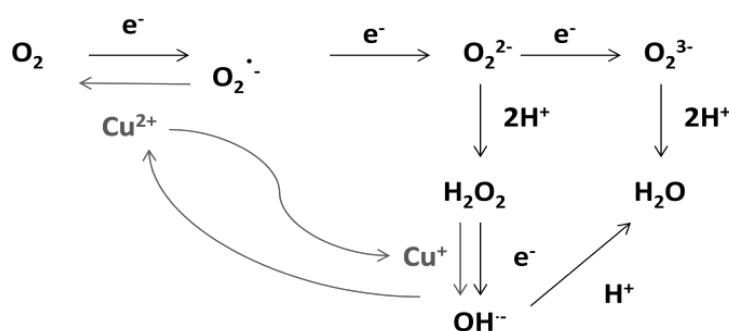
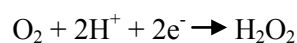


Figure 3: ROS production by multistep reduction of oxygen.(from Appel & Hirt, 2004; Gechev *et al.*,2006). Grey lines show the Haber-Weiss reactions.

1.3.1.1.3 Hydrogen peroxide (H_2O_2)

The hydrogen peroxide production represents an evolutionary strategy performed by plants. In response to various environmental stimuli there is an increase of O_2 production, which is subsequently converted to hydrogen peroxide (H_2O_2).

As the $O_2^{\cdot-}$ is relatively unstable, it can be converted to O_2 or in reaction with a proton to H_2O_2 , spontaneously or catalyzed by the enzyme superoxide dismutase (SOD):

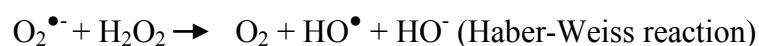
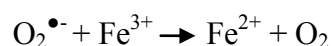


H_2O_2 is a relatively long-life ROS, it can pass through the cellular membranes and act as signalling molecule in cell-to-cell communication because it's diffusable between cells (Bienert *et al.*, 2006). It has been shown that ROS participate in various types of cell-to-cell communication in animals and plants. For example in animal cells ROS regulate the formation of intracellular gap junctions and signal transduction (Upham and Trosko, 2009) involved in apoptosis in adjacent animal cells (Widel, 2012).

1.3.1.1.4 Hydroxyl radical (HO^{\cdot})

The hydroxyl radical (HO^{\cdot}), one of the strongest oxidant, is highly reactive and interact with proteins, nucleic acids, lipids and phospholipids. HO^{\cdot} is involved in radical reactions and then is the major responsible for irreversible changes of macromolecules and damages to organelles. HO^{\cdot} has a very short time-life (10^{-9} s) and interacts exclusively in the environment surrounding the site of production.

H_2O_2 takes part in the formation of HO^{\cdot} , which is produced in the reaction between H_2O_2 and Fe^{2+} acting as a metal catalyst (Fenton reaction). The equation below represents the main way of HO^{\cdot} production:



1.3.1.1.5 Nitric oxide (NO^{\cdot})

Nitric oxide is a radical synthesized from arginine by NO synthase (NOS) in mammalian cells (Wendehenne *et al.*, 2001). In plants the system responsible for its synthesis is unknown yet. Its derivate peroxynitrite $ONOO^-$ can react with $O_2^{\cdot-}$ and induce protein modification by tyrosine nitration (Gaupels *et al.*, 2011). Although the

peroxynitrite is highly reactive and toxic in animals, it is not involved in NO-mediated cell death in plants (Delledonne *et al.*, 1998; Gaupels *et al.*, 2011).

1.3.1.2 Sources of ROS in plant cells

As mentioned above, ROS are produced by plant cells under normal conditions as products of metabolic processes and as responses to various environmental stresses. Different plant cell compartments are involved in ROS production. Chloroplasts, mitochondria and peroxisomes are considered the main ROS producers during abiotic stresses (Asada, 2006b; Bose *et al.*, 2013; Mittler, 2002; Mittler *et al.*, 2004). A schematic representation of ROS production in plant cell compartments is shown in Figure 4.

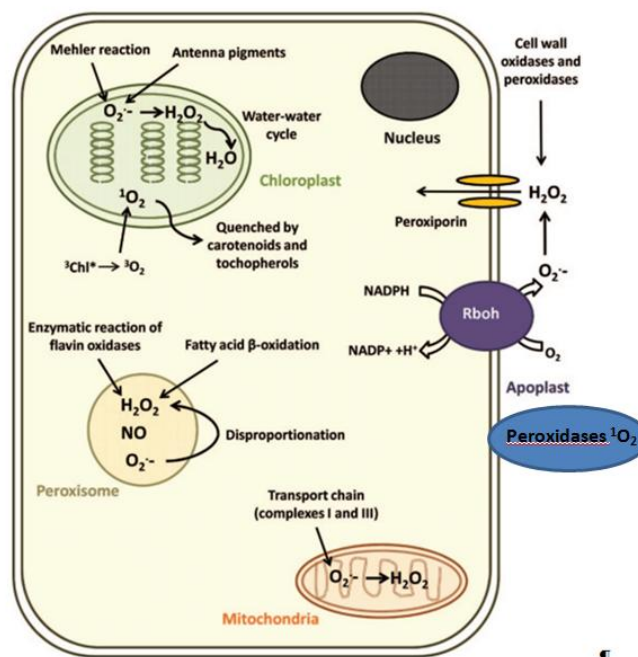


Figure 4: Sources of ROS production in plant cells (Adapted from Bose *et al.*, 2013)

1.3.1.2.1 Chloroplasts

In the light chloroplasts produce ROS through photosystem I (PSI) and PSII electron transport chain (ECT) that are the main reactions centres (Asada, 2006a; Foyer and Noctor, 2003). The antenna pigments are the main producers of O_2^- by the Mehler

reaction at PSI (Asada,2006). After, H₂O₂ is produced spontaneously or by a reaction catalyzed by SOD and then further reduced by the action of ascorbate peroxidases (APXs) in the water–water cycle (Asada, 2006). In addition singlet oxygen ¹O₂ can be produced during photosynthesis (Laloi *et al.*, 2004). It was shown an enhanced ¹O₂ generation at PSI during water stress (Miller *et al.*, 2010). The reduced CO₂ availability due to stomata closure and the excessive exposure to light are involved in this enhanced ¹O₂ generation.

1.3.1.2.2 Peroxisomes

The peroxisomes are a type of microbody with a single membrane. Peroxisomes are intracellular organelles mediating a wide variety of biosynthetic and biodegradative reactions. These organelles generate large quantities of ROS (Nyathi and Baker, 2006). Peroxisomes are also involved in senescence (Rio *et al.*, 2006) and heavy metal toxicity (Romero-Puertas *et al.*, 1999). Peroxisomes can be divided into three groups: glyoxysomes, leaf peroxisomes and not specialized peroxisomes. Glyoxysomes (specialized peroxisomes) are storage organelles involved in lipid mobilization. Like mitochondria and chloroplasts, peroxisomes produce radicals O₂⁻ due to their metabolism. There are two O₂⁻ generation sites in peroxisomes, in the organelles and in peroxisomal membranes. In the organelles the xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid. In the peroxisomal membranes, an electron transport chain composed by NADH and cytochrome b generates O₂⁻. Also the monodehydroascorbate reductase (MDHAR) generates the O₂⁻. The H₂O₂ is produced during different processes, including the photorespiration glycolate oxidase reaction, fatty acids β-oxidation and disproportionation of O₂⁻(Foyer and Noctor, 2009). Peroxisomes can also produce nitric oxide (Corpas *et al.*, 2011). Peroxisomes seem to be involved in ROS dependent response to drought. Riviero *et al.* (2007) have shown that transegenic tobacco plants expressing the isopentenyltransferase (IPT) gene, encoding an enzyme that catalyzes the rate-limiting step in cytokinin (CKs) biosynthesis, under control of a drought-induced senescence-associated-receptor-protein-kinase (SARK) promoter, had an enhanced drought tolerance (Miller *et al.*, 2010; Rivero *et al.*, 2007). In SARK-IPT transgenic plants there is an increase of

modulation of ROS metabolism genes of peroxisomal ascorbate–glutathione (AsA–GSH) cycle genes (Miller *et al.*, 2010).

1.3.1.2.3 Mitochondria

In plants mitochondria, complexes I and III are the main sites of ROS production and they are overall the main sites of ROS production (Møller, 2001), especially in non-green tissues as roots, where they are considered as the main source of ROS generation (Bose *et al.*, 2013). The enzyme alternative oxidase (AOX), contributes to H₂O₂ generation (Apel and Hirt, 2004). Moreover, mitochondria are involved in NO generation due to the reduction of nitrite via a nitrite reductase that has been shown to be dependent on the mitochondrial electron transport (Planchet *et al.*, 2005). It is known that the mitochondrial ROS production enhances during abiotic stresses, especially drought and salinity (Pastore *et al.*, 2007). During drought stress the respiration rate increases to compensate the reduced rate of chloroplast ATP synthesis, consequently ROS production in mitochondria is enhanced.

1.3.1.2.4 Apoplast

The apoplast represents an important site for H₂O₂ production in response to various abiotic stresses, such as drought and salinity (Hernandez *et al.*, 2001; Jubany-Mari *et al.*, 2009).

Their production depends on several groups of enzymes, including cell wall peroxidases and plasma membrane NADPH oxidases (e.g. AtRbohD and AtRbohF encode two major NADPH oxidases expressed in guard and mesophyll cells in Arabidopsis), that generate O₂⁻ by oxidizing NADPH and transferring the electron to oxygen (O₂) (Sagi and Fluhr, 2006). This ROS apoplastic generation is also known as “oxidative burst” during the hypersensitive responses under a pathogen attack and also regulate cell growth, development, and programmed cell death (PCD) (Foreman *et al.*, 2003; Gapper and Dolan, 2006), (Laloi *et al.*, 2007; Sagi and Fluhr, 2006; Torres *et al.*, 2002). During drought and salt stress, accumulation of H₂O₂ could be involved in acclimation responses of plant to drought and salt stresses. It was shown that the reduction of stress-induced apoplastic ROS formation is associated with a decrease in leaf elongation. This response was observed after NaCl but not osmotic treatments. It was found that the

apoplast is a presumed site of singlet oxygen $^1\text{O}_2$ production by apoplastic peroxidases (POXs) (Kawano *et al.*, 1998).

1.3.1.3 ROS scavenging during osmotic stress

As mentioned above, ROS are continuously produced as metabolic products of various pathways localized in different cellular compartments (Foyer and Noctor, 2013). Due to their reactivity, toxicity and involvement in different processes such as growth, development and programmed cell death, plants have evolved complex different ROS scavenging mechanisms that mainly consist in enzymatic and non-enzymatic pathways to control ROS levels. In plant cells, specific ROS producing and scavenging systems were found in different organelles, such as chloroplasts, mitochondria and peroxisomes. ROS-scavenging pathways from different cellular compartments are coordinated with each other (Pang and Wang, 2008).

3.1.3.1 Enzymatic control of ROS level

The antioxidative defense system comprises several enzymes such as: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutathione (AsA-GSH) cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer and Noctor, 2013). Figure 5 shows the localization of ROS generation and scavenging pathways.

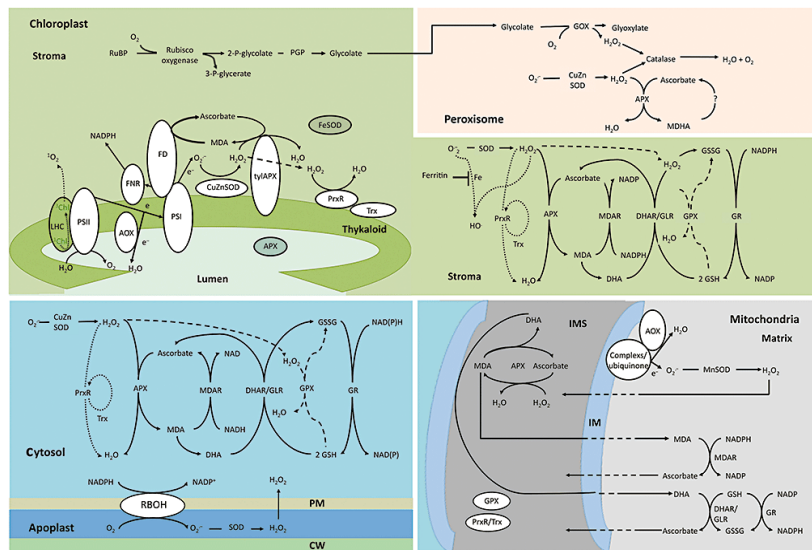


Figure 5: Localization of reactive oxygen species (ROS) generation and scavenging pathways in plant cells. The water–water cycle detoxifies $O_2^{\cdot-}$ and H_2O_2 and alternative oxidase (AOX; Immutans) reduces the production rate of $O_2^{\cdot-}$ in thylakoids [top left; in some plants iron superoxide dismutase (FeSOD) might replace CuZnSOD in the chloroplast]. ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and the stromal ascorbate–glutathione cycle. Peroxiredoxin (PrxR) and glutathione peroxidase (GPX) are also involved in H_2O_2 removal in the stroma (middle right). Excited chlorophyll (Chl) in its triplet state at the light-harvesting complex (LHC) can generate 1O_2 when the electron transport chain is over-reduced. ROS produced in peroxisomes during fatty acid oxidation, photorespiration or other reactions are decomposed by SOD, catalase (CAT) and ascorbate peroxidase (APX) (top right). SOD and other components of the ascorbate–glutathione cycle are also present in mitochondria. In addition, AOX prevents oxidative damage in mitochondria (bottom right). In principle, the cytosol contains the same set of enzymes found in the stroma (bottom left). NADPH oxidases [respiratory burst oxidase homologs (RBOHs)] are the major producers of ROS-associated signals required in a wide range of biological activities. The enzymatic components responsible for ROS detoxification in the apoplast and cell wall (CW) are only partially known, and the ROS-scavenging pathways at the vacuole and nucleus are unknown. Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. Although the pathways in the different compartments are mostly separated from each other, H_2O_2 can easily diffuse through membranes and antioxidants such as glutathione and ascorbic acid can be transported between the different compartments. DHA, dehydroascorbate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GOX, glycolate oxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate; MDAR, MDA reductase; PGP, phosphoglycolate phosphatase; PM, plasma membrane; PSI, photosystem I; PSII, photosystem II; RuBP, ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase oxygenase; Trx, thioredoxin; tyl, thylakoid. (from Miller et al.,2009)

1.3.1.3.1.2 Superoxide dismutase (SOD,1.15.1.1)

Superoxide dismutase (SOD), which dismutates O_2^- into H_2O_2 , is present in all aerobic organisms (Scandalios,1993). The enzyme SOD is classified into three groups: The Fe-SOD present mainly in chloroplasts; Mn-SOD in peroxisomes and mitochondria, Cu/Zn-SOD in the cytosol and chloroplasts.

The data of SOD enzyme activities have shown an increase under various environmental stresses such as drought and salinity. The SOD activity increase is correlated to plant capacity to face environmental stress. For instance, in case of salinity the halophytes show a greater SOD enzyme activity if compared to glycophytes (Jithesh *et al.*, 2006; Uzilday *et al.*, 2014)

1.3.1.3.1.3 Catalase (CAT,1.11.1.6)

The enzyme CAT is involved in decomposition of H_2O_2 into water and oxygen (Willekens *et al.*, 1997). The enzyme CAT has a very high turnover rate. As SOD also CAT has different isoforms, but the main one is sited in peroxisomes. The enzyme CAT is able to scavenge H_2O_2 produced by various reactions: photorespiratory oxidation, β -oxidation of fatty acids, and other enzyme systems such as XOD coupled to SOD (Corpas *et al.*, 2008; del Rio *et al.*, 1977). Some studies have shown that CAT-deficients plants had an increased susceptibility to salt, ozone and paraquat (Willekens *et al.*, 1997).

1.3.1.3.1.4 Guaiacol Peroxidase (GPX,1.11.1.7)

Guaiacol peroxidase (GPX, EC 1.11.1.7), belongs to a family of enzymes present in fungi, plants and vertebrates. These proteins contain an heme group and oxidize several substrates such as guaiacol and pyragallol in the presence of H_2O_2 (Penel *et al.*, 1992; Vianello *et al.*, 2007). Different isoenzymes of GPX were found in vacuoles, cell wall and cytosol. GPX is involved in different biosynthetic processes including lignification of cell wall, degradation of IAA and protection against abiotic and biotic stress.

1.3.1.3.1.5 Ascorbate-Glutathione Cycle

The ascorbate-glutathione (AsA–GSH) cycle plays an important role in the scavenging system for ROS. This pathway includes four enzymes such as ascorbate

peroxidase (APX), monodehydroascorbate reductase (MDsAR), dehydroascorbate reductase (DAsAR) and glutathione reductase (GR). APX reduces H_2O_2 to H_2O , prevents the accumulation of toxic level of H_2O_2 , while GR reduces oxidized (GSSG) to GSH using NAD(P)H as an electron donor. The Figure 6 schematizes the ascorbate-glutathione cycle.

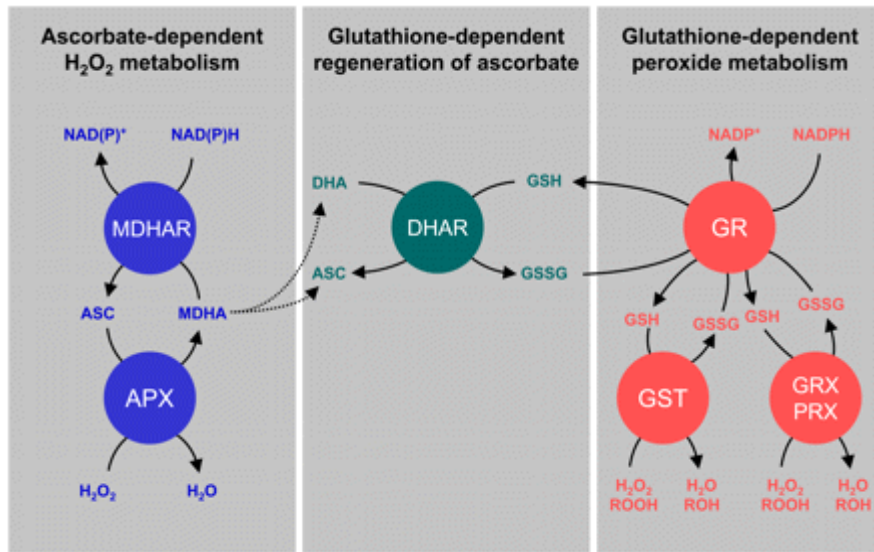


Figure 6: Ascorbate-Glutathione cycle (from Foyer and Noctor 2011)

1.3.1.3.2 Non-enzymatic control of ROS level

Ascorbate and glutathione can reduce the superoxide produced by Mehler reaction (Noctor and Foyer, 1998). Plants have evolved non-enzymatic antioxidants that together with the enzymatic pathways play an important role in ROS scavenging under normal and stress conditions for example drought. The major non-enzymatic antioxidants are mentioned below.

1.3.1.3.2.1 Glycine-Betaine

Glycine-betaine are compatible solutes accumulated in response to various environmental stresses such as drought, salt, or cold (Mahajan and Tuteja, 2005). They are associated with osmotic balance and can have a role in ROS homeostasis. Glycine-betaine stabilize and enhance both structure and function of oxygen-evolving complex of PSII against dissociation of the regulatory extrinsic proteins (the 18 kD, 23 kD and

33 kD proteins (Papageorgiou and Murata, 1995). Exogenous application of Glycine-betaine to *Arabidopsis* roots, that does not produce these compounds constitutively, has been shown to alleviate OH-generated K^+ leakage (Cuin and Shabala, 2007). Moreover, Booker *et al.* (2009) have shown that overexpression of glycine-betaine synthesizing enzyme Betaine aldehyde dehydrogenase (BADH) in *Arabidopsis* and rice (non-accumulators of glycine-betaine) results in an enhanced salinity and ozone stress tolerance in transgenic plants (Booker *et al.*, 2009)

1.3.1.3. 2.2 Proline

Proline is known to accumulate in plants in response to environmental stress and represents a solute involved in plants protection from water deficiency. Chen and Dickman (2005) demonstrated that the amino acid proline is a potent scavenger of ROS (Chen and Dickman, 2005). Moreover, this amino acid is capable to mitigate the impacts of drought, salt and temperature stress in plants. For example, proline accumulation can influence stress tolerance in multiple ways (Fig 7).

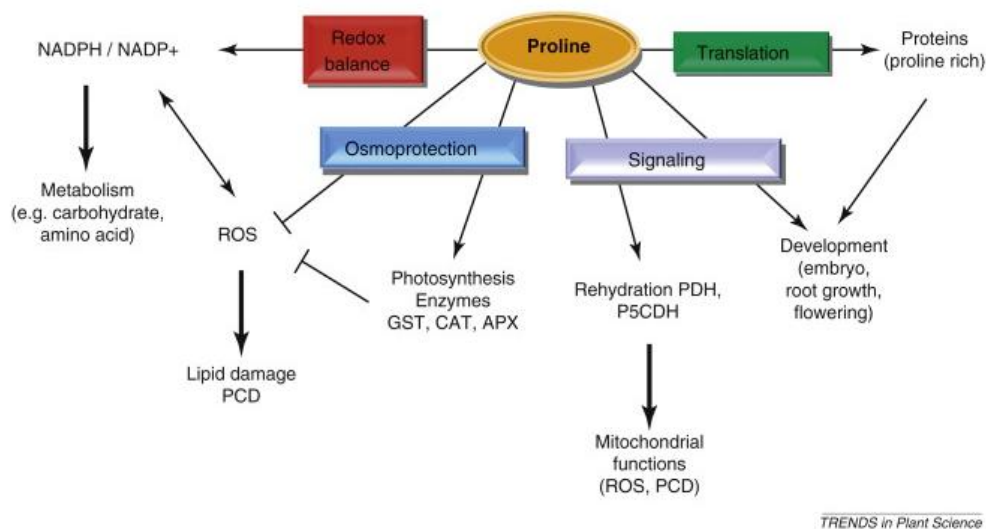


Figure 7: Multiple functions of proline in plants. Proline is used for protein synthesis, has protective functions as an osmolyte, contributes to the maintenance of the redox balance, can regulate development and is a component of metabolic signaling networks controlling mitochondrial functions, stress relief and development. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; PCD, programmed cell death (from Szabados and Savouré, 2010)

1.3.1.3. 2.3 Polyamines and other secondary metabolites

Polyamines (PA) are polycationic compounds present in most living organisms (Alcázar *et al.*, 2010). These compounds seem to be involved in plant growth and development, PCD, signaling, gene expression and adaptation to environmental stresses (Kuznetsov and Shevyakova, 2007; Takahashi and Kakehi, 2010). Recently it was shown an important role for 1,3-diaminopropane (DAP) in *A. thaliana* drought stress response (Jammes *et al.*, 2014). The 1,3-diaminopropane in plants is co-produced with H₂O₂ from metabolism of the classical polyamines. Acetylated 1,3-diaminopropane seems to have a role in adaptation to drought avoiding the complete stomatal closure to maintain CO₂ diffusion to photosynthetic tissues. Indeed, during stomata closing there is an increase in water use efficiency for the plant and the CO₂ is cut off. This diminution in CO₂ may conduce to stomatal (re)opening despite high ABA and the increased liability of dehydration, and even death (Jammes *et al.*,2014). The model proposed by Jammes *et al.* (2014) shown that during slow-soil-water availability, acetylation of DAP by acetyltransferases, identified earlier as NATA1 is involved in regulation of membrane transport mechanisms involved in readjusting CO₂-H₂O homeostasis. While an intense drought leads to suppression of NATA1 expression (Jammes *et al.*, 2014). Carotenoids are natural pigments, which are synthesized by plants, with antioxidant property, indeed they are involved in quenching of ¹O₂ and peroxy-radicals (Neubauer and Yamamoto, 1994). Other secondary metabolites such as sorbitol, mannitol, myo-inositol, ononitol or pinotol are accumulated during drought and salinity stresses (Williamson *et al.*,2002) acting as antioxidants.

1.3.2 Calcium signaling in response to osmotic stress

Calcium (Ca^{2+}) is an important second messenger in regulation of different cellular processes and Ca^{2+} dependent signaling is widely conserved among eukaryotes. (Lecourieux *et al.*, 2006; Xiong *et al.*, 2014).

In plant cells, $[\text{Ca}^{2+}]_{\text{cyt}}$ is maintained low around 100nM respect to the vacuole (200 nM) and apoplast (300 nM) and it was proposed that this mechanism may provide the possibility to quickly import calcium inside the cell when required for signalling (Lecourieux *et al.*, 2006). Changes in plant $[\text{Ca}^{2+}]_{\text{cyt}}$ are observed in response to salinity and drought (Parre *et al.*, 2007; Ranf *et al.*, 2008). The signalling pathway in which is involved Ca^{2+} is complex because it processes multiple signals simultaneously. Increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ are sensed by Ca^{2+} -sensor proteins and it transforms them in downstream pathways by binding or activating different targets.

Among the effector proteins there are calmoduline (CaM), CaM-binding proteins, Ca^{2+} -dependent protein kinases(CDPK) (Wheeler and Brownlee, 2008). The signals from CaM can induce gene expression of specific transcription factors (Tuteja and Mahajan, 2007). CDPKs were found in osmotically stressed plants, in fact a CDPK was reported to be expressed earlier and for longer in a drought tolerant species respect to the sensitive ones. In plant several isoforms of CDPKs exist and this can be explained with the need of fine tuning responses to diverse abiotic stresses.

Calcium permeable channels can be considered as the upstream elements in the Ca^{2+} -dependent signalling pathways, thus their activation is quite important. These channels are placed in plasma membrane, tonoplast, as well as in the ER (Kudla *et al.*, 2010), and their activity is coupled with Ca^{2+} -ATPase whose main function is to maintain calcium homeostasis by pumping out calcium (White, 2000). On plasma membrane also Ca^{2+} voltage dependent channels are present and their activation depend on depolarization of membrane such as depolarization-activated Ca^{2+} channel (DACC)and the hyperpolarization-activated Ca^{2+} channel (HACC) (Tuteja and Mahajan, 2007). Cytosolic calcium increase can be due also to Ca^{2+} mobilization from organelles, event mediated by second messengers, IP3or cADPR ((Hetherington and Brownlee, 2004), that acts as ligand for calcium gated channels in vacuolar or ER membranes or by Ca^{2+} -antiporters (Lecourieux *et al.*, 2006).

It has also been reported that the rise in $[Ca^{2+}]_{cyt}$ in plant could be provided by non-selective cation channels (NSCCs) that allow the cation passage across membranes with limited discrimination between cations (Demidchik and Maathuis, 2007). These NSCCs play also a crucial role in salinity stress (Demidchik and Maathuis, 2007). Indeed, Ca^{2+} blocks some NSCC and consequently reduces Na^+ influx into intact tissue to maintain a correct K^+/Na^+ ratio (Demidchik and Maathuis, 2007).

A recent study has demonstrated that Ca^{2+} -dependent wave signaling may play an important role in the process of systemic signaling in response to salt stress (Choi *et al.*, 2014). It was found that local salt stress at the root tip level leads to the spreading of a Ca^{2+} wave that propagates preferentially through cortical and endodermal cells to distal shoot tissues, triggering systemic molecular responses in the shoot. In particular, local treatment with $LaCl_3$, a putative plasma membrane channel blocker, inhibits the transmission of the Ca^{2+} waves and in parallel also inhibits the systemic molecular responses. Up to now, the Cyclic Nucleotide-Gated Channels (CNGC) and the Glu Receptors (Dodd *et al.*, 2010) involved in Ca^{2+} flux (Vincill *et al.*, 2012) are identified, but none have been shown to function in $NaCl$ -induced $[Ca^{2+}]_{cyt}$ elevation. Recently a role was proposed for plant annexin in Ca^{2+} flux under salinity (Laohavisit *et al.*, 2013). The annexin1 (AtANN1) seems to have an role in the generation of root and root epidermal $NaCl$ -induced $[Ca^{2+}]_{cyt}$ elevation.

1.3.2.1 Polyphosphoinositides (PPIs)

Polyphosphoinositides (PPIs) are present in all eukaryotes (Michell, 1975). The Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] is the best known PPI and it is the substrate for signal-activated phospholipase C (PLC), with the latter being an enzyme that hydrolyzes inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ releases Ca^{2+} from an intracellular store by activating a ligand-gated Ca^{2+} -channel (Munnik and Vermeer, 2010) which triggers several physiological changes in response to osmotic stress, including stomata closure (Bartels and Sunkar, 2005). Furthermore, in plant the first phospholipid based signalling pathway in response to osmotic stress is the PLC pathway, which is activated within minutes after both salt and water stresses (Munnik and Vermeer, 2010). Osmotically-triggered PtdIns(3,5)P₂ responses have been reported for *Chlamydomonas*, tobacco pollen tubes, epidermal

strips of pea leaves and suspension-cultured cells of tomato and alfalfa (Munnik and Vermeer, 2010; Zonia and Munnik, 2004). In mammalian cells, during osmotic stress, the PtdIns (4,5) P₂ regulates many ion channels and transporters (for example NHE1, VRAC, ENaC) (Hilgemann *et al.*, 2001; Suh and Hille, 2005; Voets and Nilius, 2007). All phosphatidylinositols phosphorylated at position 3 are synthesized by the phosphatidylinositol 3 kinase (PI3K), capable of phosphorylating inositol in position D3 (Toker, 1998). Several studies suggest that the activity of this enzyme is related with the Regulatory Volume Decrease Channel (RVDC, topic discussed more in detail in the next section). Current knowledges indicate that the PI3K plays a role as a sensor of cellular swelling, as the phosphorylation of PI in position 3 of the ring, by means of PI3K, strongly depends on the curvature of the membrane; therefore it would appear that the activity of this enzyme can be regulated by the mechanical deformation of the membrane itself due to changes in cell volume (Hübner *et al.*, 1998).

1.3.2.3 Salt Overly Sensitive (SOS)

Exposure to salinity activates the Salt Overly Sensitive (SOS) pathway. The SOS pathway is involved in removal of Na⁺ from the cytosol by activation of Na⁺/K⁺ exchangers (antiporters), which are membrane proteins localized in the vacuolar or plasmamembrane. The Ca²⁺ spike after salt stress induces the SOS pathways. SOS3 encodes amyristoylated calcium-binding protein that appears to have the function of calcium sensor in the perception of an increase of cytosolic Ca²⁺. The complex SOS3-Ca²⁺ is able to interact with the protein kinase SOS2 which phosphorylates SOS3 proteins. SOS3–SOS2 interactions recruit SOS2 to the plasma membrane, leading to activation of the downstream target SOS1, an Na⁺/H⁺ antiporter allowing extrusion of excessive Na⁺ from the cytosol (Ji *et al.*, 2013).

1.3.4 Protein kinases

1.3.4.1 MAPKs pathway

Mitogen-activated protein kinase (MAPKs) appears to be a key mechanism for intracellular signal transduction in both eukaryotic and prokaryotic. In general, the cascades of MAP kinase enzymes are characterized by the presence of three functionally related protein kinases: (i) the activation of MAP kinase requires phosphorylation of tyrosine and threonine residues, preserved in the so-called “TEY (Thr,Glu,Tyr) activation loop” by the MAPK kinases (MAPKK); (ii) in turn, the MAPKK are activated through phosphorylation of threonine residues of conserved threonine and or serine by a MAPKK kinase (MAPKKK) (Bartels and Sunkar, 2005); (iii) and finally the activated form of MAPK then migrates from the cytoplasm to the nucleus in order to directly activate transcription factors (TF). In vertebrates 14 MAP kinase genes have been identified that define 7 distinct MAP kinase pathways, involved in different abiotic and biotic stresses (Pearson *et al.*, 2001). For example, in vertebrates, TF involved in osmotic stress response is the NFAT5 that activates the transcription of genes, which are indirectly involved in cell volume regulation, such as the sodium chloride-betainecotransporter (SLC6A12)the sodium/myo-inositol cotransporter (SLC5A3) and the taurine transporter (SLC6A6) (Lee *et al.*, 2011). As in vertebrates, in plants there are several MAP kinase genes, that define distinct MAP kinase pathways vital in the signaling of different abiotic and biotic stresses. For example, in *Arabidopsis thaliana* there are MPK3, MPK4 and MPK6 kinases involved in response to shock osmotic stress. Indeed, Kim and coworkers (Kim *et al.*, 2011) have shown a role for MPK4, and observed that mpk4 loss-of-function mutants and MpK4-overexpressing transgenic plants (MpK4 OXs) produce aberrant responses following osmotic stress. In-gel kinase assays for MPK3 phosphorylation in mpk4 mutants and MpK4 OXs demonstrated that MpK4 plays an important role in osmotic-stress responses via regulation of MPK3 activity; in fact, after osmotic stress in MpK4 mutant, there is a decline in phosphorylation rate meanwhile in MpK4 OXs the level of phosphorylation remains higher, even when compared with wild type. In addition, it was also found that these components also cross-talked with ABA-dependent stress-responses genes, in particular NCED3, a gene that encodes a key regulator of the ABA signaling pathway. NCED3 gene expression is reduced in mpk4 mutants and increased

in MpK4 OXs compared with WT plants. All together, these results strongly suggest that MpK4 might mediate osmotic-stress response and promote NCED3 expression(Kim *et al.*, 2011).

1.3.5 Plant ion channels

The ion channels activity is generally associated to the earliest observed events following the perception of a stimulus. The ions (eg. K^+ , Na^+ , etc) are transferred in the direction of their electrochemical gradient, from one compartment to another. The opening/closure of these channels depends on various stimuli, there are two main types of ion channels:gated and non-gated. Under physiological conditions, ion channels perform three basic functions: (i) are involved in osmotic homeostasis (ii) the transduction of signals, amplifying and propagating electric signals for example, or carrying secondary messengers, and (iii) control of the membrane potential. The following sections describe an overview of the different classes of ion channels present in plants, focusing mainly on plasma membrane channels, on how their structures and activities are linked to other signaling events, and finally, on their roles and implications in signaling pathways leading to PCD process.

1.3.5.1 Plant cation channels

1.3.5.1.1 Potassium channels

Potassium (K^+) represents up to 10% of the total dry weight of the plant. It is the most abundant cation in the cytosol as it is compatible with the structure of proteins, even at high concentrations (Kronzucker and Britto, 2011; Maathuis and Amtmann, 1999). The cell uses this cation in several important functions such as neutralization of the anion electric control of membrane polarization and osmoregulation. Plants have a variety of transport systems for K^+ (Fig.8).

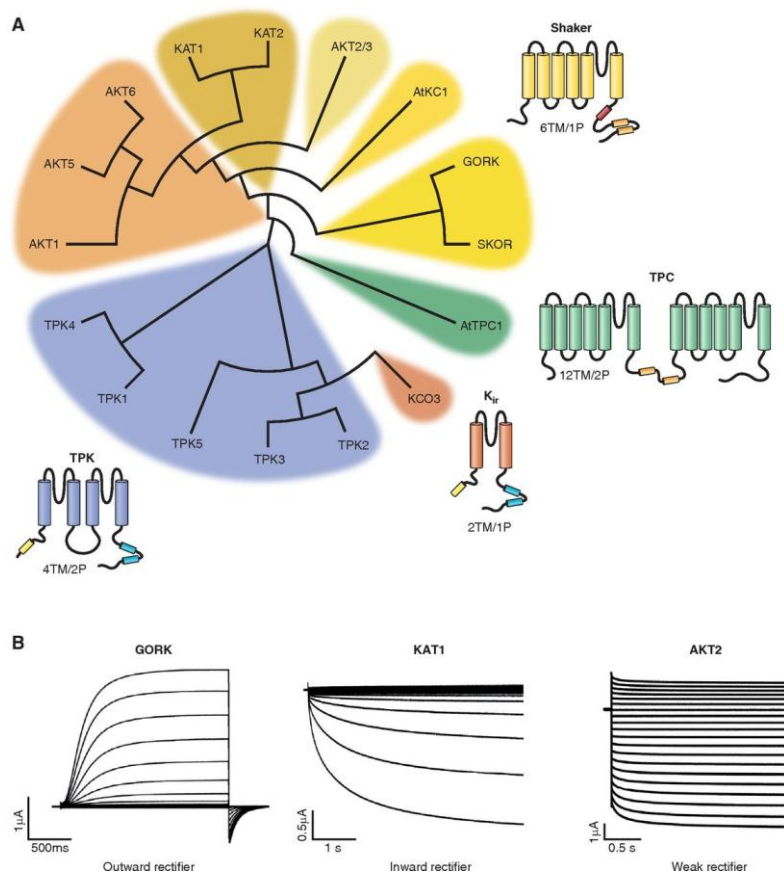


Figure 8:Phylogenetic relationship and topology of *Arabidopsis thaliana* potassium channels. A: the genome of the model plant *Arabidopsis thaliana* encodes for 15 K^+ -selective channels, which are subdivided into three structural classes: The class of voltage-dependent *Shaker*-like potassium channels (K_v), the twin pore channels (TPKs; K_{2p}), and K_{ir} -like potassium channel (KCO3). In addition, it encodes for a cation-nonspecific two-pore channel (TPC1). TM, transmembrane domain; P, pore region. B: despite their structural resemblance, plant *Shaker*-like K_v channels split up into outward (*left*), inward (*middle*), and

weak (*right*) rectifiers according to their gating properties. Typical voltage-clamp recording from heterologously (*Xenopus oocytes*) expressed Kv channels are shown (from Hedrich, 2012)

In plants, as in animal cells, the activity of potassium channels is determined by the cell membrane electrical properties. Two K^+ permeable conductances have been characterized and they differ in the voltage range by which they are activated. One is activated by membrane hyperpolarization (with potential more negative than $E_K = E_i$, thermodynamic equilibrium potential of K^+) and it is mainly involved in K^+ entry (Fig 9). This incoming rectifying conductance is commonly called IRC Inward Rectifying Conductance (Fig 10). The other K^+ conductance is activated at potential levels more positive than E_K membrane and it's involved in the efflux of K^+ out of the cell. This type of conductance is called ORC for Outward Rectifying Conductance.

$$E_i = \frac{RT}{zF} \ln \frac{[i]_{int}}{[i]_{ext}}$$

Figure 9: Nernst Equation

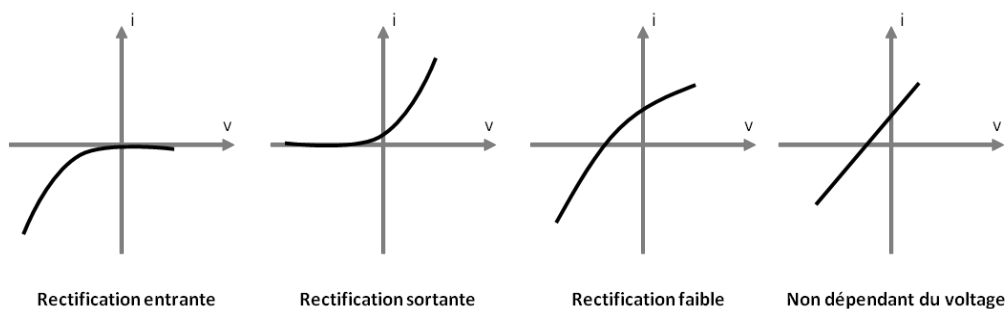


Figure 10: Relation between current and voltage (I/V). It is shown the different type of K^+ channels in plants (Adapted from Véry & Sentenac, 2002)

The ORC is characterized by slow sigmoidal activation current (Fig 8). The ion channels belonging to this family are mainly selective for K^+ if compared to Na^+ and are blocked by Ba^{2+} and tetraethylammonium (TEA). In *Arabidopsis*, the presence of ORC

was reported in guard cells, root hairs and cultured cells of different plants (Bouteau and Tran, 2012; Forestier *et al.*, 1998; Roelfsema and Hedrich, 2010). The K^+ channels are multimeric proteins. Their transmembrane subunits, named α subunits are characterized by the presence of one or two pore domains P. Three families of subunits α forming selective channels K^+ have been identified in plants areas: Shaker, TPK and Kir-like (Véry and Sentenac, 2003). They all have counterparts in animal cells.

3.5.1.2 Shaker-like potassium channels

Shaker-like potassium channels, inhibited by TEA, are divided in three groups depending on their voltage sensitivity (Véry and Sentenac, 2002). The first class is called Potassium Inward-Rectifying channels (KIR) including *AKT1*, *KAT1*, *KAT2*, and *SPIK*. They are responsible of K^+ uptake after membrane hyperpolarization. In the second class there are the potassium channels that depending in K^+ electrochemical gradients mediate both uptake and release. *AKT2* belongs to this family. The third is the Potassium Outward-Rectifying channels (KOR) including *GORK* and *SKOR* that mediate K^+ release upon membrane depolarization. The Shaker family contains nine genes and are composed of four subunits arranged around a central pore (Zimmermann and Sentenac, 1999). Seven of the nine Shaker Arabidopsis proteins were characterized so far and it was found to be highly selective for K^+ . The available information suggests that these channels are the main transport systems involved in the nutrition K^+ and / or the regulation of the K^+ equilibrium. Among the Shaker ion channels *GORK* (outward-rectifying potassium-selective channel) is expressed in guard cells and root hairs, while *SKOR* is localized in the vasculature, namely, the xylem parenchyma cells, where it is involved in solute loading in the xylem network (reviewed by, (Hedrich, 2012). Fig. 11 shows a schematic representation of the expression patterns of Arabidopsis K^+ - selective channels.

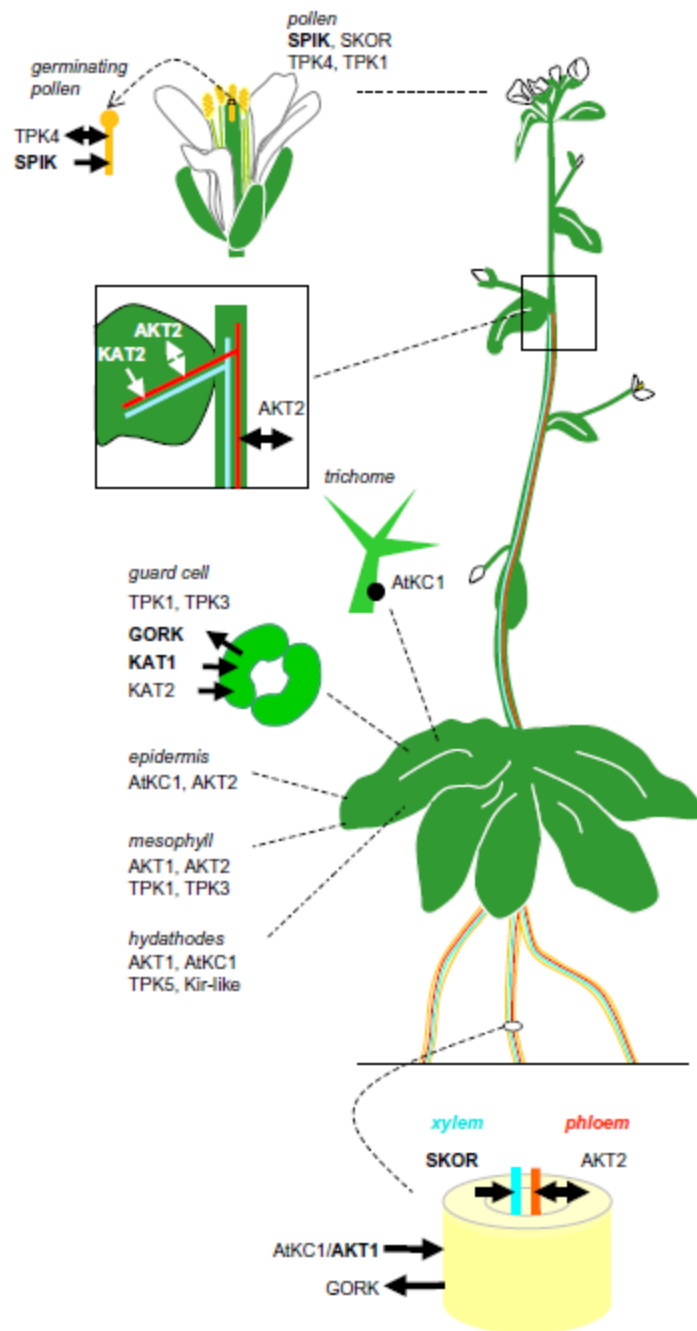


Figure 11: Expression patterns of Arabidopsis K⁺-selective channels (from Lebaudy et al.,2007).

1.3.5.1.3 Twin-Pore K^+ /KCO

In mammalian cells, the KCNK are characterized by a hydrophobic core composed by four transmembrane segments and two P domains (Fig 12). The characteristic of this class of ion channel is that they have a weak voltage activation (Patel and Honore, 2001). In Arabidopsis, five channels (TPK1, TPK2, TPK4, and TPK5 TPK6) have structural homologies to mammalian ion channels (Voelker *et al.*, 2006; Zimmermann and Sentenac, 1999). These channels are localized in the tonoplast (Czempinski *et al.*, 2002; Schönknecht *et al.*, 2002; Voelker *et al.*, 2006), except AtTPK4, which is localized at the plasma membrane and it's specific for the pollen tube (Becker *et al.*, 2004).

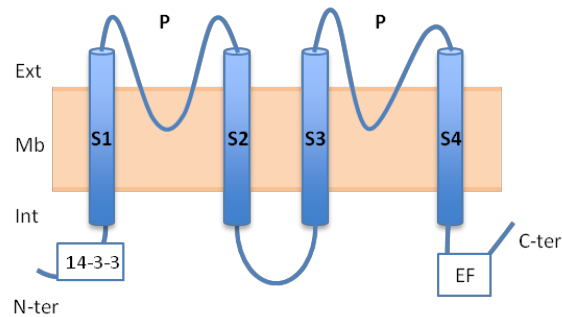


Figure 12: Structural conformation of TPK ion channels in Arabidopsis (from Lebaudy *et al.*, 2007)

1.3.5.1.4 Kir-like

In mammalian cells, this family is characterized by IRK conductance and has an hydrophobic core formed of two segments and a transmembrane domain (fig 13) .These channels don't show voltage-dependence, the inward rectification is due to a blockage of outward current by Mg^{2+} and polyamines (Ficker *et al.*, 1994; Matsuda *et al.*, 1987). In *Arabidopsis thaliana*, a channel located at the tonoplast was found (Voelker *et al.*, 2006) to share similarity with the two P domain channels (KCO family) and was therefore named KCO3 (Czempinski *et al.*, 1999).

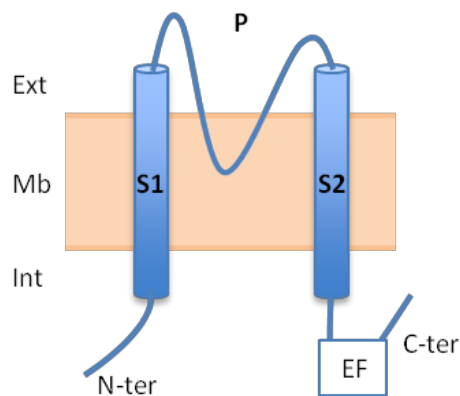


Figure 13: Structural conformation of *Kir-like* ion channels in *Arabidopsis* (Adapted from Lebaudy *et al.*, 2007)

1.3.5.1.5 Non selective cation channels (NSCC)

Non-selective cation channels are macromolecular pores at plasma membrane that allow the ions Na^+ , K^+ , Ca^{2+} fluxes.

Under saline conditions Na^+ enters in the cells through non-selective cation channels (NSCCs,) (Demidchik and Tester, 2002), depolarizing the plasma membrane (Chen *et al.*, 2007; Pandolfi *et al.*, 2010; Shabala and Cuin, 2008; Wegner *et al.*, 2011).

Even though no definitive molecular candidates have clearly emerged for NSCCs, it seems that various classes of NSCCs could be responsible for influx of Na^+ under salt stress, especially a depolarization activated class of NSCCs (Demidchik and Maathuis, 2007). There are some candidate genes belonging to family of glutamate receptors (GluR) (Lacombe *et al.*, 2001) and the family of CNGC (= Cyclic Nucleotide Gated Channel) (Mäser *et al.*, 2001). The NSCC may be activated by many factors such as the voltage (hyperpolarization or depolarization), calcium, mechanical variations, cyclic nucleotides or glutamate (Demidchik *et al.*, 2002). Most studies have focused on the NSCC of roots. They are responsible for the influx of Na^+ at the root level (Demidchik and Maathuis, 2007; Demidchik and Tester, 2002). NSCCs can therefore be subdivided according to their voltage dependence into voltage-insensitive, depolarization-activated or hyperpolarization-activated NSCCs (reviewed by (Demidchik and Maathuis, 2007)):

- ❖ Depolarization-activated NSCCs (DA-NSCCs)
- ❖ Voltage-independent NSCCs (VI-NSCCs)
- ❖ Hyperpolarization-activated NSCCs (HA-NSCCs)

The DA-NSCCs were found in *Arabidopsis* cultured cells (Cerana and Colombo, 1992), *Arabidopsis* leaf mesophyll (Shabala *et al.*, 2006), *Arabidopsis* root epidermis (Shabala *et al.*, 2006), *Arabidopsis* guard cells (Pei *et al.*, 1998), *Hordeum vulgare* root xylem (Wegner and De Boer, 1997), *Thlaspi* spp. Mesophyll cells (Piñeros and Kochian, 2003), *Arabidopsis* pollen tubes (Becker *et al.*, 2004), *Phaseolus vulgaris* seed coats (Zhang *et al.*, 2002) and *Phaseolus vulgaris* cotyledon epidermal cells (ZHANG *et al.*, 2004). The voltage dependence of DA-NSCCs is similar to outward rectifying K^+ -selective channels (ORC). The DA-NSCCs can be blocked by extracellular Ca^{2+} , TEA, nifedipine, diltiazem and verapamil (reviewed by (Demidchik *et al.*, 2002; Shabala *et al.*, 2006).

The voltage-independent NSCCs (VI-NSCCs) are insensitive to plasma membrane voltage, this suggests that they equally conduct outward and inward currents. They can be divided into two groups on the basis of blockage by cations such as Ca^{2+} , Ba^{2+} , Mg^{2+} and Zn^{2+} . This class of ion channels was found in *Aster tripolium* guard cells (Véry *et al.*, 1998), *Arabidopsis* mesophyll cells (Shabala *et al.*, 2006), *Arabidopsis* cells (Amtmann *et al.*, 1997), *Arabidopsis* pollen tubes (Becker *et al.*, 2004), expanding *Pisum sativum* leaf epidermis (Elzenga and Van Volkenburgh, 1994) and motor cells of *Samanea saman* (Yu *et al.*, 2001). DA-NSCCs and VI-NSCCs are the primary candidates for Na^+ entry.

The Hyperpolarization-activated NSCCs (HA-NSCCs) conduct mainly Ca^{2+} and were described as hyperpolarization-activated Ca^{2+} channels (HACaCs) (Demidchik and Maathuis, 2007). This class of NSCC is involved in Ca^{2+} influx important for plant elongation, growth of root hairs and cells in the elongation zone (Demidchik and Maathuis, 2007). Gelli *et al.* (Gelli *et al.*, 1997) have shown that some type of HA-NSCCs are sensitive to elicitors, this suggest that these channels may be involved in Ca^{2+} signalling, which is part of the early response to pathogen attack (Gelli *et al.*, 1997).

1.3.5.2 Anion channels

Anion currents are divided into S-type, R-type, and IRAC. Guard cells were known to have both S- and R-type anion channels (Fig 13,14)(reviewed by (Hedrich, 2012)

1.3.5.2.1 Slac

Slac1 (slow-associated Anion Channel 1) (Fig 14), was identified from *Arabidopsis* mutant, which encodes a protein from the plasma membrane in guard cells (Negi *et al.*, 2008; Vahisalu *et al.*, 2008).

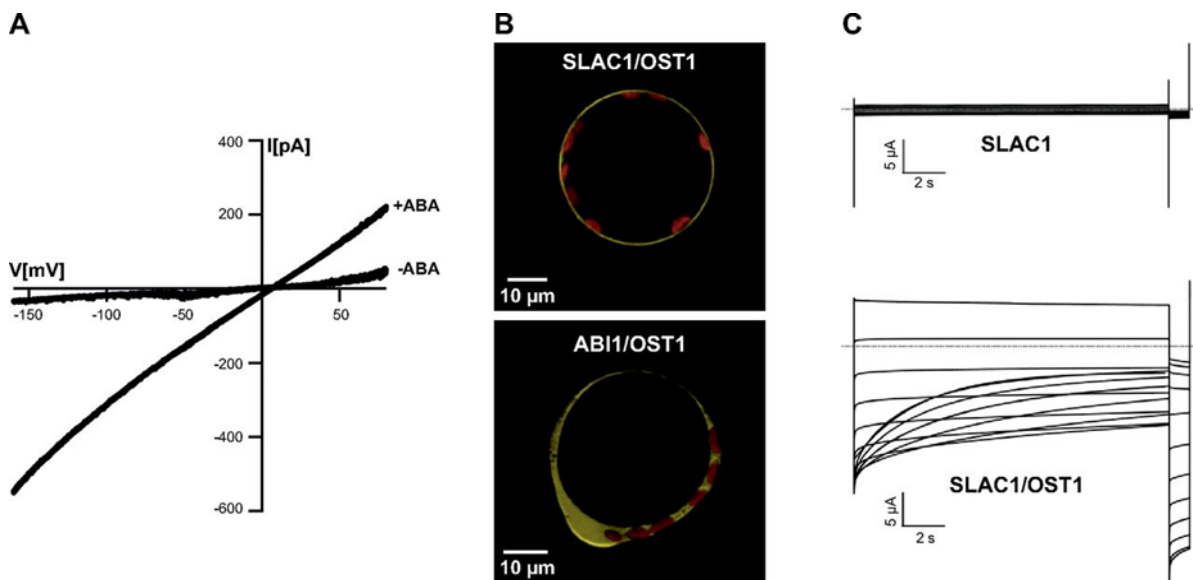


Figure 14: Anion channel SLAC1 controlled by protein kinase-phosphatase. A: S-type anion channels in the plasma membrane of *Vicia faba* guard cells are activated by stress hormone ABA. B split YFP assays: top panel, SLAC1, a guard cell plasma membrane S-type anion channel, is interacting with the SnRK2 protein kinase OST1 at the plasma membrane. Bottom panel: the cytosolic enzymes OST1 (protein kinase) and ABI1 (2C protein phosphatase) physically interact. C: two electrode voltage-clamp experiments indicate that SLAC1 activation in *Xenopus laevis* oocytes requires OST1. (from Hedrich,2012)

The mutated alleles *slac1* show insensitivity to CO₂, ABA and O₃-induced stomatal closure (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Analyses in patch-clamp the two

mutants *slac1* alleles, showed that slow anionic currents were considerably reduced, while current rapid anion channels (R-type) and permeable channels Ca^{2+} of the plasma membrane were intact in guard cells.

SLAC1 belongs to gene family composed by five members (SLAH1, 2, 3 and 4). SLAC1 gene encodes a membrane protein that has ten transmembrane domains with large N and C terminal domains (Fig 15). The SLAC proteins have low homology in their transmembrane domains with a malate transporter of fungi and bacteria of the TDT family (for Tellurite-resistance/Dicarboxylate Transporter) (Camarasa *et al.*, 2001). This protein is controlled by the torque kinase OST1 (Open stomata 1) and PP2C in stomatal response, but also by a kinase CDPK (Geiger *et al.*, 2010). These channels are the main actors of drought-induced membrane depolarization and it was shown that in root hairs they are activated during desiccation (Dauphin *et al.*, 2001).

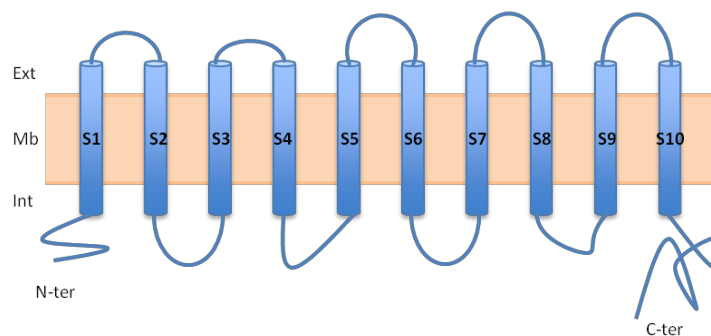


Figure 15: SLAC1 structure (from Chen *et al.*, 2010)

1.3.5.2.2 IRACs channels

The IRACs channels were found in various species of plants and algae, as in pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), *Medicago sativa* (Bouteau *et al.*, 1999), *Arabidopsis thaliana* (Bouizgarne *et al.*, 2006), barley (*Hordeum vulgare*), phytoplankton (*Coccolithus pelagicus*), seaweed *Valoniutricularis* (Tavares *et al.*, 2011) and root hairs of *Medicago sativa* (Bouteau *et al.*, 1999). These channels share a voltage-dependent inward rectification. In maize (*Zea mays*) and barley, the inward rectification is amplified by the increasing cytosolic Ca^{2+} , but the open mechanism is different (Gilliham and Tester, 2005; Köhler and Raschke, 2000). In maize, the IRAC channel is voltage-dependent (open probability increases at negative voltages). These

channels are responsible for maintaining the homeostasis of the cell anionic e.g. in the salt stress tolerance during the repolarization of the membrane potential and in regulating the osmotic stress caused by drought or excess of water. It also helps preventing strong depolarizations / hyperpolarizations of the plasma membrane. To date no molecular data are available on this type of channels.

1.3.5.2.3 R-type anion channels

The R-type anion channels (Fig 16) are characterized by rapidity of anion efflux. These channels were found in guard cells of *Vicia faba* (Raschke *et al.*, 2003) as well as the rapid anion channel GCAC1 (Guard Cell Anion Channel 1) (Geiger *et al.*, 2009). Under normal conditions R-type channels are closed, while a depolarization induced channels opening.

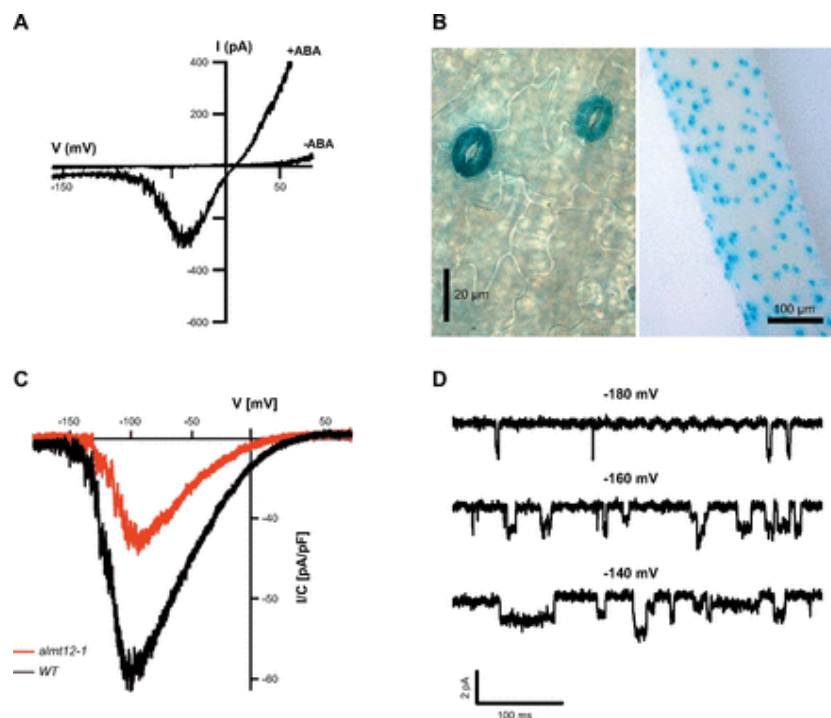


Figure 16: R-type anion channels gate like cation channels in nerve cells. A: R-type anion channels in the plasma membrane of *Vicia faba* guard cells are activated by ABA and gated open by depolarization (Hedrich, 2012)

1.3.5.3 Mechanosensitive ion channels

In bacterial and mammalian cells, the mechano-sensitive ion channels (MS) are involved in the perception of changes in pressure, touch and sound (Hamill and Martinac, 2001; Vogel and Sheetz, 2006; Zhou *et al.*, 2005). Although plants respond to a wide variety of mechanical stimuli and many mechano-sensitive ion channel activities have been characterized in plant membranes by the patch-clamp method, the molecular mechanisms of mechanical perception remain poorly understood (Haswell, 2007). In *Arabidopsis*, 10 proteins MSCs (for MechanoSensitive Channel of Small conductance) have been found, called MSL1-10 (Haswell, 2007; Kloda and Martinac, 2002; Pivetti *et al.*, 2003). The structure predictions show that MSL have six transmembrane domains and parts of N- and C-termini are cytosolic (Fig 17). MSL3 and MSL2 are involved in controlling the morphology of the organelles (Haswell and Meyerowitz, 2006; Nakayama *et al.*, 2007). Five proteins are found in the plasma membrane of root cells MSL4, 5, 6, 9 and 10 (Haswell *et al.*, 2008). The use of mutant lines *msl9* and *msl10* showed mechanical dependent channel activity with greater selectivity than the ions Ca^{2+} , Cl^- in the roots of *A. thaliana* (Haswell *et al.*, 2008).

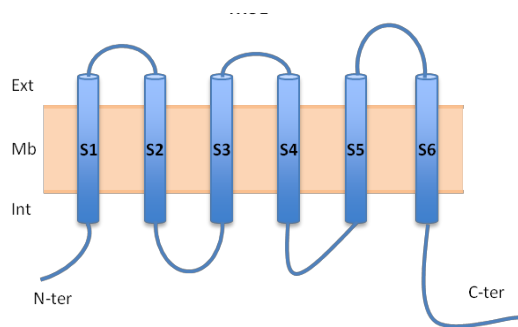


Figure 17: Mechanosensitive ion channels structure (from Lebaudy *et al.*, 2007).

1.4 Osmotic stress, cell volume regulation and cell death

Osmotic stress induces water flux across the plasma membrane, resulting in altered cellular volume and ionic strength, and severely affects cell functions.

In general, the cell hasn't a "preferred" volume to adapt to osmotic (hyper and hypo) changes. Animal cells have evolved the capacity to change their volume, which is an important factor for cells adaptation to environment challenges. Indeed, the control of volume and osmolarity (also of turgor in plants) lies at the core of cellular homeostatic networks. These volume changes are mainly driven by K^+ , Cl^- and other solutes (e.g. betaine) fluxing across the plasma membrane. In fact, a correct balance between RVI (Regulation volume increase) and RVD (Regulation volume decrease) should lead to mitigation of osmotic stress, while an excessive RVD can lead to cell death. This is an important point in cell biology that needs more studies, because it is often not very easy to understand the "switch" that lead from correct balance RVI/RVD to RVD. Therefore in this thesis we will focus on the role of ion channels in this "switch". The regulation of ion channels in apoptosis, a type of programmed cell death (PCD), was widely studied in mammalian cells (Yu and Choi, 2000), as a chronic event alters volume regulation and/or ion transporter expression, resulting in pathological developments associated with cancer (Pedersen *et al.*, 2013). The key mechanism that links ion channels, cell volume and PCD is the Apoptosis Volume Decrease (AVD) and involve K^+ and/or Cl^- fluxes (Fig 18).

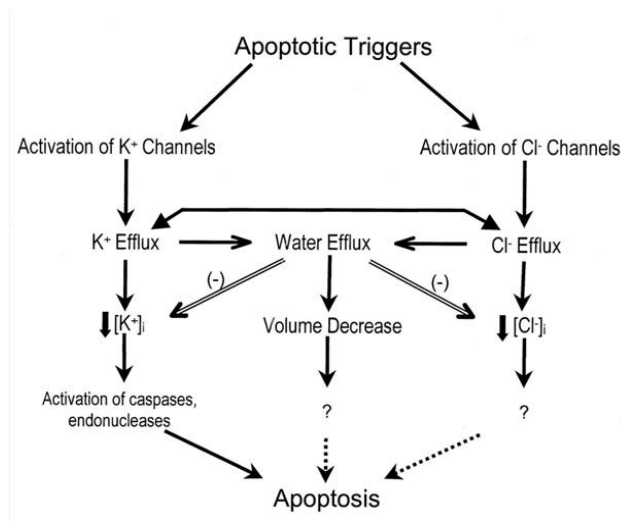


Figure 18: Mechanism of Apoptosis volume decrease involved in apoptosis (from Yu & Choi,2000).

Particularly, the diminution of intracellular K^+ leads to the caspases activation, since the intracellular K^+ promotes the catalytic activity of caspases and nucleases (Kroemer *et al.*, 1998; Perregaux and Gabel, 1994).

In animal cells, it was shown that the volume regulation could protect cells from apoptosis (Maeno *et al.*, 2000; Maeno *et al.*, 2006; Reinehr *et al.*, 2007). Numata *et al.* (Numata *et al.*, 2007) suggested that the transition receptor channel has a role in cell volume regulation because the calcium increase, which leads to the activation of K^+ and Cl^- calcium. In mammalian cells, the $I_{Cl,swell}$ (a commonly name used for classification of the chloride anion channels) is one of the major pathways responsible for RVD (Hoffmann *et al.*, 2009; Lang *et al.*, 2007), but the molecular characteristics of channels underlying the $I_{Cl,swell}$ (normally involved by cell swelling resulting from exposure to extracellular hypotonic solution) (Catacuzzeno *et al.*, 2014) still remain unknown.

In plant, K^+ efflux and its involvement in PCD was widely studied in response to salinity stress (as osmotic challenge) and biotic stress (i.e. in response to harpins). Indeed, in *A.thaliana* roots the K^+ efflux in response to salinity stress is necessary to lead PCD, (Demidchik *et al.*, 2010), and anion efflux coupled with potassium efflux have an important role in plant cell volume regulation. In *A.thaliana* cells the harpine N (HrpNea) produced by plant pathogenic bacteria lead to the activation of K^+ currents in the plasma membrane: K^+ outward rectifying currents (KORC) (Reboutier *et al.*, 2007). Recently, in *A. thaliana* was also reported a delayed KORC activation in response to ozone (O_3) (Tran *et al.*, 2013b). The exposure of *A.thaliana* suspension to O_3 induced an acute cell death (Kadono *et al.*, 2010). This cell death is characterized and involved in an early activation of anion channel (Kadono *et al.*, 2010) and a delayed activation of K^+ outward currents (Tran *et al.*, 2013a). In animals, cell shrinkage involved dysfunction of ionic homeostasis and consequently progression of cell death (e.g. caspase and nuclease activation) (Bortner and Cidlowski, 2004; Yu and Choi, 2000). It was shown that ozone induced a ion channel dependent caspase-like activities involved in early events participating to development of O_3 -induced PCD (Tran *et al.*, 2013b).

During biotic stress, the anion efflux was described as an early event induced by interaction plant/pathogens in the hypersensitive response (Garcia-Brugger *et al.*, 2006) induced by harpins (Reboutier *et al.*, 2007), thaxtamine, oxalic acid (Errakhi *et al.*,

2008) or cryptogaine (Gauthier *et al.*, 2007; Wendehenne *et al.*, 2001). For example, it was found that the oxalic acid or cryptogein induced cell death depending on the activation of anion channels. It was also found that ozone induces cell shrinkage by an early activation of anion channels that leads to cell death (Kadono *et al.*, 2010).

1.5 Programmed Cell Death

The Programmed Cell Death (PCD) is an essential part of plant development and responses to abiotic and biotic stress (Williams *et al.*, 1992). This type of cell death is a genetically programmed process characterized by distinct morphological characteristics. Different types of PCD have been described in plants, which has led to a call for a detailed classification of cell death events (Van Doorn *et al.*, 2011). Typical peculiarities of PCD in plants frequently include the fragmentation of the DNA by specific nucleases (DNA laddering), condensation and shrinkage of the cytoplasm, release of cytochrome *c* from mitochondria, elevation of the cytosolic calcium concentration ($[Ca^{2+}]_{\text{cyt}}$), generation of reactive oxygen species (ROS), and an activity increase of caspase-like enzymes (Tsiatsiani *et al.*, 2011; Van Doorn *et al.*, 2011). PCD has effectively been proved to occur in response to various abiotic stresses (Kadono *et al.*, 2010; Tran *et al.*, 2013b; Van Doorn *et al.*, 2011) comprising drought and salinity (Huh *et al.*, 2002).

1.6 Aim of this thesis

The aim of this thesis was to better understand the role of ion channels during ionic- and non-ionic hyperosmotic stress leading to Programmed Cell Death (PCD). In the first study, BY2-tobacco cells was chosen as cellular model study and *Arabidopsis thaliana* in the second and third. Cultured cells represent a good model system to study abiotic and/or biotic stresses, in fact they are characterized by high omogeneity and high growth rate. *A.thaliana* suspension cells allow working on mutants. Moreover, they are a good tool for studies at cellular levels comprising microelectrode voltage clamp. The microelectrode voltage clamp is a method that allows the measurements of ion flow through a membrane as an electrical current in intact cells, preserving their cell wall, unlike the patch clamp that requires the use of isolate protoplasts (Bouteau and Tran, 2012). The main objectives of this work were to compare and understand the role of the ion currents induced by saline and non-saline hyperosmotic stress leading to PCD and search for putative regulators of hyperosmotic-induced PCD in plants

Chapter 2

**Early events involved in hyperosmotic
stress induced PCD in tobacco BY2 cells**

Chapter 2 Early events involved in hyperosmotic stress induced PCD in tobacco BY2 cells

2.1 Introduction

Ionic and non-ionic hyperosmotic stresses have severe effects on plant growth (Tester and Davenport, 2003). Salt stress induces complex and wide series of cellular responses that may result in the induction of signalling events that lead to PCD (Huh *et al.*, 2002; Shabala, 2009). Also drought was shown to induce PCD (Duan *et al.*, 2010). In this study, by using BY2 cells, we compared the early events induced by ionic and non-ionic hyperosmotic stress and their relation(s) with ion channels regulation and PCD. Ion channels activity was recorded by using the microelectrodes voltage-clamps techniques (dSEVC). With this technique it's possible to measure transmembrane potential changes and ion channels activity from intact cultured cells preserving their cell wall. This is a point of great importance in plant cells, since the cell wall could not allow a volume increase as observed in animal cells (cf RVI, §1.4). The figure 19 shows a plant cell sustained by a microfunnel and impaled by a microelectrode allowing dSEVC recording.

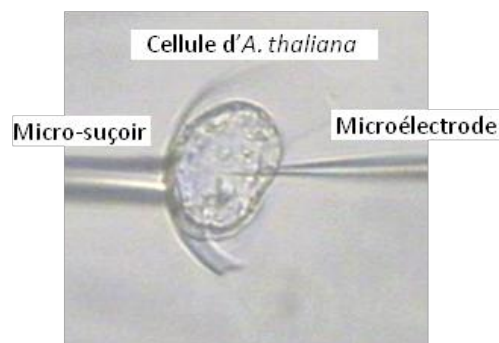


Figure 19: Typical st up of plant cells impalement for voltage clamp measurements.

In case of ionic hyperosmotic stress (addition of NaCl) it was possible to record plasma membrane depolarization due to influx of Na^+ and in case of non-ionic hyperosmotic stress (addition of sorbitol), we observed a hyperpolarization due to a decrease in anions currents. If a relation could be observed between Na^+ current and NaCl-induced PCD, no link was observed between anion current decrease and the sorbitol induced PCD.

Chapter 2 Early events involved in hyperosmotic stress induced PCD in tobacco BY2 cells

2.2 Deciphering early events involved in hyperosmotic stress-induced programmed cell death in tobacco BY-2 cells

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RESEARCH PAPER

Deciphering early events involved in hyperosmotic stress-induced programmed cell death in tobacco BY-2 cells

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Abstract

Hyperosmotic stresses represent one of the major constraints that adversely affect plants growth, development, and productivity. In this study, the focus was on early responses to hyperosmotic stress- (NaCl and sorbitol) induced reactive oxygen species (ROS) generation, cytosolic Ca²⁺ concentration ([Ca²⁺]_{cyt}) increase, ion fluxes, and mitochondrial potential variations, and on their links in pathways leading to programmed cell death (PCD). By using BY-2 tobacco cells, it was shown that both NaCl- and sorbitol-induced PCD seemed to be dependent on superoxide anion (O₂⁻) generation by NADPH-oxidase. In the case of NaCl, an early influx of sodium through non-selective cation channels participates in the development of PCD through mitochondrial dysfunction and NADPH-oxidase-dependent O₂⁻ generation. This supports the hypothesis of different pathways in NaCl- and sorbitol-induced cell death. Surprisingly, other shared early responses, such as [Ca²⁺]_{cyt} increase and singlet oxygen production, do not seem to be involved in PCD.

Key words: Calcium, hyperosmotic stress, mitochondria, NaCl, *Nicotiana tabacum*, non-selective cation channels, programmed cell death, reactive oxygen species.

Introduction

Salt stress is known to have severe effects on plant growth and development (Tester and Davenport, 2003). High salinity leads to ionic, osmotic, and oxidative stress in plants (Zhu, 2001) that may result in the induction of signalling events that lead to programmed cell death (PCD) in higher plants (Huh *et al.*, 2002; Lin *et al.*, 2006; Shabala, 2009; Wang *et al.*, 2010) and algae (Affenzeller *et al.*, 2009). Such PCD could be

regarded as a salt adaptation mechanism (Huh *et al.*, 2002). Drought, which consists at least in part of a hyperosmotic stress, was also shown to induce PCD in plants (Duan *et al.*, 2010). PCD is an active cellular process that facilitates the removal of unwanted or damaged cells and is essential for cellular differentiation and tissue homeostasis (van Doorn *et al.*, 2011). PCD has effectively been proved to occur in response

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to various abiotic stresses (Kadono *et al.*, 2010; van Doorn *et al.*, 2011). Different types of PCD with overlapping morphological and physiological hallmarks have been described in plants, which has led to a call for a detailed classification of cell death events (van Doorn, 2011; van Doorn *et al.*, 2011). Although the delineation between the different PCD types of sometimes remains difficult (van Doorn *et al.*, 2011), typical hallmarks of PCD in plants frequently include the fragmentation of the DNA by specific nucleases (DNA laddering), condensation and shrinkage of the cytoplasm, release of cytochrome *c* from mitochondria, elevation of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), generation of reactive oxygen species (ROS), and an activity increase of caspase-like enzymes (van Doorn, 2011; Tsiatsiani *et al.*, 2011).

Although early events reported in responses to ionic and non-ionic hyperosmotic stress such as a transient $[Ca^{2+}]_{cyt}$ increase (Xiong *et al.*, 2002; Donaldson *et al.*, 2004; Lin *et al.*, 2006; Kim *et al.*, 2007; Parre *et al.*, 2007; Ranf *et al.*, 2008), generation of ROS (Zhu, 2001; Xiong *et al.*, 2002; Lin *et al.*, 2006; Zhang *et al.*, 2013), or up-regulation of protein kinases (Zhang *et al.*, 2013) seemed to be shared in plants, some other responses are specific to one of these stresses. Under saline conditions, Na^+ enters the cells through non-selective cation channels (NSCCs; Demidchik and Tester, 2002), depolarizing the plasma membrane (Chen *et al.*, 2007; Hua *et al.*, 2008; Shabala and Cuin, 2008; Pandolfi *et al.*, 2010; Wegner *et al.*, 2011). In contrast, isotonic mannitol or sorbitol solutions cause significant membrane hyperpolarization (Li and Delrot, 1987; Zingarelli *et al.*, 1999; Shabala *et al.*, 2000; Shabala and Lew, 2002). The DNA laddering, due to endonuclease release through permeable transition pores (PTPs) leading to mitochondria depolarization (Huh *et al.*, 2002; Lin *et al.*, 2006) occurred in NaCl- but not in sorbitol-stressed cells (Affenzeller *et al.*, 2009). Thus, although some common events are induced upon osmotic stress, multiple signal transduction pathways are involved in the response to ionic and non-ionic hyperosmotic treatments (Donaldson *et al.*, 2004; Parré *et al.*, 2007).

In this study, it was shown using Bright Yellow 2 (BY-2) cells that both ionic and non-ionic hyperosmotic stresses effectively induced early singlet oxygen (1O_2) generation and an 1O_2 -dependent influx of Ca^{2+} , which are not involved in PCD processes. The PCD observed in response to NaCl and sorbitol seemed to be dependent on delayed superoxide anion ($O_2^{\cdot-}$) generation by NADPH-oxidase, this last being linked to Na^+ influx through NSCCs and mitochondrial dysfunction only in the case of NaCl hyperosmotic stress.

Materials and methods

Cell culture conditions

Nicotiana tabacum L. BY-2 suspension cells were grown in Murashige and Skoog (MS) medium, pH 5.8 augmented with 30 g l^{-1} sucrose and 0.2 mg l^{-1} 2,4 D (Pauly *et al.*, 2001). Cells were maintained at $22 \pm 2\text{ }^\circ\text{C}$, under continuous darkness and continuous shaking (gyratory shaker) at 120 rpm. Cell suspensions were subcultured weekly using a 1:15 dilution. All experiments were performed at $22 \pm 2\text{ }^\circ\text{C}$ using log-phase cells (6 d after subculture) maintained in their culture medium. Cell density was $\sim 4 \times 10^5\text{ cells ml}^{-1}$.

Osmolality changes

The osmolality changes were systematically obtained by addition of $50\text{ }\mu\text{l}$ of sorbitol or NaCl from various stock solutions. For the measurement of extracellular medium osmolality changes after NaCl or sorbitol treatment, $100\text{ }\mu\text{l}$ of supernatant of cell suspensions treated with NaCl or sorbitol, were determined by the freezing depression method using an Automatic Micro-Osmometer Type 15 (Löser Messtechnik, Berlin, Germany).

Cell viability assays

Hyperosmosis-induced cell death in the cell suspension culture was determined by staining the dead cells with the vital dye Evans blue (0.005%, w/v) by mixing and incubating the cells and the dye for 10 min. Then stained cells were observed under a microscope. When appropriate, a 15 min pre-treatment with pharmacological effectors was done prior to NaCl or sorbitol exposure. Cells were counted under a microscope and cells that accumulated Evans blue were considered dead. At least 500 cells were counted for each independent treatment, and the procedure was repeated at least three times for each condition.

Monitoring of ROS production

The production of ROS was monitored by the chemiluminescence of the *Cypridina* luciferin analogue (CLA) as previously described (Kadono *et al.*, 2006, 2010). CLA is known to react mainly with $O_2^{\cdot-}$ and 1O_2 with light emission (Nakano, 1986), and allows measurement of extracellular ROS in plant cells (Tran *et al.*, 2013). Chemiluminescence from CLA was monitored using an FB12-Berthold luminometer (with a signal integrating time of 0.2 s). For data analysis, the luminescence ratio (L/L_{basal}) was calculated by dividing the intensity of CLA luminescence (L) by the luminescence intensity before treatment (L_{basal}). The ROS scavengers 1,2-dihydroxybenzene-3,5-disulphonic acid disodium salt (Tiron), 1,4-diazabicyclo[2.2.2]octane (DABCO), and salicylhydroxamic acid (SHAM) were added 5 min before NaCl and sorbitol treatment. Other inhibitors were added to the cell suspension 30 min before NaCl and sorbitol treatment.

Aequorin luminescence measurements

The $[Ca^{2+}]_{cyt}$ variations were recorded in a BY-2 cell suspension expressing the aequorin gene. Aequorin was reconstituted by overnight incubation in MS medium supplemented with 30 g l^{-1} sucrose and $2.5\text{ }\mu\text{M}$ native coelenterazine. Cell culture aliquots ($500\text{ }\mu\text{l}$) were transferred carefully into a luminometer tube, and the luminescence counts were recorded continuously at 0.2 s intervals with a luminometer. Treatments were performed by pipette injection of $50\text{ }\mu\text{l}$ of the effectors (NaCl or sorbitol). The residual aequorin was discharged by addition of $500\text{ }\mu\text{l}$ of a 1 M CaCl_2 solution dissolved in 100% methanol. The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of calcium measurement was performed by using the equation: $pCa = 0.332588(-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by the total remaining counts (Knight *et al.*, 1996). The results are expressed in micromolar Ca^{2+} and correspond to the mean \pm SD of 3–5 independent experiments.

Voltage clamp measurements

Experiments were conducted on 6-day-old cells maintained in their culture medium to limit stress (main ions in MS medium 28 mM NO_3^- , 16 mM K^+). Individual cells were immobilized by a microfunnel ($\sim 50\text{--}80\text{ }\mu\text{m}$ outer diameter) and controlled by a micromanipulator (WR6-1, Narishige, Japan). Impalements were carried out with a piezoelectric micromanipulator (PCS-5000, Burleigh Inst., USA) in

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a chamber (500 μ l) made of Perspex. Voltage clamp measurements of whole-cell currents from intact cultured cells presenting a stable running membrane potential were carried out using the technique of the discontinuous single voltage clamp microelectrode (dSEVC; Finkel and Redman, 1984). In this technique, both current passing and voltage recording use the same microelectrode. Interactions between the two tasks are prevented by time-sharing techniques (sampling frequency 1.5–3 kHz). Microelectrodes were made from borosilicate capillary glass (Clark GC 150F, Clark Electromedical, Pangbourne Reading, UK) pulled on a vertical puller (Narishige PE11, Japan). Their tips were <1 μ m diameter; they were filled with 600 mM KCl, and had electrical resistances between 20 M Ω and 50 M Ω with the culture medium. The capacity compensation of the microelectrode amplifier (Axoclamp 2A, Molecular Devices, Sunnyvale, CA, USA) was set to a subcritical level to produce the fastest electrode response. The relatively large size of the cells ensured a sufficiently high membrane time constant despite a relatively low input resistance (~40 M Ω). Specific software (pCLAMP 8) drives the voltage clamp amplifier. Voltage and current were simultaneously displayed on a dual input oscilloscope (Gould 1425, Gould Instruments Ltd, Hainault, UK), digitalized with a Digidata 1322A (Molecular Devices). In whole-cell current measurements, the membrane potential was held to the value of the resting membrane potential. Current recordings were obtained by hyperpolarizing pulses from -200 mV to +80 mV (20 mV, 2 s steps of current injection, 6 s of settling time). It was systematically checked that cells were correctly clamped by comparing the protocol voltage values with those really imposed. Only microelectrodes presenting a linear relationship were used.

Confocal microscopy

Confocal imaging was performed using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Germany) equipped with a \times 63 oil immersion objective. To analyse the NaCl influx, the sodium indicator Sodium Green was used (Molecular Probes, USA). The BY-2 tobacco cells were pre-incubated for 15 min with an NSCC inhibitor and then incubated with 200 mM NaCl for 1 h. Sodium Green indicator (10 μ M) was added to the solution 30 min after the beginning of the salt treatment. After incubation with NaCl and Sodium Green indicator, the BY-2 cells were washed with phosphate-buffered saline (PBS) buffer. The excitation wavelength was set at 514 nm, and the emission was detected at 530 \pm 20 nm.

Mitochondrial membrane potential measurement

Six-day-old BY-2 suspension cells were collected and washed by filtration in a suspension buffer containing 50 mM HEPES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 10 mM glucose, pH 7.0 (Errakhi *et al.*, 2008). After treatment, cells were stained with the mitochondrial membrane potential probe JC-1 by incubating 2 ml of cell suspensions for 15 min (24 $^{\circ}$ C in the dark) with 2 μ g ml⁻¹ JC-1 (3 μ M). JC-1 was dissolved and stored according to the manufacturer's instructions. Treated cells without prior washing were subjected to analysis using a Hitachi F-2000 fluorescence spectrophotometer. The excitation wavelength used was 500 nm. Fluorescence signals were collected using a band pass filter centred at 530 nm and 590 nm.

Chemicals

All chemical products were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), except JC1 and Sodium Green indicator which were from Molecular Probes (Saint Aubin, France). Stock solution of diphenyliodonium chloride (DPI; 10 mM) was dissolved in dimethylsulphoxide (DMSO) in order to obtain a 0.01% final concentration of DMSO. This DMSO concentration did not induce any change in ROS or [Ca²⁺]_{cyt} levels (not shown). All other chemicals were dissolved in water.

Statistical analysis

Data were analysed by analysis of variance (ANOVA), and the mean separation was achieved by Newman and Keuls multiple range test. All numerical differences in the data were considered significantly different at the probability level of $P \leq 0.05$.

Results

Hyperosmotic changes induce cell death in *N. tabacum* BY-2 suspension-cultured cells

The impact of NaCl and sorbitol additions on osmolality changes in BY-2 medium was first evaluated and it was found that the concentrations of NaCl (200 mM) and sorbitol (400 mM) most frequently used in this study showed almost the same osmolality shifts (Table 1). These shifts in osmolality induced by 400 mM sorbitol or 200 mM NaCl led to the death of a part of the cell population, dead cells displaying large cell shrinkage (Fig. 1A), the hallmark of the PCD process (van Doorn, 2011). Cell death scoring at various concentrations of sorbitol and NaCl showed the time- and dose-dependent progression of death (Fig. 1B, C), half of the cells being dead after 4 h at 400 mM sorbitol and 200 mM NaCl. In order to confirm whether this cell death was due to an active process requiring active gene expression and cellular metabolism, BY-2 cell suspensions were treated with actinomycin D (AD), an inhibitor of RNA synthesis, or with cycloheximide (Chx), an inhibitor of protein synthesis, each at 20 mg ml⁻¹, 15 min prior to 200 mM NaCl or 400 mM sorbitol exposure. In both cases, AD and Chx significantly reduced cell death (Fig. 1D). These results indicated that this cell death required active cell metabolism, namely gene transcription and *de novo* protein synthesis. Taken together, these data showed that saline or non-saline hyperosmotic stress induced a rapid PCD of a part of the *N. tabacum* BY-2 suspension cell population.

The kinetics of some early events classically detected upon saline stress or drought, namely an increase in cytosolic Ca²⁺, ion flux variations, ROS production, and mitochondrial membrane depolarization, were then followed, and it was checked how they could be involved in PCD induced by hyperosmotic stress.

Sorbitol- and NaCl-induced ROS generation

To study the effect of sorbitol on production of ROS in BY-2 cell suspension culture, the chemiluminescence of CLA, which indicates the generation of O₂⁻ and ¹O₂, was

Table 1. Osmolality changes in the medium after treatment with NaCl and sorbitol

	Medium	NaCl (mM)			Sorbitol (mM)		
		100	200	300	200	400	600
Osmolality (mosmol)	182	369	558	944	375	605	952

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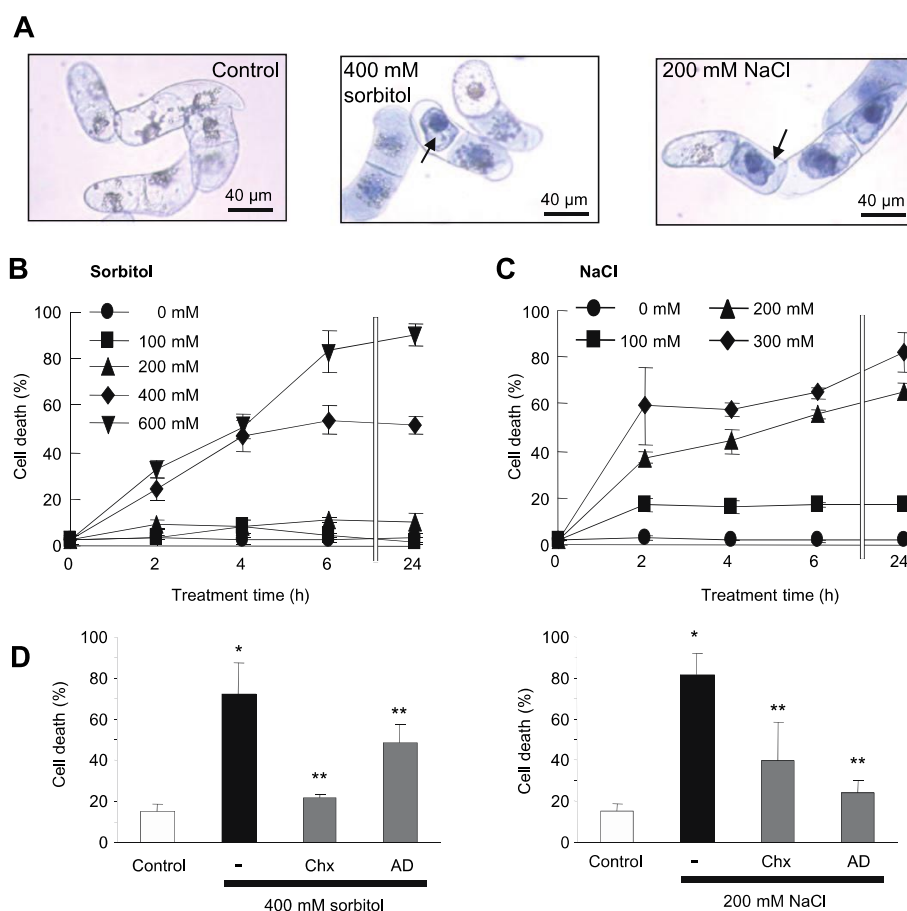


Fig. 1. NaCl- and sorbitol-induced cell death in tobacco BY-2 cells. (A) Light micrographs of BY-2 cultured cells stained with Evans blue 2 h after incubation with 400 mM sorbitol (centre) or 200 mM NaCl (right) compared with control cells maintained in their medium (left). Arrows indicate the cell shrinkage. (B) Effect of incubation time and concentration of sorbitol or NaCl on the extent of cell death. (C) Effect of pre-treatment with actinomycin D (AD; 20 $\mu\text{g ml}^{-1}$) or cycloheximide (Chx; 20 $\mu\text{g ml}^{-1}$) on sorbitol- and NaCl-induced cell death. Each data point and error bar reflect the mean and SD, respectively, of at least three independent replicates. *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$. (This figure is available in colour at *JXB* online.)

used. Addition of 400 mM sorbitol to BY-2 cell suspension culture resulted in transient production of ROS that reaches the maximal level immediately after treatment (Fig. 2A). This sorbitol-induced ROS generation was dose dependent (Fig. 2B) and could be blocked using DABCO, an $^1\text{O}_2$ scavenger, but not Tiron, an $\text{O}_2^{\cdot-}$ scavenger (Fig. 2A, C). Addition of 200 mM NaCl to BY-2 cell suspension culture also resulted in transient production of ROS that reaches the maximal level immediately after NaCl treatment (Fig. 2D, E). In the case of sorbitol, only DABCO was able to decrease the NaCl-induced CLA chemiluminescence (Fig. 2D, F). Thus, in both cases the early increase in CLA chemiluminescence seemed to be dependent on $^1\text{O}_2$ generation but not on

$\text{O}_2^{\cdot-}$ generation. SHAM, an inhibitor of peroxidase (POX) (Kawano *et al.*, 1998; Hossain *et al.*, 2013), which could be responsible for extracellular $^1\text{O}_2$ generation (Kawano *et al.*, 1998; Kanofsky, 2000; Guo *et al.*, 2009), was thus used. Pre-treatment of the BY-2 cell suspension culture with 5 mM SHAM only slightly reduced the increase in CLA chemiluminescence induced by 400 mM sorbitol (Fig. 2C) but significantly reduced that induced by 200 mM NaCl (Fig. 2F). This suggests the involvement of POX, at least in NaCl-induced $^1\text{O}_2$ generation.

The impact of ROS pharmacology on NaCl- and sorbitol-induced PCD (Fig. 1) was further checked. DABCO, the $^1\text{O}_2$ scavenger, failed to decrease sorbitol- (400 mM) and

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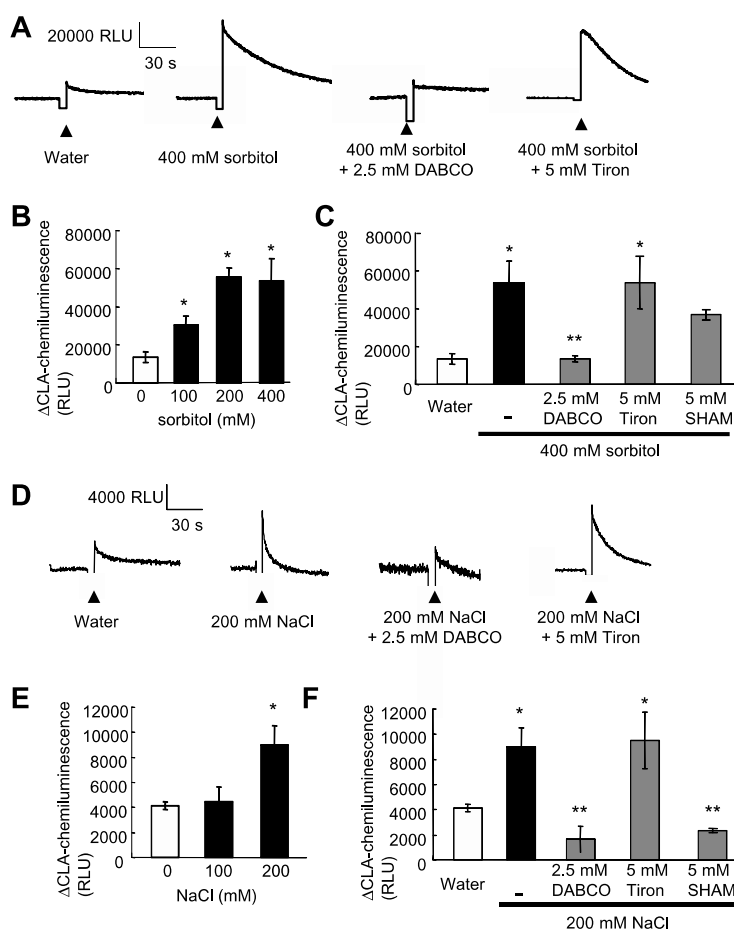


Fig. 2. Induction of rapid ROS generation in tobacco BY-2 cells by sorbitol or NaCl. (A) Typical kinetics of the sorbitol-induced increase in CLA chemiluminescence reflecting the production ROS and modulation by ROS scavengers. (B) Effect of the concentration of sorbitol on ROS generation, (C) Modulation of sorbitol-induced ROS generation by DABCO, a scavenger of singlet oxygen, Tiron, a scavenger of the superoxide anion, or salicylhydroxamic acid (SHAM), an inhibitor of peroxidase, (D) Typical kinetics of the NaCl-induced increase in CLA chemiluminescence and modulation by ROS scavengers. (E) Effect of the concentration of NaCl on ROS generation. (F) Modulation of NaCl-induced ROS generation by DABCO, Tiron, or SHAM. Each data point and error bar reflect the mean and SD, respectively ($n=5$). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

NaCl- (200mM) induced cell death and even increased NaCl-induced cell death after 2h of treatment (Fig. 3A, B). For Tiron, the $O_2^{\cdot-}$ scavenger, there was no effect after 2h but a decrease in cell death could be observed after 4h treatment with NaCl (Fig. 3B). Thus, the hyperosmotic stress-induced cell death seemed not to be dependent on 1O_2 generation but on $O_2^{\cdot-}$ generation. Thus a possible delayed $O_2^{\cdot-}$ generation after treatment with 400mM sorbitol or 200mM NaCl was searched for. After such hyperosmotic stress, increases in CLA chemiluminescence could be detected (Fig. 3C). In both cases of CLA chemiluminescence increases, the maximum chemiluminescence levels were reached after 1h and then decreased

to the control level after 4h, the decrease being more rapid upon NaCl treatment (Fig. 3C). It is noteworthy that these increases occurred before cell death reached the plateau level (Fig. 1B, C). These increases in CLA chemiluminescence could be inhibited by pre-treatment with Tiron, but also with 10 μ M DPI, an inhibitor of NADPH-oxidase (Fig. 3D), suggesting that the generation $O_2^{\cdot-}$ through enhancement of NADPH-oxidase activity was involved in the delayed ROS generation after treatment with sorbitol and NaCl (Fig. 3C). In agreement with this, 10 μ M DPI could also significantly reduce both sorbitol- and NaCl-induced cell death after 4h (Fig. 3A, B).

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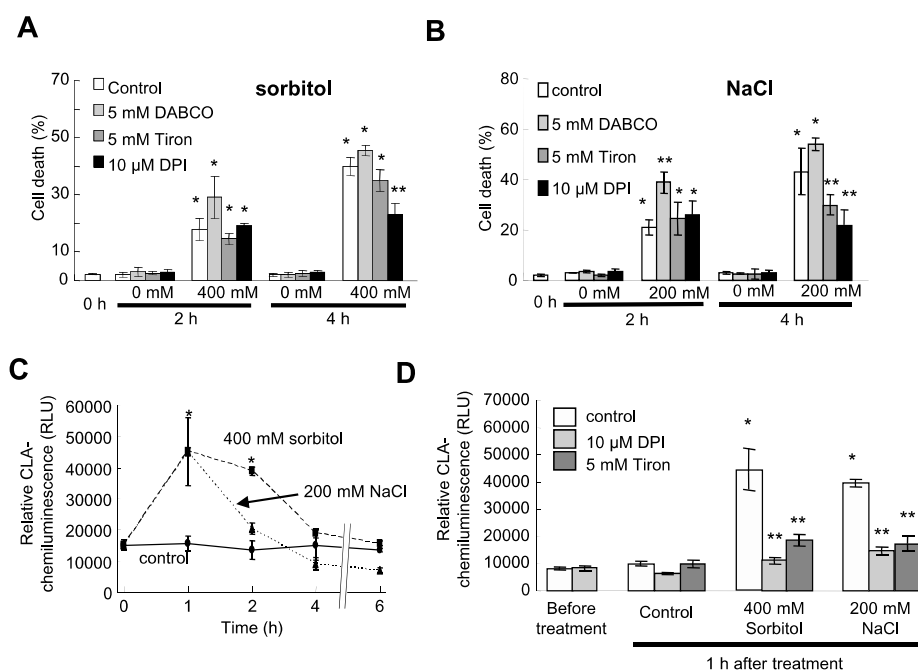


Fig. 3. Effects of ROS scavengers on NaCl- or sorbitol-induced cell death in BY-2 cells. (A) Effect of Tiron, DABCO, or DPI, an NADPH-oxidase inhibitor, on cell death induced by 400mM sorbitol after 2h or 4h treatment. (B) Effect of Tiron, DABCO, or DPI on cell death induced by 200mM NaCl after 2h or 4h treatment. (C) Time course of CLA chemiluminescence during 6h treatment with 400mM sorbitol or 200mM NaCl. (D) Inhibition of sorbitol and NaCl-induced delayed ROS generation by Tiron or DPI. Each data point and error bar reflect the mean and SD, respectively ($n=3$). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

Sorbitol and NaCl induce a rapid change in $[Ca^{2+}]_{cyt}$

The changes in $[Ca^{2+}]_{cyt}$ were monitored by the Ca^{2+} -dependent emission of blue light from aequorin (Knight *et al.*, 1996). Treatment of BY-2 cells with sorbitol (400mM) resulted in a rapid transient increase in aequorin luminescence (Fig. 4A) reflecting an increase in $[Ca^{2+}]_{cyt}$ of $0.145 \pm 0.035 \mu M$ ($n=23$). This increase could be inhibited by the presence of Ca^{2+} channel blockers, $LaCl_3$ and $GdCl_3$, when Ca^{2+} internal store inhibitors (U73122 and dantrolene, Meimoun *et al.*, 2009) showed no significant inhibitory effects (Fig. 4B), indicating that the sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ is mainly due to influx of Ca^{2+} across the plasma membrane through Ca^{2+} channels. As the generation of 1O_2 by sorbitol occurred immediately upon hyperosmotic stress (Fig. 2A), the effect of ROS pharmacology on the sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ was further tested. The 1O_2 scavenger DABCO strongly reduced the $[Ca^{2+}]_{cyt}$ increase compared with that in control cells; the POX inhibitor SHAM showed lower efficiency, while no differences were seen with the $O_2^{\cdot-}$ scavenger Tiron (Fig. 4A, C). These results strongly suggested that sorbitol-induced Ca^{2+} uptake through Ca^{2+} channels occurs downstream of the sorbitol-induced 1O_2 generation.

In the same way, treatment of BY-2 cells with NaCl (200mM) resulted in a transient increase in aequorin luminescence (Fig. 4D), reflecting an increase in $[Ca^{2+}]_{cyt}$ of $0.217 \pm 0.059 \mu M$ ($n=23$). As for sorbitol, inhibitors of Ca^{2+} release from intracellular organelles (U73122 and dantrolene) failed to suppress the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ whereas Ca^{2+} channel blockers $LaCl_3$ and $GdCl_3$ were efficient at reducing the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 4E). In a similar manner to what was observed for sorbitol, this increase was shown to be inhibited by the presence of DABCO (Fig. 4F) but less efficiently by SHAM, whereas Tiron seemed even to increase this Ca^{2+} influx (Fig. 4D, F). The effect of the Ca^{2+} channel inhibitor La^{3+} on NaCl- and sorbitol-induced cell death in BY-2 suspension-cultured cells was then studied. Lanthanum (500 μM) failed to decrease sorbitol- (400mM) and NaCl- (200mM) induced cell death and even increases this cell death after 2h of treatment (Fig. 4G), as observed for DABCO with NaCl (Fig. 3B). These data are in agreement with the link observed between the immediate 1O_2 generation inducing an influx of Ca^{2+} upon sorbitol or NaCl stress and further suggest that these early induced events are not involved in a pathway leading to PCD.

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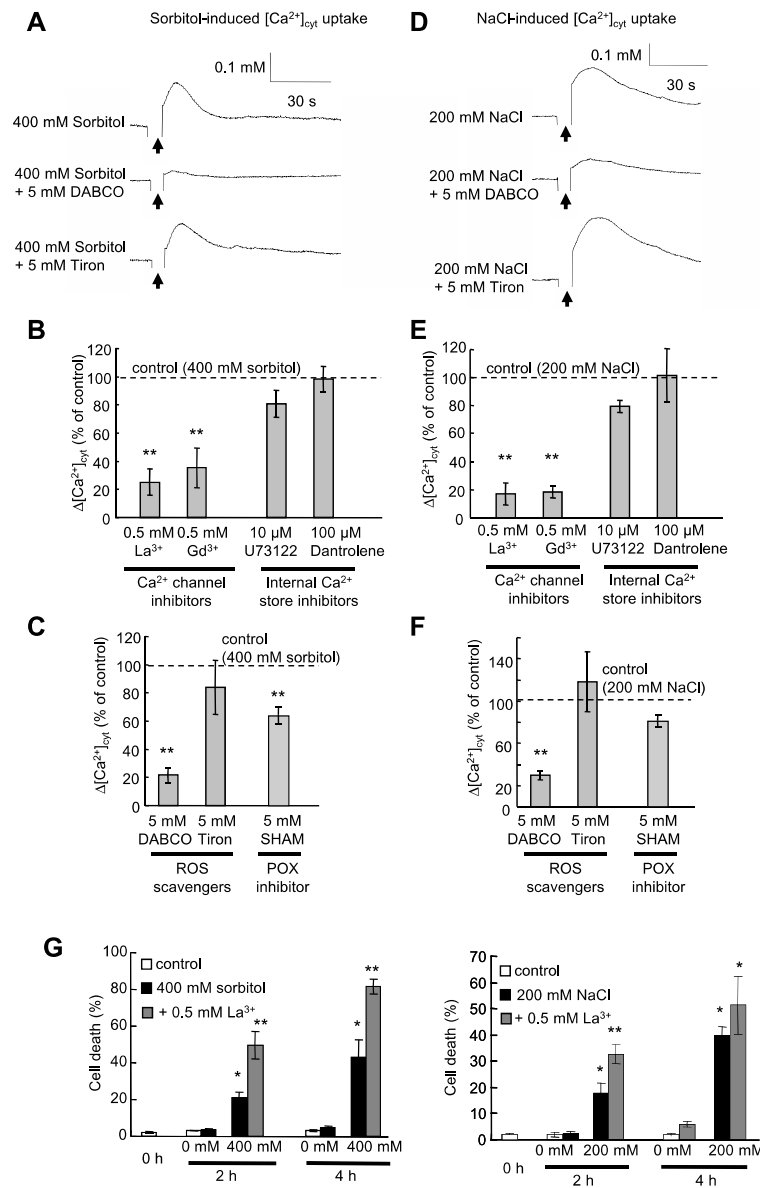


Fig. 4. Induction of $[Ca^{2+}]_{cyt}$ increase in aequorin-expressing tobacco BY-2 cells by sorbitol or NaCl. (A) Typical kinetics of sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ and modulation by ROS scavengers. (B) Modulation of sorbitol-induced $[Ca^{2+}]_{cyt}$ increase by the calcium channel blockers La^{3+} or Gd^{3+} (500 μ M each) and the internal stock release inhibitors U73122 (100 μ M) and dantrolene (10 μ M). (C) Modulation of sorbitol-induced $[Ca^{2+}]_{cyt}$ increase by DABCO, a scavenger of singlet oxygen, Tiron, a scavenger of anion superoxide, or salicylhydroxamic acid (SHAM), an inhibitor of peroxidase. (D) Typical kinetics of the NaCl-induced increase in $[Ca^{2+}]_{cyt}$ and modulation by ROS scavengers. (E) Modulation of the NaCl-induced $[Ca^{2+}]_{cyt}$ increase by La^{3+} , Gd^{3+} (500 μ M each), U73122 (100 μ M), or dantrolene (10 μ M). (F) Modulation of NaCl-induced $[Ca^{2+}]_{cyt}$ increase by DABCO, Tiron, or SHAM. (G) Effect of La^{3+} on cell death induced by 200 mM NaCl (left) or 400 mM sorbitol (right) after 2 h or 4 h treatment. Each data point and error bar reflect the mean and SD, respectively ($n=5$). *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$.

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Hyperosmotic constraints induce change in membrane potential and ion channel activities

Saline and non-saline hyperosmotic stresses are well known to modify the plasma membrane potential (V_m) of cells (Teodoro *et al.*, 1998; Zingarelli *et al.*, 1999; Shabala and Cuin, 2008; Wegner *et al.*, 2011). By using an electrophysiological technique (dSEVC), the impact of NaCl and sorbitol on BY-2 cultured cell membrane potential was investigated. In control conditions in culture medium, the V_m of BY-2 cells was -21.1 ± 2.2 mV ($n=15$). In MS medium, the main ions are 16 mM K^+ and 28 mM NO_3^- ; thus the equilibrium potential estimated for K^+ , E_K , is about -46 mV ($[K^+]_{out}=16$ mM with $[K^+]_{in}$ estimated at 100 mM). The equilibrium potential estimated for NO_3^- is about -25 mV ($[NO_3^-]_{out}=28$ mM with $[NO_3^-]_{in}$ estimated at 5 mM). As previously observed with cultured cells of *Arabidopsis thaliana* (Kadono *et al.*, 2010; Tran *et al.*, 2013) or tobacco (Gauthier *et al.*, 2007), the occurrence of anion currents in most of the BY-2 cells in their culture medium could explain the mean polarization of around -20 mV recorded in control and non-stressed conditions. The mean control value of these currents at -200 mV and after 1.8

s of voltage pulse was -1.12 ± 0.2 nA ($n=11$). These currents were shown to be sensitive to structurally unrelated anion channel inhibitors, 9-anthracene carboxylic acid (9-AC) and glibenclamide (gli) (Supplementary Fig. S1 available at *JXB* online), reinforcing the hypothesis of an anionic nature for these currents. Addition of NaCl to suspension cultures resulted in a significant membrane depolarization (Fig. 5A) when sorbitol induced a hyperpolarization of the cells (Fig. 5A), clearly indicating a difference between saline and non-saline hyperosmotic stress. The sorbitol-induced hyperpolarization was correlated with a decrease in anion current (Fig. 5B) when the NaCl-induced depolarization was correlated with a large increase in whole-cell ion current (Fig. 5B). The positive shifts of the reversal potential of the current upon addition of NaCl are in accordance with a current carried by Na^+ .

The influx of Na^+ through the plasma membrane by NSCCs was the most probable reason for the cell depolarization (Demidchick *et al.*, 2002a, b), this Na^+ uptake was further checked using the Na^+ -sensitive fluorescent probe Sodium Green (Wegner *et al.*, 2011). An accumulation of fluorescence in the cytoplasm of the cells could be observed after

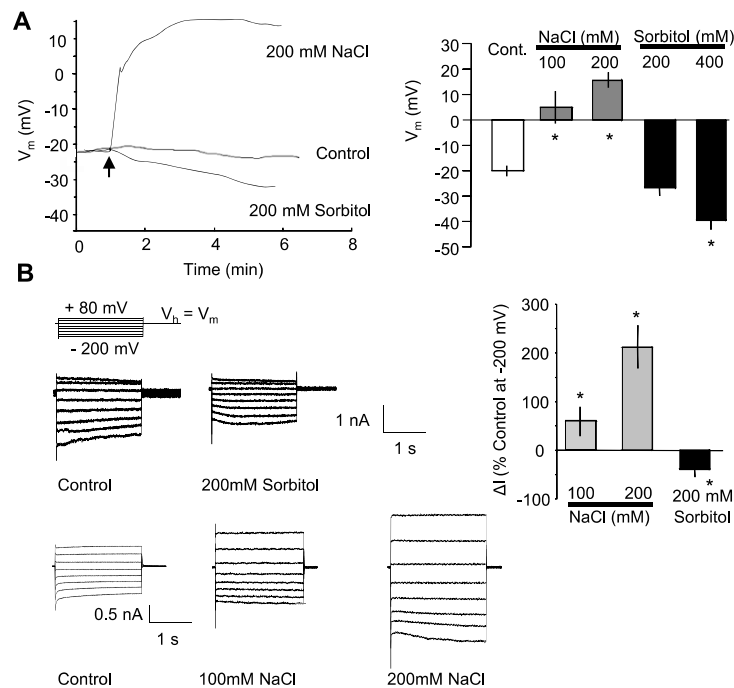


Fig. 5. (A) Typical modulation of BY-2 cultured cell plasma membrane (PM) potential variations observed in response to NaCl or sorbitol. (B) Mean values of PM potentials recorded a few minutes after treatment with NaCl (100 mM or 200 mM) or sorbitol (200 mM or 400 mM). (C) Typical changes in whole-cell current profiles after treatments with sorbitol (up) or NaCl (down). The protocol was as illustrated; the holding potential (V_h) was V_m . (D) Mean values of whole-cell current variations (recorded at -200 mV and 1.8 s) after treatment with NaCl or sorbitol. Current variations are given as a percentage of the control level before treatments. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. *Significantly different from controls, $P < 0.05$.

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1 h treatment with 200 mM NaCl (Fig. 6A). This fluorescence could be reduced by using pre-treatments with inhibitors of NSCC, 5 mM tetraethylammonium chloride (TEA⁺) or 1 mM quinine (Demidchick *et al.*, 2002a) (Fig. 6A). Addition of 1 mM quinine or 5 mM TEA⁺ also allowed reduction of the NaCl-induced increase in currents (Fig. 6B, C) in BY-2 cells, as did verapamil (100 μ M), another potent inhibitor of NSCCs (Demidchick *et al.*, 2002a). The impact of these NSCC blockers on the extent of NaCl-induced cell death was thus further tested. The NSCC blockers were efficient at reducing the cell death induced by 200 mM NaCl (Fig. 6D), suggesting that NSCC activation is related to the NaCl-induced cell death. As this cell death was also dependent on a delayed O₂⁻ generation by NADPH-oxidase (Fig. 3), the effect of the earliest activation of NSCCs by NaCl on this delayed ROS production was also checked. As for cell death, the NSCC blockers were efficient at reducing this NaCl-dependent delayed ROS production (Fig. 6E), suggesting that NaCl influx participates in the O₂⁻ generation. Interestingly, the calcium channel blocker La³⁺ did not allow the delayed ROS generation to be decreased, confirming the hypothesis of the induction of different pathways in response to NaCl stress.

Mitochondrial depolarization is involved in NaCl- but not sorbitol-induced cell death

The role of mitochondria is well recognized in salinity tolerance (Jacoby *et al.*, 2011), and dysfunction of mitochondria, leading to cytochrome *c* release upon salt stress, was shown to induce PCD in *Thellungiella halophila* suspension-cultured cells (Wang *et al.*, 2010). Mitochondria are effectively pivotal in controlling cell life and PCD, through complex mechanisms that culminate in opening of PTPs leading to mitochondrial membrane potential ($\Delta\Psi_m$) loss (Vianello *et al.*, 2007). It was thus checked whether NaCl and sorbitol lead to a decrease in $\Delta\Psi_m$ in the present model. In untreated cells, the JC-1 fluorescence ratio of mitochondria displaying a high $\Delta\Psi_m$ versus mitochondria presenting a low $\Delta\Psi_m$ was greatly superior to 1 (Fig. 7A). This ratio decreased in a time-dependent manner upon addition of NaCl (200 mM), indicating that NaCl induced a significant decrease of $\Delta\Psi_m$ in some mitochondria when sorbitol (400 mM) induced an increase of $\Delta\Psi_m$ during the first 30 min, the ratio reaching the control value after 2 h (Fig. 7A). Since a mitochondrial $\Delta\Psi_m$ decrease during cell death was reported to be due to the formation of the mitochondrial PTPs (Vianello *et al.*, 2007), the effect of cyclosporin A (CsA), an inhibitor of PTPs, was tested on NaCl-induced mitochondrial depolarization. A pre-treatment with CsA significantly reduced the NaCl-induced mitochondrial depolarization after 15 min (Fig. 7B), indicating that mitochondrial PTPs were involved in NaCl-induced mitochondrial depolarization. Moreover, pre-treatment with CsA significantly inhibited NaCl-induced cell death (Fig. 7C), indicating that PTP formation could participate in NaCl-induced cell death. As the activation of NSCCs occurs rapidly upon NaCl stress (Fig. 5A) and is involved in ROS generation (Fig. 6E), the impact of sodium influx on $\Delta\Psi_m$ was investigated by using NSCC inhibitors. A significant reduction of NaCl-induced

mitochondrial depolarization was observed after pre-treatment with verapamil (Fig. 7B). These data show that (i) two different pathways could be involved in the hyperosmotic stress-induced cell death; and (ii) the ROS generation dependent on Na⁺ influx could participate in the decrease in $\Delta\Psi_m$ during NaCl-induced cell death.

Discussion

As expected from previous studies (Huh, 2002; Wang, 2010), the death of a part of the BY-2 cell population characterized by large cell shrinkage, a hallmark of the PCD process (van Doorn, 2011), was observed in response to NaCl-induced hyperosmotic stress. The extent of cell death was time and dose dependent, reaching about half of the population in 4 h with 200 mM NaCl. In order to check whether this cell death was due to an active mechanism requiring active gene expression and cellular metabolism, BY-2 cell suspensions were treated with AD, an inhibitor of RNA synthesis, or with Chx, an inhibitor of protein synthesis, prior to NaCl exposure. AD and Chx significantly reduced the NaCl-induced cell death. These results indicated that this cell death required active cell metabolism, namely gene transcription and *de novo* protein synthesis. The same behavior, namely time- and dose-dependent cell death characterized by cell shrinkage and requiring active metabolism using iso-osmotic concentrations of sorbitol, was observed here. This suggests that non-ionic hyperosmotic stress, like ionic hyperosmotic stress, could induce PCD in BY-2 cells as previously observed in various animal cell lines (Murata *et al.*, 2002; Galvez *et al.*, 2003; Niswander and Dokas, 2007). It was then checked whether some early events classically detected during hyperosmotic stress responses and PCD in plant could be involved in this cell death, namely ROS production, an increase in cytosolic Ca²⁺, ion flux variations, and mitochondrial membrane depolarization.

The earliest response observed after exposure of BY-2 cells to NaCl was an immediate peak of ROS. Addition of Tiron (a scavenger of O₂⁻) did not significantly reduce NaCl-induced ROS generation, whereas DABCO (a scavenger of ¹O₂) avoided this production. Although ¹O₂ formation is likely to occur during the exposure to high light intensities (Krieger-Liszkay, 2004), it was also found that different enzymes including POXs could produce extracellular ¹O₂ in animals (Kanofsky, 2000; Stief, 2003; Tarr and Valenzano, 2003) and in plant cells (Kawano *et al.*, 1998; Guo *et al.*, 2009). As the studied cultured cells were non-photosynthetic, the POX inhibitor SHAM was further tested (Kawano *et al.*, 1998; Hossain *et al.*, 2013), and this could reduce the early NaCl-induced ROS generation effectively. Thus, it would appear that these early transient ¹O₂ generations are not specific to ionic or non-ionic hyperosmotic stress.

In the case of both NaCl and sorbitol treatments, rapid transient [Ca²⁺]_{cyt} increases in BY-2 cells were observed, as previously described in various models (Knight *et al.*, 1997; Donaldson *et al.*, 2004; Lin *et al.*, 2006; Parre *et al.*, 2007; Ranf *et al.*, 2008). In BY-2 cells, these [Ca²⁺]_{cyt} increases were inhibited by the calcium channel blockers La³⁺ and

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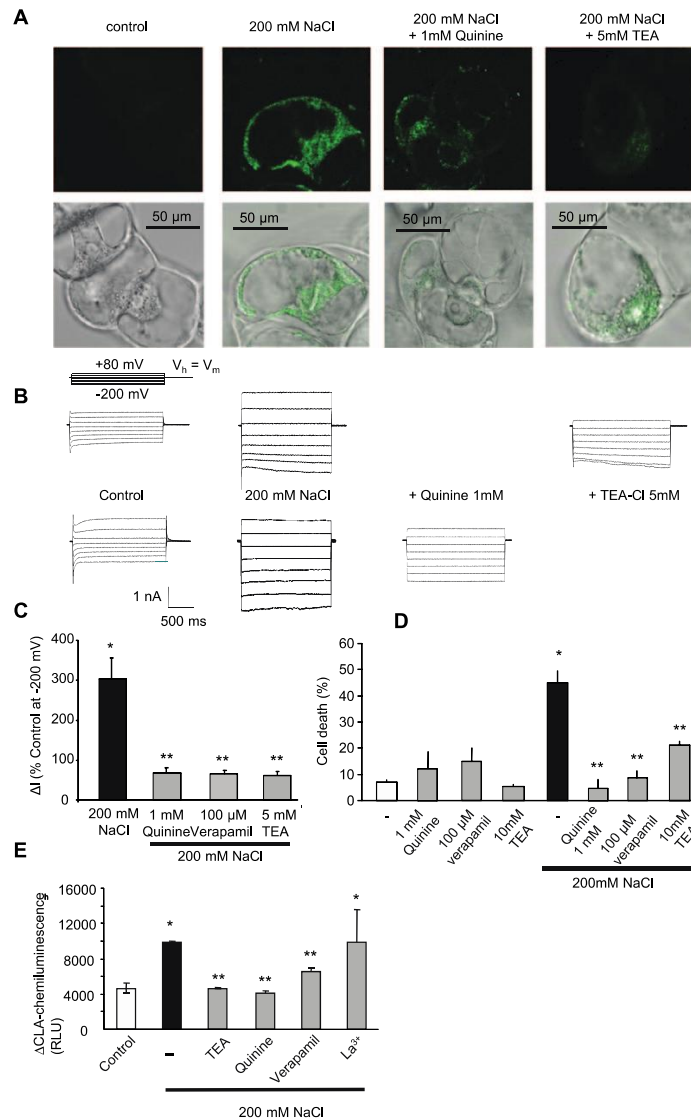


Fig. 6. (A) Confocal imaging of NaCl accumulation in BY-2 cells after 1 h treatment with 200 mM NaCl using the Sodium Green fluorescent probe (left). Decrease in fluorescence in cells pre-treated with quinine (1 mM) or TEA⁺ (5 mM), blockers of non-selective cation channels (NSCCs), prior to NaCl treatment (right). Corresponding bright field images are shown on the line below. Each image is representative of symptoms observed in at least three independent experiments. (B) Variations of whole-cell currents recorded before and after addition of 200 mM NaCl and subsequent addition of 1 mM quinine or 5 mM TEA⁺. The protocol was as illustrated; the holding potential (V_h) was V_m . (C) Mean values of whole-cell current variations (recorded at -200 mV and 1.8 s) after treatment with 200 mM NaCl with or without the NSCC blockers quinine (1 mM), TEA⁺ (5 mM), or verapamil (200 μ M). Current variations are given as a percentage of the control level before treatments. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. (D) Effect of pre-treatments with the NSCC blockers on NaCl-induced cell death. The data correspond to means of at least four independent replicates, and error bars correspond to the SD. (E) Effect of pre-treatments with the NSCC blockers or with La^{3+} (500 μ M) on NaCl-induced delayed ROS generation and cell death. The data correspond to means of at least five independent replicates, and error bars correspond to the SD. *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl- or sorbitol-treated cells, $P < 0.05$. (This figure is available in colour at *JXB* online.)

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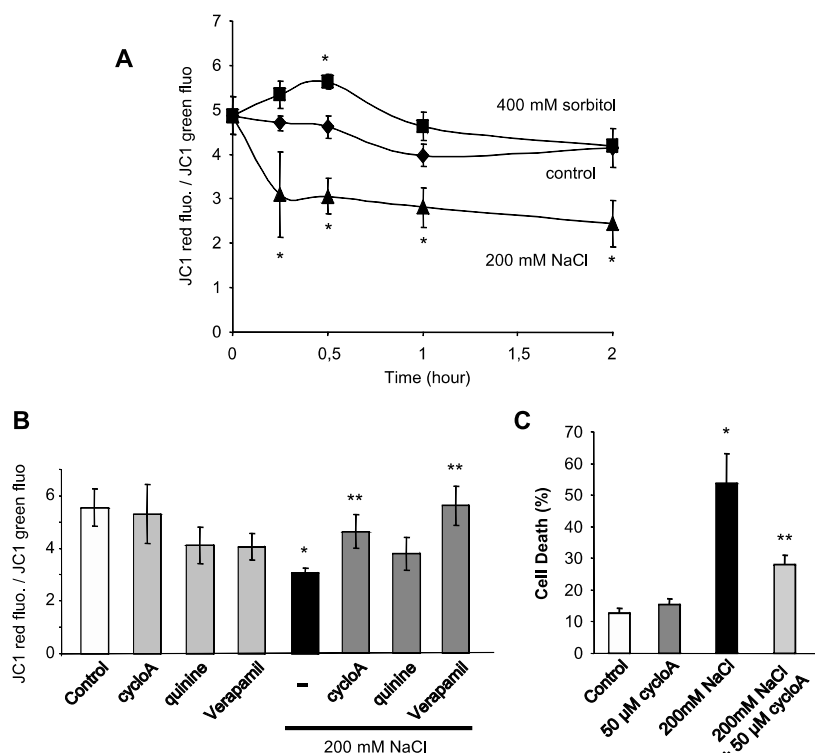


Fig. 7. (A) Variations of mitochondrial membrane potential ($\Delta\Psi_m$) of BY-2 tobacco cells after treatments with 200 mM NaCl or 400 mM sorbitol. (B) Effect of 50 μ M cyclosporin A (CsA), an inhibitor of the permeability transition pore, 1 mM quinine, or 200 μ M verapamil, inhibitors of NSCCs, on $\Delta\Psi_m$ variation induced by 200 mM NaCl after 15 min. The data reflect the means \pm SE of at least four independent experiments. (C) Effect of 50 μ M CsA, 1 mM quinine, or 200 μ M verapamil on cell death induced by 200 mM NaCl. The data reflect the means \pm SE of at least three independent replicates. *Significantly different from controls, $P < 0.05$; **significantly different from the NaCl-treated cells, $P < 0.05$.

Gd^{3+} but not by dantrolene and U73122, inhibitors of calcium-induced calcium release channels and of the inositol triphosphate receptor, respectively, known to be efficient in plants (Meimoun *et al.*, 2009). This suggests that the $[Ca^{2+}]_{cyt}$ increase was not due to Ca^{2+} release from intracellular Ca^{2+} stores, but to an influx through plasma membrane-permeable Ca^{2+} channels. Although rapid, the Ca^{2+} influxes happened after the 1O_2 generation since DABCO pre-treatments strongly decreased these influxes, suggesting that they were dependent on the generation of 1O_2 . Accordingly, SHAM decreased the Ca^{2+} influxes to a lower extent and Tiron was unable to decrease them. This confirms the hypothesis that an 1O_2 -dependent Ca^{2+} influx was induced by sorbitol and NaCl and highlights the role of 1O_2 as a signalling molecule (Fischer *et al.*, 2013).

Perturbation of Ca^{2+} homeostasis in plant cells, as well as in animal cells, has been described as a prerequisite for PCD (Grant *et al.*, 2000; Davis and Distelhorst, 2006; Lecourieux *et al.*, 2006). It was not possible to ascertain that in the BY-2 population all cells respond with a Ca^{2+} increase in the face of

hyperosmotic stress. However, if only non-dying cells respond with a Ca^{2+} increase, this Ca^{2+} increase has no role in inducing PCD. In other words, if only dying cells (or even all cells) respond with a Ca^{2+} increase, since La^{3+} was inefficient in decreasing NaCl- and sorbitol-induced cell death, and even enhanced this cell death, it could suggest that Ca^{2+} influx could participate in cell protection. After perception of salt stress, the Ca^{2+} spike generated in the cytoplasm of root cells is known to activate the Salt Overly Sensitive (SOS) signal transduction cascade to protect the cells from damage due to excessive ion accumulation (Ji *et al.*, 2013). *SOS3* encodes a myristoylated calcium-binding protein that appears to function as a primary calcium sensor to perceive the increase in cytosolic Ca^{2+} triggered by Na^+ excess that has entered the cytoplasm. Upon binding to Ca^{2+} , *SOS3* is able to interact with and activate the protein kinase *SOS2* which phosphorylates *SOS3* proteins. *SOS3*–*SOS2* interactions recruit *SOS2* to the plasma membrane, leading to activation of the downstream target *SOS1*, an Na^+/H^+ antiporter allowing extrusion of excessive Na^+ from the cytosol (Ji *et al.*, 2013). However, in

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the present model, neither of these early linked events, Ca^{2+} increase and $^1\text{O}_2$, seemed to be involved in PCD.

On the other hand, Tiron, a scavenger of $\text{O}_2^{\cdot-}$, and DPI, an inhibitor of NADPH-oxidase, decreased the NaCl- and sorbitol-induced cell death and the delayed generation of ROS. This indicated that the delayed and more sustained $\text{O}_2^{\cdot-}$ generation from NADPH-oxidase activity could play a central role in the death of these cells. Several reports implicate NADPH-oxidase activity in production of ROS in salinity stress, with the ROS resulting in Ca^{2+} influx. In the present model, no effect of DPI on early stress-induced Ca^{2+} increase could be detected (Supplementary Fig. S2 available at *JXB* online), in accordance with the delayed DPI-sensitive ROS generation. However, from the present data, a delayed increase in Ca^{2+} linked to NADPH-oxidase activity cannot be excluded, but this increase in Ca^{2+} should be La^{3+} independent, since La^{3+} could not decrease cell death. It is obvious that sorbitol-induced ROS generation could not depend on Na^+ influx and thus possibly other hyperosmotic-induced events (e.g. NO production) could participate in NADPH-oxidase-dependent ROS generation observed in response to sorbitol and NaCl. However, upon NaCl stress, the delayed ROS generation could be decreased by quinine and verapamil, putative inhibitors of NSCCs (Demidchik *et al.*, 2002a, b). Even though no definitive molecular candidates have clearly emerged for NSCCs, it seems that various classes of NSCCs could be responsible for influx of Na^+ under salt stress, especially a depolarization-activated class of NSCCs (Demidchik and Maathuis, 2007). A rapid and large depolarization of the BY-2 cells could be recorded upon NaCl addition due to an increase in a current sensitive to quinine and verapamil but also to TEA^+ , an inhibitor of K^+ channels known to block some NSCCs (Demidchik *et al.*, 2002a). It could also be verified that the accumulation

of Na^+ in the cell was decreased upon pre-treatment with quinine or TEA^+ , strongly suggesting that NSCCs were responsible for Na^+ influx into BY-2 cells. Moreover, these NSCC blockers were efficient in decreasing the NaCl-induced cell death, highlighting the toxic effect of Na^+ as previously reported (Huh *et al.*, 2002; Affenzeller *et al.*, 2009; Wang *et al.*, 2010). It is further noticeable that La^{3+} , a potent inhibitor of some NSCCs (Demidchik *et al.*, 2002a, b), was unable to decrease NaCl-induced cell death as well as the delayed ROS generation, whereas the NSCC blockers quinine, verapamil, and TEA^+ failed to decrease the NaCl-induced Ca^{2+} increase (Supplementary Fig. S3 available at *JXB* online). This indicates that the Ca^{2+} influx was completely dissociated from Na^+ influx through NSCCs. Moreover, sorbitol- and NaCl-induced Ca^{2+} influxes presented the same characteristics and were induced upon hyperpolarization in the case of sorbitol and depolarization in the case of NaCl, suggesting the voltage independence of the transporter involved. Further studies will be needed to determine if putative ligand-activated calcium channels such as cyclic nucleotide-gated channels or glutamate receptors could be responsible for these Ca^{2+} influxes in BY-2 cells; however, a putative candidate could be the calcium regulatory protein annexin (Laohavisit *et al.*, 2013).

Concerning ion flux regulation in response to sorbitol, a hyperpolarization of the BY-2 cells due to the decrease of anion currents was observed, as previously described in different models (Pennarun and Maillot, 1988; Teodoro *et al.*, 1998; Shabala *et al.*, 2000). Although such a regulation of anion current was shown to be involved during PCD induced by HrpN_{ca} , a hypersensitive elicitor from *Erwinia amylovora* (Reboutier *et al.*, 2005), it did not seem to be involved in sorbitol-induced PCD since bromotetramisole, an activator of anion channels, failed to limit sorbitol-induced PCD (data

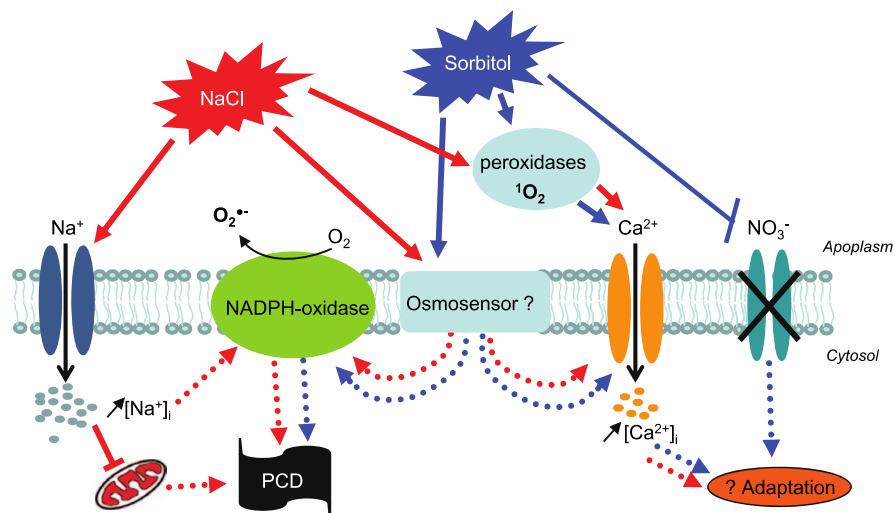


Fig. 8. Possible pathways induced by NaCl and sorbitol leading to cell death of tobacco cells. (This figure is available in colour at *JXB* online.)

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not shown), in contrast to the previously observed effect of $HrpN_{ea}$ (Reboutier *et al.*, 2005).

Although ROS-activated outward-rectifying K^+ channels (KORCs) were shown to be involved in salt-induced PCD (Demidchik *et al.*, 2003, 2010), it was not possible to detect rapid activation of KORCs in response to NaCl or sorbitol. However, an activation of KORCs after addition of NaCl cannot be excluded, this current being masked in the large Na^+ current recorded. On the other hand, KORC activation could also be delayed, as was recently reported in response to O_3 (Tran *et al.*, 2013).

Finally, one of the most important differences observed between sorbitol- and NaCl-induced PCD in BY-2 cells was the role of mitochondria. Sorbitol did not affect mitochondrial polarization and even slightly and transiently stimulated its polarization, whereas NaCl induced a large mitochondrial depolarization. This depolarization was probably involved in the formation of the PTP since CsA, an inhibitor of PTP, could reduce the NaCl-induced decrease in $\Delta\Psi_m$, as previously described (Wang *et al.*, 2010) and also the NaCl-induced cell death. This could be related to observation made on *Micrasterias denticulate* (Affenzeller *et al.*, 2009) for which a typical laddering of the DNA was only observed for NaCl-stressed cells but not for sorbitol-treated cells. The endonuclease responsible for this laddering could be released from mitochondria through PTPs (Vianello *et al.*, 2007). It is to be noted that the inhibitors of NSCCs, verapamil, quinine, and TEA^+ , were also efficient in limiting the loss in $\Delta\Psi_m$, suggesting a direct role for cytosolic Na^+ -induced mitochondrial dysfunction.

As already described in numerous models, in BY-2 cells overlapping responses exist in response to ionic and non-ionic hyperosmotic stress and, among them, a PCD process. However, some overlapping responses such as the early transient 1O_2 generation responsible for an influx of Ca^{2+} did not seem to be involved in PCD progress, while other shared responses such as the delayed NADPH-oxidase stimulation were important for these processes (Fig. 8). Specific responses also seemed to be involved in the PCD processes since upon NaCl stress, NADPH-oxidase stimulation was at least partly due to Na^+ influx through NSCCs, which also seemed to be responsible for mitochondrial depolarization not observed after the sorbitol challenge. Further studies will be needed to characterize fully the pathways leading to PCD induced by these hyperosmotic stresses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Typical anion current recorded in control conditions in BY-2 cells.

Figure S2. NaCl- and sorbitol-induced $[Ca^{2+}]_{cyt}$ increase in aequorin-expressing-tobacco BY-2 cells after a pre-treatment with the inhibitor of NADPH-oxidase DPI (20 μ M).

Figure S3. NaCl-induced $[Ca^{2+}]_{cyt}$ increases in aequorin expressing-tobacco BY-2 cells after pre-treatments with the NSCC blockers quinine, verapamil, or TEA^+ .

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Chapter 3

Dual responses of cultured plant cells to hyperosmotic stress

Chapter 3: Dual responses of cultured plant cells to hyperosmotic stress

3.1 Introduction

In the previous section we observed the early responses to ionic- and non-ionic hyperosmotic stress leading to PCD. It was shown that in response to ionic- and non-ionic hyperosmotic stresses, the early $^1\text{O}_2$ generation and subsequent $[\text{Ca}^{2+}]_{\text{cyt}}$ increase were not involved in PCD, while a delayed O_2^- generation could be involved in the events leading to PCD. One of the most important differences observed between non-ionic and ionic hyperosmotic stresses induced PCD in BY2 tobacco cells was the role of NSCCs in case of NaCl treatment. In fact, Na^+ influx through NSCC causes a mitochondrial depolarization probably involved in PCD. After sorbitol treatment we observed a hyperpolarization correlated with a decrease in anion currents. However the cell shrinkage observed after sorbitol treatment, could not be explained by the early anion current decrease. Since anion current increase was reported to participate in toxins and ozone induced cell shrinkage and death (Errakhi *et al.*, 2008; Gauthier *et al.*, 2007; Kadono *et al.*, 2010) and in addition biphasic regulation of anion fluxes were previously reported in response to non-ionic hyperosmotic stress (Shabala *et al.*, 2000; Teodoro *et al.*, 1998), we investigated if a putative delayed increase in anion currents could participate to non-ionic hyperosmotic-induced cell death. Because SLAC channel allows long term efflux of anion and it's a candidate for the anion current evoked during such PCD (Kadono *et al.*, 2010), we used *A. thaliana* cultured cells due to the availability of the SLAC1 mutant (Negi *et al.*, 2008).

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3.2 Dual responses of cultured plant cells to hyperosmotic stress

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Dual responses of cultured plant cells to hyperosmotic stress

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Running title: Dual role of anion channel in responses to sorbitol

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Abstract

Hyperosmotic-stress represent one of the most serious abiotic factors that limit plant development. In this study we showed dual responses of cultured plant *Arabidopsis thaliana* cultured cells to sorbitol-induced hyperosmotic stress. A first set of events, namely cytosolic Ca^{2+} increase, singlet oxygen production or hypopolarization due to anion channel activity decrease could participate to signalisation or osmotic adjustment allowing cell adaptation and survival, when a second set of events, namely superoxide anion ($\text{O}_2^{\cdot-}$) generation by NADPH-oxidase and anion channel activation could participate in PCD development of a part of the cell population raising the question of how a survival pathway and a death pathway could be induced by the same hyperosmotic conditions.

Key words: anion channels, *Arabidopsis thaliana*, hyperosmotic stress, programmed cell death

Abbreviations: 9-AC, 9-anthracen carboxylic acid; AD, actinomycin D; AVD, apoptosis volume decrease; RVI, regulatory volume increase; RVD, regulatory volume decrease; PM, plasma membrane BAPTA, $[\text{Ca}^{2+}]_{\text{cyt}}$, cytosolic Ca^{2+} concentration; Chx, cycloheximide; $\Delta\Psi_m$, mitochondrial membrane potential; FDA, fluorescein diacetate; Gli, glibenclamide; PCD, programmed cell death; ROS, reactive oxygen species; V_m , resting membrane potential.

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Introduction

Most of the living organisms have to support shift in extracellular osmolality which lower the water potential of the cellular external medium during their life. The influence of such change is determined both by its strength and duration and by the interaction between this shift and the genetic peculiarities of the organism. The way the organism respond to such change can be a matter of life or death since there always exists the so-called “stability limit”. Each deviation out of this stability limit of the live system results in stress, which to a different degree disturbs its functional activity. For plants, drought represent some of the major stress that should adversely affect their development. Plants can tolerate drought by maintaining sufficient cell turgor to allow metabolism to continue under increasing water deficits. This tolerance involves osmotic adjustment as the water potential of the cell’s environment decreases, enabling water uptake (Munns, 1988). Hyperosmotic change due to addition of osmotica such as sorbitol, are thus frequently used to simulate drought (Verslues *et al.*, 2006). Such addition makes it harder for plants to extract water, simulating what happens in drying soil. In such conditions, a rapid modulation of the activities of their plasma membrane ion transport systems generally allows counteracting perturbations due to the hyperosmotic change, as observed whatever the cell types, from bacteria to metazoans, fungi, and plants. Early modulation of K^+ and Cl^- flux (Li and Delrot, 1987; Shabala *et al.*, 2000; Shabala and Lew, 2002) and regulation of H^+ -ATPase activity (Meijer *et al.*, 2002) supposed to participate to osmotic adjustment were effectively reported in plants, although the inhibition of anion efflux was shown to be restored rapidly (PENNARUN and Maillot, 1988; Teodoro *et al.*, 1998). Recently we showed that sorbitol-induced hyperosmotic stress could induce the programmed cell death (PCD) of part of a plant cell culture population (Monetti *et al.*, 2014b) . We thus decide to use such model to study the question of how the same initial perturbation could cause opposite physiological responses, survival or death. The sorbitol-induced PCD in BY2 cell culture were shown to be dependent on delayed production of reactive oxygen species (ROS) (Monetti *et al.*, 2014b). In this study we focused on the

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role of anion channel regulation since (i) H₂O₂ could stimulate anion channel activity in our cells (Kadono *et al.*, 2010), (ii) regulation of anion fluxes seemed to be complex and time dependent under hyperosmotic condition (PENNARUN and Maillot, 1988; Teodoro *et al.*, 1998), (iii) these channels were shown to play fundamental roles in plant cell PCD or by reduction of their activity (Reboutier *et al.*, 2007; Reboutier *et al.*, 2005) either by increasing this activity (Errakhi *et al.*, 2008; Gauthier *et al.*, 2007; Kadono *et al.*, 2010; Tran *et al.*, 2013b) and (iv) they are involved in response to hyperosmotic stress in animal cells (Dezaki *et al.*, 2012; Lang *et al.*, 1998). Our data showed upon hyperosmotic stress a biphasic regulation of anion channel activity occurs, and that delayed sustained anion current increase through SLAC1 channels could participate in the deviation of the stability limit leading to the death of part of the cell population.

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Materials and Methods

Cell culture conditions

Arabidopsis thaliana L. cell suspensions of the cell line T87 (Axelos *et al.*, 1992) were maintained in Gamborg culture medium complemented with 20 g.L⁻¹ sucrose, 2 mg.L⁻¹ 2,4 D, 0.1 mg.L⁻¹ kinetin at 22 ± 2 °C under continuous white light (40 μE.m⁻².s⁻¹) with continuous shaking (gyratory shaker at 120 rpm), as previously described (Kadono *et al.*, 2010). Cell suspensions were sub-cultured weekly using a 1:10 dilution. All experiments were performed at 22 ± 2 °C using log-phase cells (4 days after sub-culture). Cell density was about 3.10⁴ cells mL⁻¹. For the freshly prepared cell suspensions derived from *Arabidopsis* Col 0 and *slac1* mutant, the seeds were sterilized in 1% (w/v) sodium hypochlorite and allowed to germinate on sterilized MS agar plates containing vitamin B5, but lacking 2,4-dichlorophenoxy acetic acid (2,4-D). The seedlings were grown on agar plates under a 12/12 h light/dark regime at 23 ± 1 °C for three weeks. Excised tissues from harvested seedlings were transferred onto agar medium containing 0.2 mg/ml 2,4-D to promote callus formation. Microcalli in suspension were initiated by addition of cut pieces of the resulting microcalli. Calli are maintained on Gamborg culture medium complemented with 20 g.L⁻¹ sucrose, 2 mg.L⁻¹ 2,4 D, 0.1 mg.L⁻¹ kinetin and agar 0.8%. The suspension cells were obtained after about 2 months and 5-6 subculture in 1 L round bottom flasks containing 350 ml liquid Gamborg culture medium (pH 5.8). Only the smallest calli were selected at each subculture to obtain suspensions culture as thin as possible.

Osmolality changes

The osmolality changes were systematically obtained by addition of 50 μL of sorbitol from various stock solutions. Measurement of osmolality changes after sorbitol treatment were determined using 100 μL supernatant of cell suspensions and the freezing depression method using an Automatic Micro-Osmometer Type 15 (Löser Messtechnik, Berlin, Germany).

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Cell viability assays

Sorbitol-induced cell death in the cell suspension culture was quantified using fluorescein diacetate (FDA) as previously described (Reboutier *et al.*, 2007). Briefly, after the appropriate treatment, 1 mL of cell suspension was gently stirred with a magnetic stirrer before FDA was added to a final concentration of 12 μM . The fluorescence increase was monitored over a 120 s period using a F-2000 spectrofluorimeter (Hitachi High-Technologies Corporation, Japan). Results are presented as the percentage of cell death = (slope of treated cells/slope of non treated cells) \cdot 100 \pm SE.

Cell viability was also determined by staining the dead cells with the vital dye Evans blue (0.005%, w/v) by mixing and incubating the cells and the dye for 10 min. Then stained cells were observed under microscope. When appropriate a pretreatment of 15 min with pharmacological effectors was done prior to sorbitol exposures. Cells were counted under a microscope and cells that accumulated Evans blue were considered dead. At least 500 cells were counted for each independent treatment and the procedure was repeated at least three times for each condition. All the pharmacological agents tested were added 5 min before sorbitol treatment

Voltage clamp measurements

Experiments were conducted on 4-day-old cells maintained in their culture medium to limit stress (main ions in Gamborg medium after 4d 9 mM K^+ , 11 mM NO_3 (Reboutier *et al.* 2002)). Individual cells were immobilized by a microfunnel (approximately 50 to 80 μm outer diameter) and controlled by a micromanipulator (WR6-1, Narishige, Japan). Impalements were carried out with a piezoelectric micromanipulator (PCS-5000, Burleigh Inst., USA) in a chamber (500 μl) made of perpeX. Voltage-clamp measurements of whole-cell currents from intact cultured cells presenting stable running membrane potential were carried out using the technique of the discontinuous single voltage-clamp microelectrode (Finkel and Redman, 1984). In this technique, both current passing and voltage recording use the same microelectrode. Interactions between the two tasks are prevented by time-sharing techniques (sampling frequency 1.5 to 3 kHz).

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Microelectrodes were made from borosilicate capillary glass (Clark GC 150F, Clark Electromedical, Pangbourne Reading, UK) pulled on a vertical puller (Narishige PEII, Japan). Their tips were less than 1 μm diameter; they were filled with 600 mM KCl, and had electrical resistances between 20 and 50 M Ω with the culture medium. The capacity compensation of the microelectrode amplifier (Axoclamp 2A, Molecular Devices, Sunnyvale, USA) was set to a sub-critical level to produce the fastest electrode response. The relatively large size of the cells ensured a sufficiently high membrane time constant despite a relatively low input resistance (about 40 M Ω). Specific software (pCLAMP 8) drives the voltage clamp amplifier. Voltage and current were simultaneously displayed on a dual input oscilloscope (Gould 1425, Gould Instruments Ltd, Hainault, UK), digitalised with a Digidata 1322A (Molecular Devices, Sunnyvale, USA). In whole-cell current measurements the membrane potential was held to the value of the resting membrane potential. Current recordings were obtained by various polarizing pulse protocols. We systematically checked that cells were correctly clamped by comparing the protocol voltage values with those really imposed. Only microelectrodes presenting a linear relationship were used.

Monitoring of ROS Production

The production of ROS was monitored by the chemiluminescence of the *Cypridina* luciferin analog (CLA) as previously described (Kadono *et al.*, 2010; Kadono *et al.*, 2006). CLA is known to react mainly with $\text{O}_2^{\cdot-}$ and $^1\text{O}_2$ with light emission (Nakano, 1998) and allows measuring extracellular ROS in plant cells (Tran *et al.*, 2013b). Chemiluminescence from CLA was monitored using a FB12-Berthold luminometer (with a signal integrating time of 0.2 s). The ROS scavengers 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt (Tiron), 1,4-diazabicyclo[2.2.2]octane (DABCO) and inhibitor of the NADPH-oxidase diphenyleneiodonium chloride (DPI), were added 5 min before sorbitol treatment.

Aequorin luminescence measurements

The $[\text{Ca}^{2+}]_{\text{cyt}}$ variations were recorded with *A. thaliana* cell suspension expressing the aequorin gene (Brault *et al.*, 2004). Aequorin was reconstituted by overnight

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incubation in Gamborg medium supplemented with 2.5 μM native coelenterazine. Cell culture aliquots (500 μL) were transferred carefully into a luminometer tube, and the luminescence counts were recorded continuously at 0.2 second intervals with a luminometer. Treatments were performed by pipette injection of 50 μL of sorbitol. The residual aequorin was discharged by addition of 500 μL of a 1 M CaCl_2 solution dissolved in 100% methanol. The resulting luminescence was used to estimate the total amount of aequorin in each experiment. Calibration of calcium measurement was performed by using the equation: $\text{pCa} = 0.332588(-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by total remaining counts (Knight *et al.*, 1996). Results are expressed in μM of Ca^{2+} and correspond to the mean \pm SD of three to five independent experiments. The Ca^{2+} channel blocker La^{3+} and Ca^{2+} chelator BAPTA were added 5 min before sorbitol treatment.

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pH measurements of the culture medium

Extracellular pH was measured directly in the medium (Brault *et al.*, 2004). The experiments were run simultaneously in 6 flasks (control and tests) each containing 2 g FW for 10 mL of suspension medium under continuous orbital shaking (60 rpm). Simultaneous changes in pH were measured from suspension cells presenting an initial pH around 5.6 by using ELIT 808 ionometer with pH sensitive combined electrodes functioning in parallel.

Chemicals

All chemical products were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Stock solution of DPI (10 mM) was dissolved in DMSO in order to have 0.01 % DMSO in final concentration. All other chemicals were solved in water.

Statistical analysis

Data were analyzed by variance analysis (ANOVA), and the mean separation was achieved by Newman and Keuls multiple range test. All numeric differences in the data were considered significantly different at the probability level of $p \leq 0.05$.

Results

*Sorbitol-induced hyperosmotic changes induce cell death in *A.thaliana* cultured cells*

We first evaluated the impact of sorbitol additions on osmolality changes in *A. thaliana* cell medium (Table 1). The shifts in osmolality induced by 200 mM sorbitol did not induced a large cell death when 400 and 600 mM sorbitol led to the death of more of the half of the cell population (Figure 1A), dead cells displaying a large cell shrinkage (Figure 1B) the hallmark of the PCD process (Van Doorn *et al.*, 2011). The cell death scoring at various concentrations of sorbitol further showed a time dependent progression (Figure 1A). The cell death reached a plateau in about 6h after the treatment with 400 mM sorbitol, about half of the cells remaining alive whatever the cell death detection used, FDA or Evans blue staining (Figure 1A,C). In order to confirm whether these cell death was due

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to an active process requiring active gene expression and cellular metabolism, cell suspensions were treated with actinomycin D (AD), an inhibitor of RNA synthesis, or with cycloheximide (Chx), an inhibitor of protein synthesis, at 20 mg.mL⁻¹ each, 15 min prior to 400 mM sorbitol exposures. In both cases, AD and Chx significantly reduced cell death (Figure 1C). These results indicated that this cell death required active cell metabolism, namely gene transcription and *de novo* protein synthesis. Taken together, these data suggest that sorbitol induced a rapid PCD of a part of the *arabidopsis* suspension cell population.

	Medium	Sorbitol (mM)		
	-	200	400	600
Osmolality (mosmol)	128	330	524	762

Table 1: Osmolality changes in medium after treatment with sorbitol.

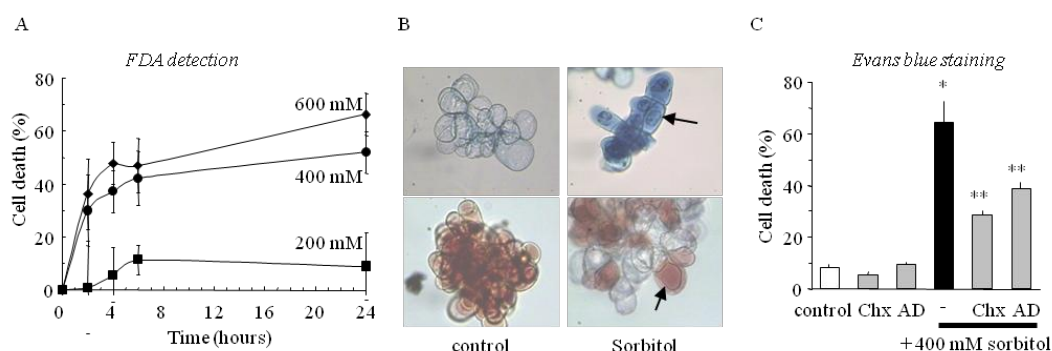


Figure 1: Sorbitol-induced cell death *A.thaliana* cells (A) Time course of the dose dependent cell death induced by sorbitol treatment. (B) Light micrographs of *A. thaliana* cells treated with 400 mM sorbitol for 6 hours and stained with Evans blue (upper panel) or neutral red (lower panel). (C) Effect of pretreatment with actinomycin D (AD, 20 µg/ml) or cycloheximide (Chx, 20 µg/ml) on a 400 mM sorbitol-induced cell death. Each data point and error bar reflect the mean and SD respectively of at least 5 independent replicates. * significantly different from controls, P <0.05 and ** significantly different from the sorbitol treated cells, P <0.05.

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Sorbitol induces transient changes in membrane potential and anion channel activity

Non-saline hyperosmotic stresses are well known to modify plasma membrane potential (V_m) of cells (Monetti *et al.*, 2014b; Shabala and Cuin, 2008; Teodoro *et al.*, 1998; Wegner *et al.*, 2011; Zingarelli *et al.*, 1999). By using an electrophysiological technique (dSEVC) we searched for the impact of sorbitol on arabidopsis cultured cell membrane potential. In control conditions in their culture medium, the V_m of cells was -34 ± 10 mV ($n=39$) similar to those we founded in previous studies ((Brault *et al.*, 2004; Kadono *et al.*, 2010; Tran *et al.*, 2013a). Addition of sorbitol induced a hyperpolarization of the cells (Fig. 2A) reaching its maximal value in 15 ± 5 minutes ($n=11$) after the treatment as previously reported in other models (Li and Delrot, 1987; Monetti *et al.*, 2014b; Shabala and Lew, 2002; Zingarelli *et al.*, 1999). This hyperpolarisation was followed by a slow repolarisation during 40 minutes for the longer recordings we could maintain (Fig 2A). Because it is difficult to record free-running PM potential time-courses more than 1h, we further compared PM potentials of cell populations exposed to 400 mM sorbitol during 20 min and 2h. After 20 min cell polarization distribution clearly showed a shift to hyperpolarization (Figure 2C) with a mean PM potential of -52 ± 9 mV ($n=22$). After 2h the cell polarization distribution showed a shift to depolarization with a mean PM potential of -20 ± 12 ($n=38$). However, two populations seemed to appear one being polarized like the control, another one more depolarized (Figure 2C) suggesting that the repolarization (Fig. 2A) sometimes goes on in depolarization for some cells.

In Gamborg medium after 4d of culture, the main ions are 9 mM K^+ , 11 mM NO_3^- (Reboutier *et al.* 2002), thus, the equilibrium potential estimated for K^+ , E_K is about -60 mV ($[K^+]_{out} = 9$ mM with $[K^+]_{in}$ estimated at 100 mM). The equilibrium potential estimated for NO_3^- is of about -30 mV ($[NO_3^-]_{out} = 11$ mM with $[NO_3^-]_{in}$ estimated at 3 mM). As previously observed with cultured cells of *Arabidopsis thaliana* (Reboutier *et al.* 2002, Kadono *et al.* 2010, Tran *et al.* 2013), the occurrence of anion currents in most of the *A. thaliana* cells in their culture medium could explain their mean polarization around -30 mV we recorded in control and non-stressing conditions. The mean control value of these currents at -

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200 mV and after 1.8s of voltage pulse was of -1.73 ± 0.2 nA (n=11). These currents presents characteristics of slow type anion channel (SLAC) (Reboutier *et al.*, 2002) (Supplemental Figure 1) and were shown to be sensitive to structurally unrelated anion channel inhibitors, 9-anthracen carboxylic acid (9-AC) (Brault *et al.*, 2004)(Supplemental Figure 1) and glibenclamide (Kadono *et al.*, 2010; Reboutier *et al.*, 2005) reinforcing the hypothesis of an anionic nature for these currents.

The sorbitol-induced PM-variations were correlated with anion current variations. A decrease in anion currents was observed during the first 20 min (Fig. 2B,D). The hyperpolarization seemed thus correlated with this decrease in anion currents, these currents reaching a mean of -0.72 ± 0.24 nA (n=11). After 2h, two populations could be discriminate (Fig. 2D), one corresponded to depolarized cells and presented an increase in anion currents to a mean of -3.3 ± 1.4 nA (n=6), the second one corresponded to depolarized cells and maintained reduced anion currents with a mean of -0.66 ± 0.12 nA (n=5).

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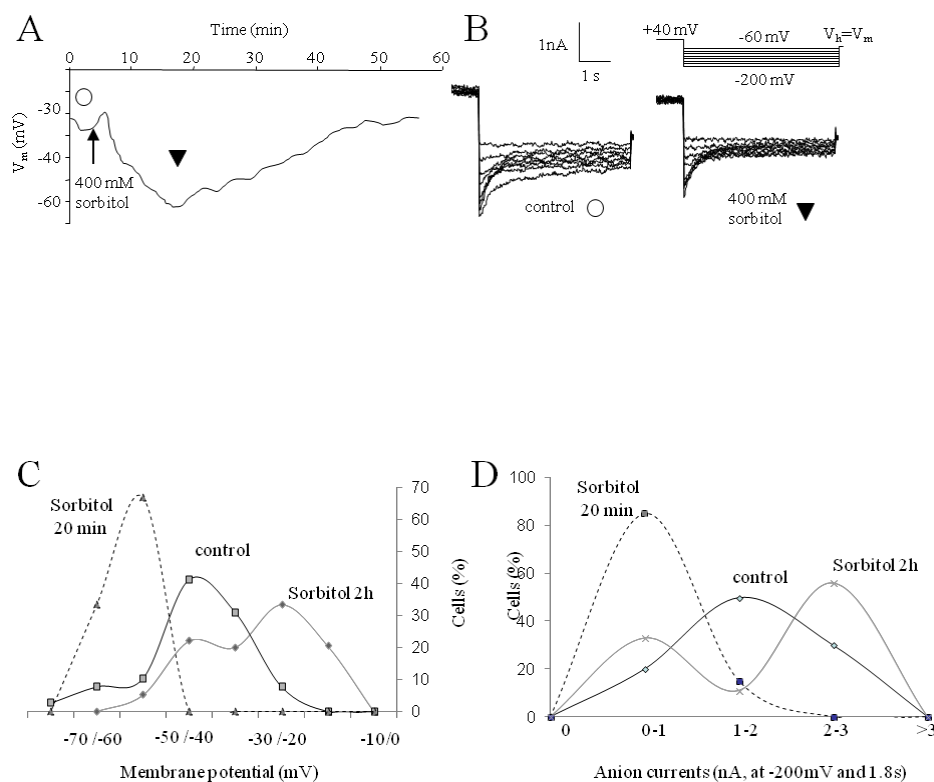


Figure 2: Biphasic regulation of *A. thaliana* cell polarization by 400 mM sorbitol (A) Typical modulation of cell plasma membrane (PM) potential variations observed in response to sorbitol. (B) Typical changes in whole cell current profiles after treatments with 400 mM sorbitol. The protocol was as illustrated, holding potential (V_h) was V_m . (C) Distribution of PM potentials recorded in control conditions, 20 min and 2 hours after treatment with 400 mM sorbitol. (D) Mean values of whole cell current variations (recorded at -200 mV and 1.8 s) in control conditions, 20 min and 2 hours after treatment with 400 mM sorbitol with or without 9-AC (200 μ M).

Anion channels are involved in sorbitol-induced PCD

In order to check whether the delayed increase in anion channel activity could be involved in the process leading to cell death of half of the population after a 400 mM sorbitol treatment, cells were pre-treated with the anion channel inhibitors 9-AC or glibenclamide (200 μ M each), before the addition of 400 mM sorbitol. Cell death was then quantified 6 hours after the treatment. These two inhibitors contributed to decrease the sorbitol-induced cell death (Fig. 3A). To further get insight into the molecular nature of the ion channel responsible for the anion

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currents, we further test the extent of cell death induced by 400 mM sorbitol on freshly prepared wild type and *SLAC1* mutant (Geiger *et al.*, 2010; Vahisalu *et al.*, 2008) cultured cells. Although the basal cell death was higher in freshly prepared cultured cells, the sorbitol induced cell death was avoided in *SLAC1* mutant (Fig. 3B) strongly suggesting that *SLAC1* channels were involved in the sorbitol induced pathway leading to cell death.

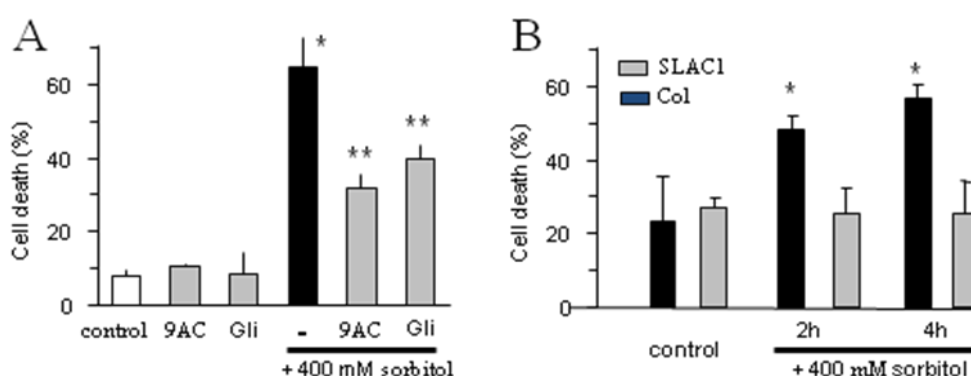


Figure 3: Anion channels are involved in sorbitol-induced PCD. (A) Effect of the anion channel blockers 9-AC and glibenclamide (200 μ M each) on cell death induced by 400 mM sorbitol after 4h treatment. (B) Effect of 400 mM sorbitol on cell death extent in freshly prepared cultured cells from the wild type (Col) and *SLAC1* mutant. The data correspond to means of at least 5 independent replicates and error bars correspond to SD. * significantly different from controls, $P < 0.05$.

Avoiding of the sorbitol induced cell death

Since we recorded an early hyperpolarization and anion channel activity decrease in most of the cells (Figure 2D) when depolarization and anion channel activity increase seem related to sorbitol-induced PCD (Figure 2E,F), we further test the impact of sorbitol removal before the increase in anion currents. We thus rinsed the cells 30 min after treatment with sorbitol with Gamborg medium free of sorbitol or with Gamborg medium with 400 mM sorbitol. When sorbitol was removed 30 min after the treatment no cell death development was observed when sorbitol was maintained in the medium the cell death extent was the same as the one of the control with sorbitol (Fig. 4) confirming that early hyperpolarization and anion channel activity decrease were not involved in PCD.

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We then tried to follow in our model cells the kinetics of some early events classically detected upon hyperosmotic stress, namely increase in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$), ROS production, H^+ -ATPase activity, upon challenge with 400 mM sorbitol, a concentration inducing cell death in half of the cell population to analyse their putative involvements in sorbitol-induced PCD .

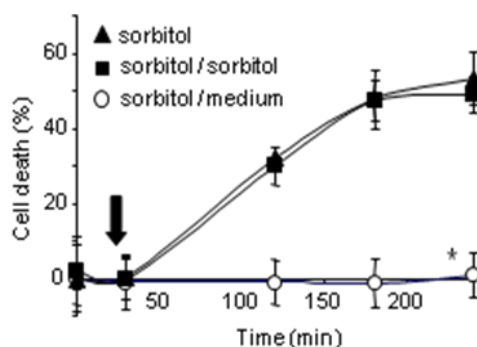


Figure 4. Impact of sorbitol (400 mM) removal after 30 min on the *A. thaliana* cell death extent. Cells were rinsed 30 min after treatment with sorbitol with Gamborg medium free of sorbitol (Sorbitol/medium) or with Gamborg medium with 400 mM sorbitol (Sorbitol/sorbitol) and the cell death progression was compared with sorbitol treated cells without rinsage as control (Sorbitol). Each data point and error bar reflect the mean and SD respectively of at least 3 independent replicates. * significantly different from controls, $P < 0.05$.

Changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ are not involved in the sorbitol-induced cell death

The changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ were monitored by the Ca^{2+} -dependent emission of blue light from aequorin (Knight *et al.*, 1996). Treatment of arabidopsis cells with 400 mM sorbitol resulted in a rapid transient increase in aequorin luminescence (Fig. 5A) reflecting an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ of about $0.6 \mu\text{M}$. This short lived increase was considerably inhibited when cells were pre-treated with the PM Ca^{2+} channel inhibitor La^{3+} or with the Ca^{2+} chelator BAPTA and then stressed with sorbitol 400 mM (Fig. 5A). This indicates that the sorbitol-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was mostly due to influx of Ca^{2+} across the plasma membrane through Ca^{2+} channels. In order to check whether the influx of Ca^{2+} was involved in the induction of the cell death, the effect of 400 mM sorbitol was tested on cell death in presence or absence of La^{3+} or BAPTA. Even if the pre-treatment of *A. thaliana* cells with Ca^{2+} channel inhibitor or with BAPTA induced a slight increase in cell

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death, the sorbitol-induced cell death did not significantly decrease after such pre-treatments (Fig 5B). These results strongly suggest that the rapid Ca^{2+} influx induced by sorbitol is not involved in the pathway leading to cell death.

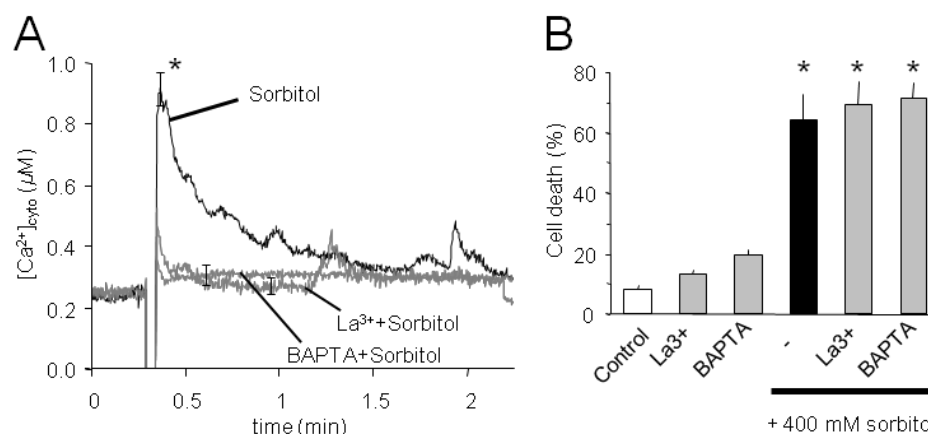


Figure 5: Induction of $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in aequorin expressing-*A. thaliana* cells by 400 mM sorbitol. (A) Typical kinetic of sorbitol-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and inhibition by the calcium channel blocker LaCl_3 (500 μM), and the calcium chelator BAPTA (1 mM). Each data point and error bar reflect the mean and SD, respectively ($n = 5$). (B) Effect of a pre-treatment with La^{3+} (500 μM) or BAPTA (1 mM) on cell death induced after 6 hours in presence of 400 mM sorbitol. Each data point and error bar reflect the mean and SD respectively of at least 3 independent replicates. * significantly different from controls, $P < 0.05$.

Sorbitol-induced ROS generation

To study the effect of sorbitol on production of ROS in arabidopsis cell culture we used the chemiluminescence of CLA which indicates the generation of $\text{O}_2^{\cdot-}$ and $^1\text{O}_2$. Addition of sorbitol to cell suspension culture resulted in transient production of ROS that reaches the maximal level immediately after treatment (Fig. 6A). This sorbitol-induced ROS generation was dose-dependent (Fig. 6B) and could be blocked using DABCO, a $^1\text{O}_2$ scavenger, but not Tiron, a $\text{O}_2^{\cdot-}$ scavenger (Fig. 6C,D). Thus, the early increase in CLA-chemiluminescence seemed to be dependent on $^1\text{O}_2$ generation but not on $\text{O}_2^{\cdot-}$ generation. We further checked the impact of the DABCO on sorbitol-induced PCD. This scavenger of $^1\text{O}_2$ failed to decrease sorbitol-induced cell death (Fig. 6E), but 10 μM diphenyleneiodonium chloride (DPI), an inhibitor of the NADPH-oxidase, decreased the sorbitol-

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induced cell death (Fig. 6E). Since no early $O_2^{\bullet-}$ generation were detected (no effect of Tiron, Fig. 6C,D), we thus further searched for a possible delayed NADPH-oxidase dependent $O_2^{\bullet-}$ generation after treatment with 400 mM sorbitol. We effectively could detect after such hyperosmotic stress an increase in CLA-chemiluminescence (Fig. 6F). The chemiluminescence drastically increased after 30 min and reached a maximal level after 1h to decrease to control level after 2h (Fig. 6F). The increase in CLA-chemiluminescence could be reduced by pretreatment with 10 μ M DPI (Fig. 6G) suggesting that the generation $O_2^{\bullet-}$ through enhancement of NADPH oxidase activity were involved in the delayed ROS generation after treatment with sorbitol.

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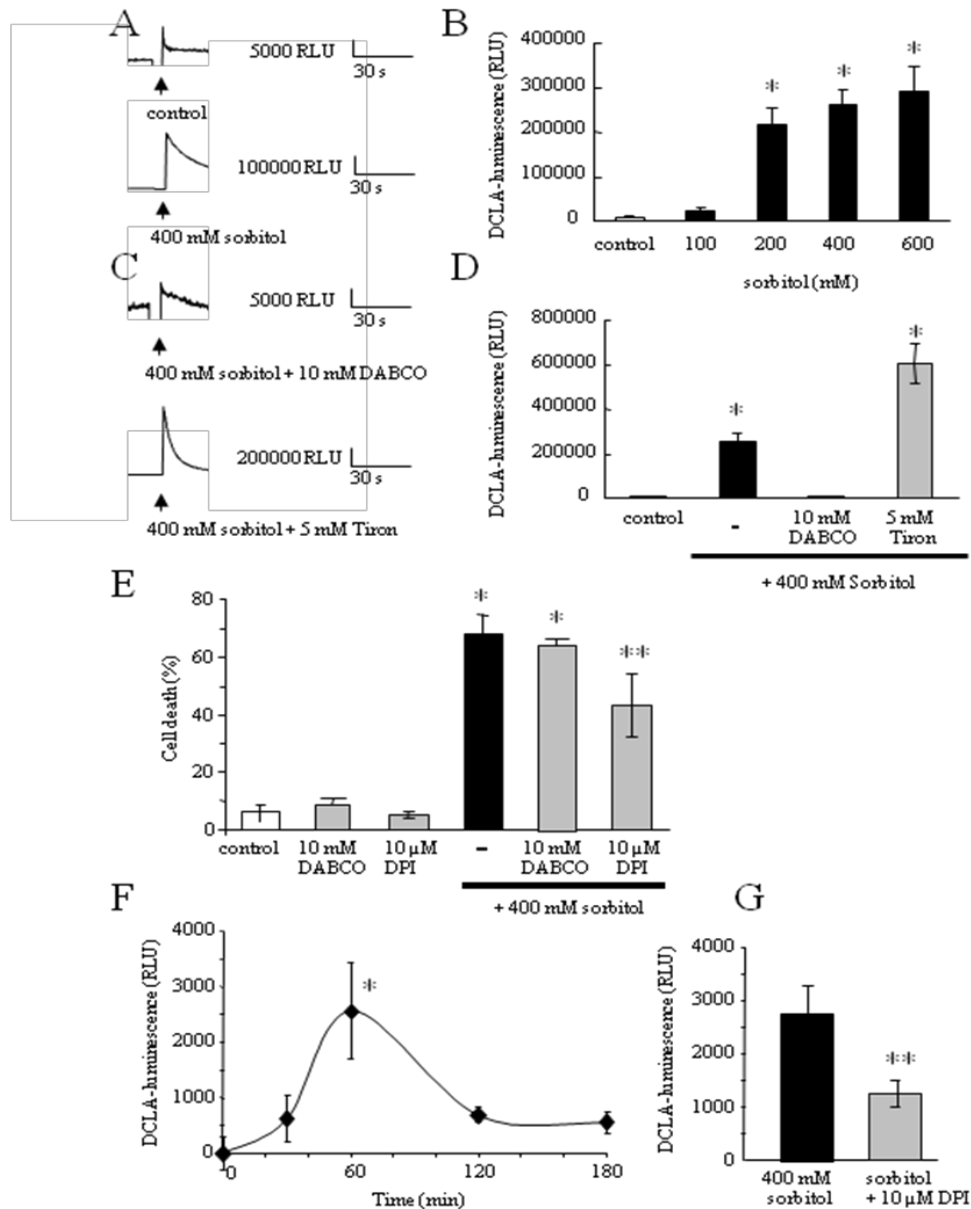


Figure 6. Induction of ROS generation in *A. thaliana* cells by sorbitol. (A) Typical kinetic of sorbitol-induced early increase in CLA-chemiluminescence reflecting the production ROS. (B) Effect of concentration of sorbitol on early ROS generation. (C) Modulation of sorbitol-induced ROS generation by DABCO, scavenger of singlet oxygen or Tiron, scavenger of anion superoxide. (D) Effect of DABCO or DPI, a NADPH oxidase inhibitor, on cell death induced by 400 mM sorbitol after 4h treatment. (E) Time course of CLA chemiluminescence during 6h treatment with 400 mM sorbitol. (F) Inhibition of sorbitol-induced delayed ROS generation by DPI. Each data point and error bar reflect the mean and SD, respectively (n = 5). * significantly different from controls, P < 0.05 and ** significantly different from the sorbitol treated cells, P < 0.05.

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Discussion

In this study we confirmed using *A. thaliana* cultured cells that sorbitol-induced hyperosmotic treatments lead to different early cellular responses previously reported such as, transient $[Ca^{2+}]_{cyt}$ increase (Donaldson *et al.*, 2004; Kim *et al.*, 2007; Lin *et al.*, 2006; Parre *et al.*, 2007; Ranf *et al.*, 2008; Xiong *et al.*, 2002), production of ROS (Lin *et al.*, 2006; Xiong *et al.*, 2002; Zhang *et al.*, 2013; Zhu, 2001) and early modulation of ion fluxes responsible for plasma membrane hyperpolarization (Li and Delrot, 1987; Shabala *et al.*, 2000; Shabala and Lew, 2002). As also expected from previous studies (Huh *et al.*, 2002; Monetti *et al.*, 2014a; Wang *et al.*, 2010) the death of a part of *A. thaliana* cell population was observed depending on the sorbitol concentration and the duration of treatment. This cell death presents hallmarks of PCD since cells it requires active gene expression and *de novo* protein synthesis. Indeed, treatment with AD, an inhibitor of RNA synthesis, or with Chx, an inhibitor of protein synthesis, prior the exposure to sorbitol significantly reduced the extent of cell death. The early events recorded with *A. thaliana* suspension cells were not involved in this PCD since Ca^{2+} chelator (BAPTA), Ca^{2+} channel inhibitor (La^{3+}) or singlet oxygen scavenger (DABCO) failed to reduced the sorbitol-induced PCD as observed on BY-2 cells (Monetti *et al.* 2014). It is further noteworthy that removal of the sorbitol after 30 min, thus after the induction of these early responses and the initial hyperpolarization of the cells by sorbitol, allows to block the PCD progress, comforting the idea that these early events were not involved in PCD development. Sorbitol-induced PCD was effectively showed to depend on a delayed superoxide anion generation probably through NADPH-oxydase, both being reduced upon pretreatment with the inhibitor of NADPHoxydase DPI, as also observed on BY2 cells (Monetti *et al.* 2014). This delayed ROS generation could participate in activation of anion channels since they were shown to be activated by H_2O_2 in *A. thaliana* cultured cells (Kadono *et al.*, 2010). Effectively by following the kinetics of cell polarization and anion current intensity after the addition of 400 mM sorbitol we could observed that, after the initial hyperpolarization and decrease of anion currents recorded for more than 80% of

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the cells, about 60% the cell population showed a large increase in anion current and depolarized when 40% of cells maintained low anion currents and repolarized to the control level. Since anion current increase was reported to be key event in PCD induced by abiotic stress like ozone (Kadono *et al.* 2010) or biotic stress (Errakhi *et al.*, 2008; Gauthier *et al.*, 2007), we further test the effect of structurally unrelated anion channels blockers (gli and 9-AC) efficient in our model (Reboutier *et al.*, 2005) (Supplemental fig. 1) on sorbitol induced PCD. Both inhibitors allowed to reduce the sorbitol-induced cell death. Moreover, cultured cells of the SLAC1 mutant impaired in slow type anion channels (Geiger *et al.*, 2009; Vahisalu *et al.*, 2008) did not showed any increase in cell death upon treatment with 400 mM sorbitol strongly suggesting that slow type anion channels were involved in this PCD. Thus two different behaviors occur in the *A. thaliana* cell population and it seems the stability limit could depend on cells since for a same stress strength and duration (eg. 400 mM sorbitol during 4h, about 60% of the population died).

Among the early event we recorded in response to sorbitol addition, the initial decrease in anion current observed in our model could be considered as an event participating to osmotic adjustment since it allows maintaining anion in the cells and it is noteworthy that this regulation is observed in most of the cells. Moreover the increase in mitochondrial membrane potential ($\Delta\psi_m$) (supplemental fig. 2) suggests an increase in metabolic activity wich could be correlated to adaptation to hyperomotic conditions. The singlet oxygen production and Ca^{2+} influx could be related to signalization process (Monetti *et al.* 2014). Thus in our model (Fig 7), the first early reponses to sorbitol observed could allow the cells to survive to the hyperomotic conditions.

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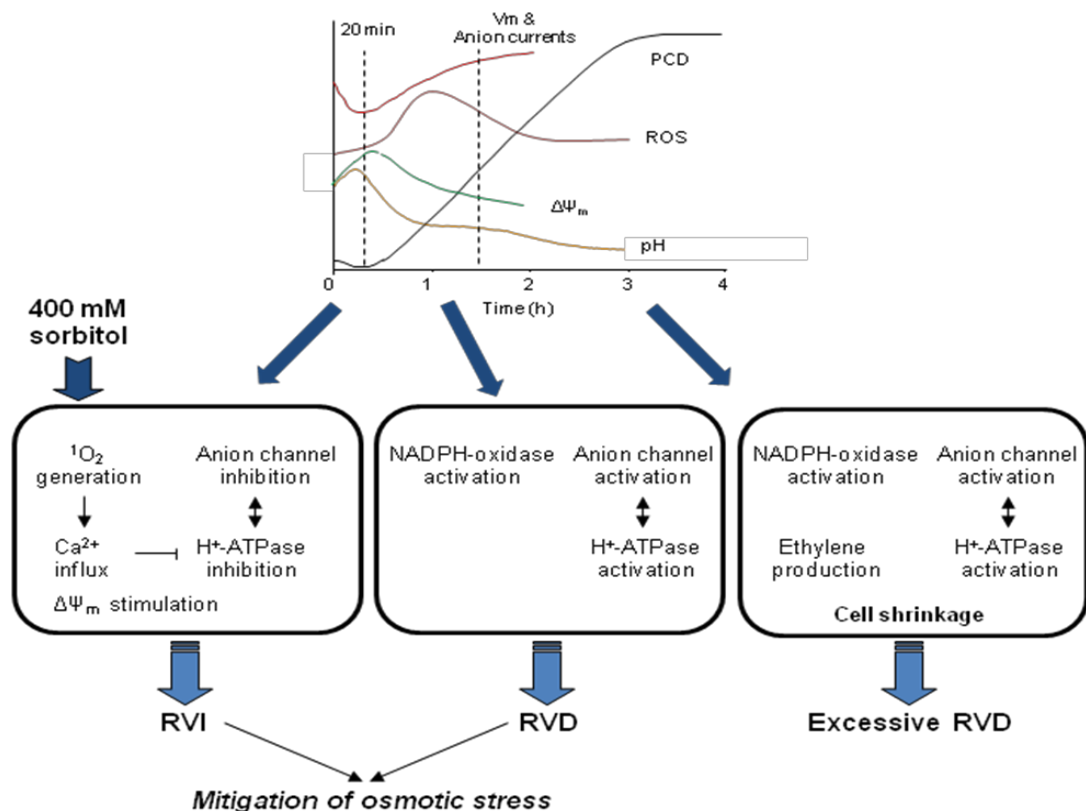


Figure 7: Events involved in sorbitol induced cell death

These data are reminiscent of what described in animal cells for which a rapid regulatory volume increase (RVI) occurs in response to hyperosmotic-induced cell shrinkage (Burg *et al.*, 2007). This RVI is associated with regulation of ion transport system to participate to osmotic adjustment. However in our model when the delayed ROS generation and delayed anion channel increase observed in half the cells seemed to be the result of a death pathway induced in cells for which the stability limit was overcome. In some animal cells RVI could be followed by switch to apoptosis volume decrease (AVD) which involved anion efflux and leads to apoptosis (Maeno *et al.*, 2000), a well know form of PCD. Although RVI and AVD cannot be taking place at the same time, it is supposed that parallel activation of survival and death pathways could be induced by hyperosmotic stress but the latter develops slower and becomes detectable at a relatively late stage (Cheong *et al.*, 2010) when RVI "dysfunction" led to AVD and apoptosis (Subramanyam *et al.*, 2010). Further study will be needed to describe precisely the

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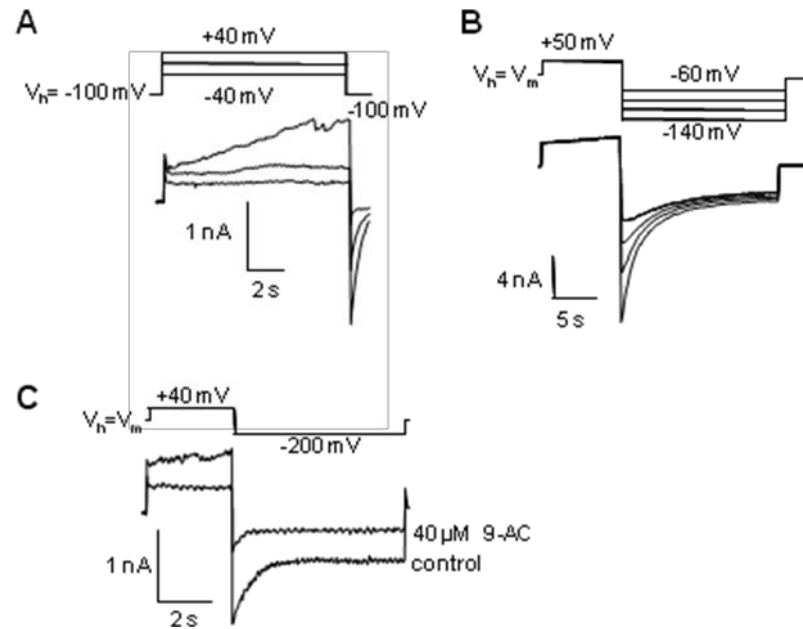
stability limit of the cells, and what control the switch between osmotic adjustment and PCD induction in plant cells submitted to hyperosmotic conditions.

Acknowledgement

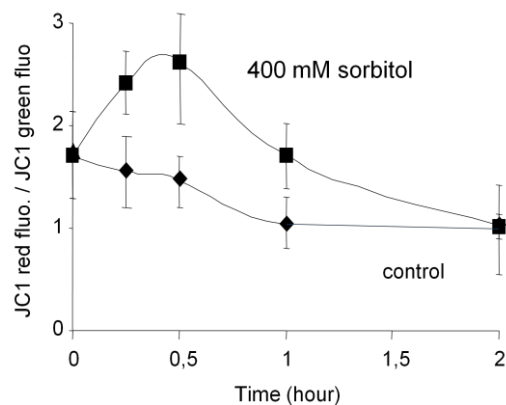
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Supplementary materials



Supplementary figure 1: Slow activation of current upon depolarization of the cell (A). Slow deactivation of current upon hyperpolarization of the cell (B). Inhibition of the current by the anion channel blocker 9-anthracen carboxylic acid (C).



Supplementary figure 2: Variations of mitochondrial membrane potential ($\Delta\psi_m$) of *A. thaliana* cells after treatments with 400 mM sorbitol.

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Chapter 4

FaRP-like peptides as osmoregulator in plant

Article published in *Frontiers in Endocrinology*

Chapter 4: FaRP-like peptides as osmoregulator in plant

4.2 Introduction

In addition of understanding the role of the ion currents during saline and non-saline hyperosmotic-induced PCD, we aimed to search for putative regulators of such PCD in plants. Numerous cellular features are conserved in eukaryotic cells (Baluška and Mancuso, 2009; Grémiaux *et al.*, 2014). Since most of the living organisms, from bacteria to metazoans, fungi and plants have to face hyperosmolarity and the establishment of an appropriate response can be a matter of life or death, we investigated if some regulators belonging to another kingdom could be efficient in regulating plant response to hyperosmotic stress. The importance of small peptides belonging to the FMRFamide-like peptides (FLPs) family have been shown to participate in osmoregulation in metazoans (Khan *et al.*, 1999; López-Vera *et al.*, 2008). These FLPs were shown to target various ion channels in response to osmotic shock (Vandorpe *et al.*, 1994). Thus we searched if putative FLPs could exist in plant and be involved in physiological processes related to hyperosmotic stress responses.

Chapter 4: FaRP-like peptides as osmoregulator in plant

4.3 Could FaRP-like peptides participate in regulation of hyperosmotic stress responses in plants?

François Bouteau, Yann Bassaglia, Emanuela Monetti, Daniel Tran, Sandra Navet, Stefano Mancuso, Hayat El-Maarouf-Bouteau and Laure Bonnaud-Ponticelli



Could FaRP-like peptides participate in regulation of hyperosmotic stress responses in plants?

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The ability to respond to hyperosmotic stress is one of the numerous conserved cellular processes that most of the organisms have to face during their life. In metazoans, some peptides belonging to the FMRFamide-like peptide (FLP) family were shown to participate in osmoregulation via regulation of ion channels; this is, a well-known response to hyperosmotic stress in plants. Thus, we explored whether FLPs exist and regulate osmotic stress in plants. First, we demonstrated the response of *Arabidopsis thaliana* cultured cells to a metazoan FLP (FLRF). We found that *A. thaliana* express genes that display typical FLP repeated sequences, which end in RF and are surrounded by K or R, which is typical of cleavage sites and suggests bioactivity; however, the terminal G, allowing an amidation process in metazoan, seems to be replaced by W. Using synthetic peptides, we showed that amidation appears unnecessary to bioactivity in *A. thaliana*, and we provide evidence that these putative FLPs could be involved in physiological processes related to hyperosmotic stress responses in plants, urging further studies on this topic.

Keywords: *Arabidopsis thaliana*, drought, FaRP-like peptides, osmotic stress, stomata

INTRODUCTION

Most of the living organisms from bacteria to metazoans, fungi, and plants have to face hyperosmolarity (i.e., an external osmolarity that is higher than the physiological range) during their lifetime, and the establishment of an appropriate response can be a matter of life or death. Whatever the cell types, they are generally able to counteract volume perturbations following a shift in extracellular osmolarity by rapidly modulating the activities of their plasma membrane ion transport systems (1, 2). Several major hormones that respond to osmotic stress have been identified in metazoans, vertebrates to arthropods, and plants (3–9), but it is only more recently that the importance of small peptides in different regulatory mechanisms has been pointed out in metazoans and plants (10, 11). In numerous metazoans (mollusks, annelids, nematodes, and vertebrates), peptides belonging to the FMRFamide-like peptides (FLPs) family have been shown to participate in osmoregulation (12, 13). Moreover, FLPs were shown to target various ion channels, among them the membrane sodium channels, such as the amiloride-sensitive FMRFa-activated sodium channel (FaNaCh) in invertebrates (13), or the structurally related acid-sensing sodium channels (ASICs) in vertebrates (14). These ligand-gated or pH sensitive- Na^+ channels are involved in Na^+ permeability and associated water transport, which makes them

critical determinants of cell volume regulation (14). In sensory neurons of *Aplysia*, chloride currents are evoked by FMRFa via the cGMP cascade (15). In the same type of neurons, FMRFa also modulates the probability of opening and closing of S-type K^+ channels, a stretch-activated channel involved in response to osmotic shock (16).

For plant, drought-induced osmotic stress and salinity represent some of the major constraints that adversely affect growth, development, and biomass production. Numerous cellular responses and proteins have been reported to be conserved between plant and animal cells (17–19). Among them are ion channels (20), and their involvement in response to hyperosmotic stress (2, 21–23). Recent works implicate small signaling peptides in developmental processes in plants (11), but to our knowledge, until now, no study has described the presence of FLPs in viridiplantae. Thus, we addressed the hypothesis that FLPs exist in plants and participate in their physiological responses to hyperosmotic stress. Moreover, since many discoveries with direct relevance to animal biology have been elaborated using plants (17), this topic could also be relevant for metazoan biology by bringing new insight in FLPs structures, functions, and evolution.

MATERIALS AND METHODS

CELL CULTURE CONDITIONS

Arabidopsis thaliana L. cell suspensions were freshly prepared from calli of the cell line T87 (24), which was generated from the ecotype Columbia plant. They were maintained in Gamborg

Abbreviations: FaRPs, FMRFamide related peptides; FLPs, FaRP-like peptides; PM, plasma membrane; V_m , plasma membrane potential.

culture medium complemented with 20 g L⁻¹ sucrose, 2 mg L⁻¹ 2,4 D, 0.1 mg L⁻¹ kinetin at 22 ± 2°C under continuous white light (40 μE m⁻² s⁻¹) with continuous shaking (gyratory shaker at 120 rpm), as previously described (24, 25). Cell suspensions were sub-cultured weekly using a 1:10 dilution. All experiments were performed at 22 ± 2°C using log-phase cells (4 days after sub-culture). Cell density was about 3.10⁴ cells mL⁻¹.

ELECTROPHYSIOLOGY

Cells were impaled in the culture medium with borosilicate capillary glass (Clark GC 150F) micropipettes (resistance: 50 MΩ when filled with 600 mM KCl). Main ion concentrations in the medium after 4 days were 9 mM K⁺, 11 mM NO₃⁻ (26). Individual cells were voltage-clamped using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) as previously described (24).

HYPEROSMOSIS TEST AND CELL VIABILITY ASSAYS

Pretreatments of 15 min with the various plants putative FLPs were done prior to the induction of a hyperosmotic stress by a 400 mM sorbitol exposure (duration: 6 h). Hyperosmosis-induced cell death in the cell suspension culture was determined after staining the dead cells with Evans blue (0.005%, w/v) for 10 min. Cells were counted under a microscope and cells accumulating Evans blue were considered to be dead. At least 500 cells were counted for each independent treatment and the procedure was repeated at least three times for each condition.

MEASUREMENT OF INTRACELLULAR ROS LEVEL

For measuring reactive oxygen species (ROS) generation, we used the CellROX[®] Deep Red Reagent (Molecular probes). The cell-permeant dye is non-fluorescent in a reduced state, and exhibits bright fluorescence upon oxidation by ROS. The cells were pre-incubated for 15 min with 100 μM of peptides and then incubated with 400 mM Sorbitol during 1 h. The cells were incubated with 5 μM CellROX Deep Red for 30 min before recording and then were washed with phosphate-buffered saline buffer. The excitation wavelength was set at 640 nm, and the emission was detected at 665 nm (27). The fluorescence intensity of the cells was measured with a Tecan Infinite 200 Spectrophotometer.

SEEDLINGS CULTURE

Arabidopsis thaliana L. seedlings were grown in an environmentally controlled chamber (8 h photoperiod, under 100 μmol photons m⁻² s⁻¹ at the leaf level, 24 ± 2°C) and plants were weekly watered.

PREPARATION OF EPIDERMAL STRIPS

Arabidopsis thaliana leaves from 4 to 6 weeks old plants were harvested 1 h after the beginning of the light period. Epidermal strips were carefully prepared from abaxial epidermis then placed cuticle side-down on microscope slides covered with medical adhesive (Dow Corning 355, Peters surgical) and immediately floated in 10 mM MES pH 6.1, 50 mM KCl, 1 mM CaCl₂ (opening buffer) under white light (40 μmol photons m⁻² s⁻¹), or in 10 mM MES pH 6.1, 10 mM KCl, 1 mM CaCl₂ (closing buffer) in dark, for 3 h before future treatments.

STOMATAL APERTURE MEASUREMENTS

Epidermal strips were analyzed with a Laborlux S (Leica, Germany) microscope (×400). For quantifying, microscope fields were digitalized with a Kappa CF11DSP (Nikon, Japan) digital camera. The width of the stomatal aperture was measured using the image analysis software Metreio Kappa Image Base (Kappa, Germany). The pore width from at least 65 stomata from 2 leaves was measured per treatment and pooled together for statistical analysis. Data are expressed as micrometer and are means ± SE.

CHEMICALS

Synthetic peptides (purity >95%) were purchased from Proteogenix (Oberhausbergen, France) and diluted in water.

IN SILICO ANALYSIS

Putative plant FLP precursors were detected using a blastp search against the protein sequence database at the NCBI and TAIR, using FMRF–FMRF, FLRF–FLRF, or ILRF–ILRF as query. Only sequences showing more than three repeats were considered. Further sequences were obtained using these results as query using blastp against the UniProtKB database. All sequences were also blasted using tblastn against *A. thaliana* ESTs database at the NCBI and TAIR. When possible, the corresponding genes were localized using the EnsemblPlant database. Some representative sequences are presented in Table 1.

STATISTICS

Significant differences between treatments were determined by the Mann and Whitney test, and *P* values <0.05 were considered significant.

RESULTS AND DISCUSSION

FLRFa-INDUCED HYPERPOLARIZATION AND ION CURRENT REGULATIONS IN *ARABIDOPSIS THALIANA* CELLS

FMRFamide (FMRFa) is a cardioexcitatory peptide that was first isolated from the nervous system of the clam, *Macrocallista nimbosa* (28), and is active as a tetrapeptide only in mollusks and annelids. Other active tetrapeptides have been identified in lophotrochozoans; these include FLRFa, YLRFa, or YMRFa. In view of the well-known effects of FLPs on ion channel regulation in metazoan cells, we first checked for the putative effect of FLRFa, a typical metazoan FLP, on plasma membrane polarization and ion channel regulations in cultured cells of the model plant *A. thaliana* by using single electrode voltage clamp (24). In control conditions (in culture medium), the cell plasma membrane potential (*V*_m) of cells was -33 ± 4 mV (*n* = 20) – similar to those we observed in previous studies (25, 26, 29). Addition of 100 μM FLRFa induced a hyperpolarization of the cells of -8 ± 1.5 mV (*n* = 4, Figure 1A). These FLRFa-induced hyperpolarizations were correlated with a decrease in inward currents (Figures 1B,E) that we previously described as anion currents (25, 26, 30), and an increase in time dependent outward rectifying currents (Figures 1B,E), previously described as K⁺ outward currents [KORC, (24, 26, 30)]. It is noteworthy that these ion current regulations are the same as those observed in response to a shift in osmolarity (128–330 mOsm induced by addition of 200 mM sorbitol in the cell culture medium; Figures 1C–E). Inhibition

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Table 1 | Identification of some putative pro-peptides and their genes in *Arabidopsis thaliana*.

Name	Prot id			EnsemblPlants – TAIR10				
	UniProt	gb	gi	Gene localization	EST	Repeats	Transposon	Tandem repeats
F26F24.19	Q9LR27_ARATH	AAF87013.1	9295707	NF	NF	NF	NF	NF
F4N2.6	Q9LQB1_ARATH	AAF27054.1	6730633	1:25976374–25976607	No	Yes	Type II	Yes
F1L3.6	Q9LNR8_ARATH	AAF79458.1	8778450	1:5965755–5966750	TC290437	Yes	Type II	Yes
At2g42050 hypothetical protein	P93742_ARATH	AAB63539.1	1871179	2:17546143–17546559	No	Yes	Type II	Yes
Unnamed protein product (BAB01828)	Q9LS63_ARATH	BAB01828.1	9293925	3:11226216–11226473	No	Yes	Type II	Yes
F22O6_210	Q9SVC3_ARATH	CAB43442.1	4886286	3:19428300–19428917	No	Yes	Type II	Yes
F19F18.60 = At4g37570	Q9SZF1_ARATH	CAB38296.1	4468982	4:17655176–17655730	TC297785	Yes	Type II	Yes
Unnamed protein product (BAB09258)	Q9FGB7_ARATH	BAB09258.1	9758805	5:14153439–14154089	No	Yes	Type I	Yes

The gene coordinates are given regarding TAIR10 genome assembly (accessible at EnsemblPlants <http://plants.ensembl.org/>) as chromosome:extend. The EST, repeats, transposon, and tandem are the automatic annotations given during the automatic annotation procedure. NF, not found.

of outward anion currents is a process by which ion leakage is decreased, and thus, results in rapid adaptation to hyperosmotic condition by ion accumulation (31–33). The activation of KORC favoring K⁺ efflux previously reported in other models (34, 35) is opposite to ion accumulation but could be a part of an initial signaling that could result in osmotic regulation (34). In view of these data, we searched for putative plant gene(s) coding for these peptides in gene and protein databases.

PUTATIVE CANDIDATE GENES FOR FLPs SYNTHESIS IN *ARABIDOPSIS THALIANA*

A FLP can be defined as a peptide that ends in RFa while a FaRP is a peptide homologous to FMRFa in metazoans (36, 37). The number of FLPs identified is increasing with the availability of genome and transcriptome databases and the development of constrained algorithm to search for them (37, 38). The length of these peptides ranges from 4 to 52 amino-acids and 37% of those found do not exceed 10 amino-acids (36). Active peptides are cleaved-out of a pro-peptide; the amino-acids allowing this cleavage are the basic amino-acids K or R, either alone or as a dimer (39) (Figure 2A). In FaRPs, the terminal cleavage site (R/K) is preceded by a G, allowing the amidation of the peptide: the XXRFamide form is biologically active, whereas a non-amidated peptide is considered to be inactive. Regarding FLP, the structure [KR](X)_nRFG[KR] appears to be the most common organization (Figure 2A). Nevertheless, the amino-acid before the RF ends may vary between peptides and/or organisms. Espinoza et al. (38) have shown that the distribution and the type of amino-acids (the order and the respective position) of each is not random. Although the specificity of each peptide seems to be very high and adapted to each species [see for example (40–42)], the structure and the relationships between structure (composition) and function of the genes, as well as the biochemical characteristic of the couple ligand/receptor, are not clearly established.

By exploring genomic and transcriptomic databases, we found in *A. thaliana*, several putative genes that may allow the production of pro-peptides including repeated FLPs peptide sequences. Some representative sequences are listed in Table 1 and illustrated in Figure 2. As underlined in Table 1, most of these sequences are not annotated as ESTs in the automatic genome annotations, probably because of the “repeat masker” step. Instead, they are interpreted to be transposable elements. But several similar sequences were found in *A. thaliana* ESTs database, suggesting that these genes could in fact be expressed (for example: EG509196 and EG509184). It is noteworthy that the ESTs are issued from *Arabidopsis* stressed with several factors, including salinity, an osmotic stress.

The sequences in Figure 2A were initially identified using a poly-ILRF query and all of them showed the characteristic properties of pro-peptides: tandem repeats and cleavage sites. Each sequence of the alignment in Figure 2B presents 3–12 repeats ending with RF. In these sequences, the IL[RK]F feature is the most abundant. The ILRF sequence is one classical ending identified among the 23 groups of longer metazoan FaRPs (38). Conventional cleavage sites (R or K) are present, suggesting a RENMIL[R,K]FWR peptide sequence. As observed in other genes/species, all the repeats are not followed and/or preceded by a putative cleavage site; these unbreakable repeats are often interpreted as non-functional.

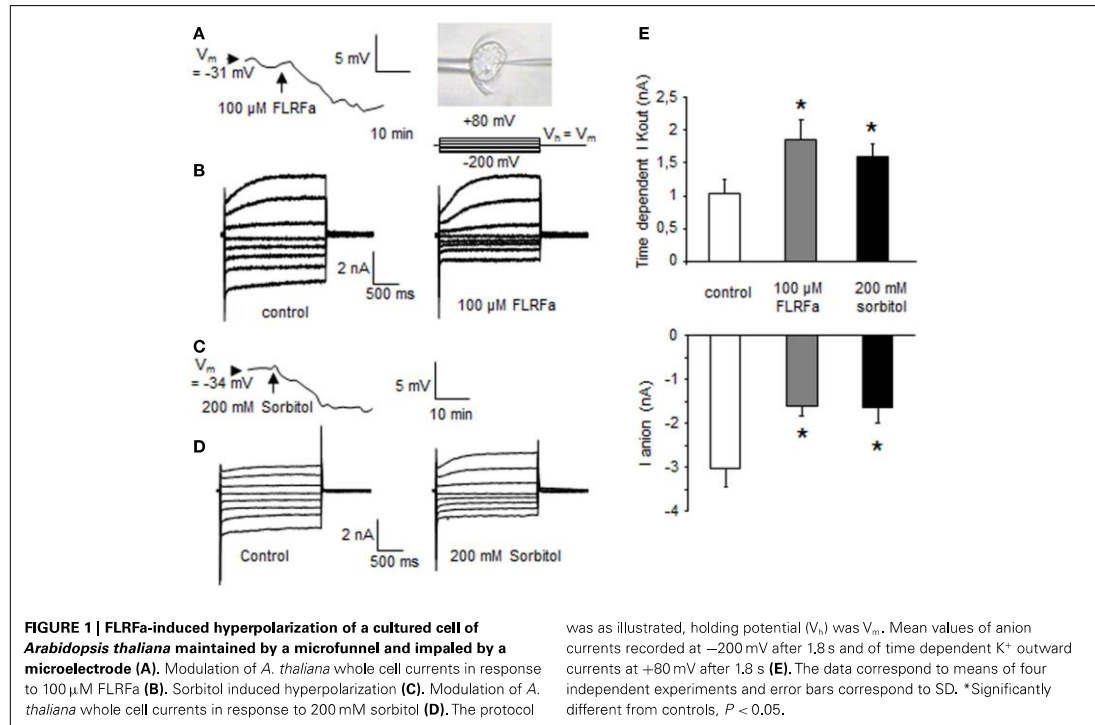
Other repeated peptides with FLPs-like structures have been evidenced. Figure 2C illustrates a group of sequences rich in KI[MIST]GLRFWR and also presenting other putative peptides. Each of these five sequences are found on different chromosomes, indicating that they correspond to different genes.

The peptide ILRF (and YLRF) is found in several combinations of metazoan peptides, suggesting that this peptide could be functional, at least in metazoans (37). This observation, combined with biological effect of tetrapeptide in *Arabidopsis* cells shown above,

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Bouteau et al.

Putative FLPs-induced plant responses



strongly suggests that functional bioactive FLPs could be synthesized in *Arabidopsis*, as in metazoans. Surprisingly, practically none of the putative peptides observed in *Arabidopsis* ended with a G, but with a W instead. This suggests that in *Arabidopsis*, amidation does not occur when the peptide is generated. This raises the question of the activity of these molecules in *Arabidopsis*, as amidation appears to be necessary to their bioactivity in metazoans.

The putative presence of RF-amide peptides in plants is, at the moment, based on genomic and transcriptomic databases. Future work should aim at characterizing the presence of translated peptides. However, because of the similarity with the metazoan peptides observed in the peptide ILRF found and whatever the differences around the cleavage site, we have explored their activity in *Arabidopsis* cells.

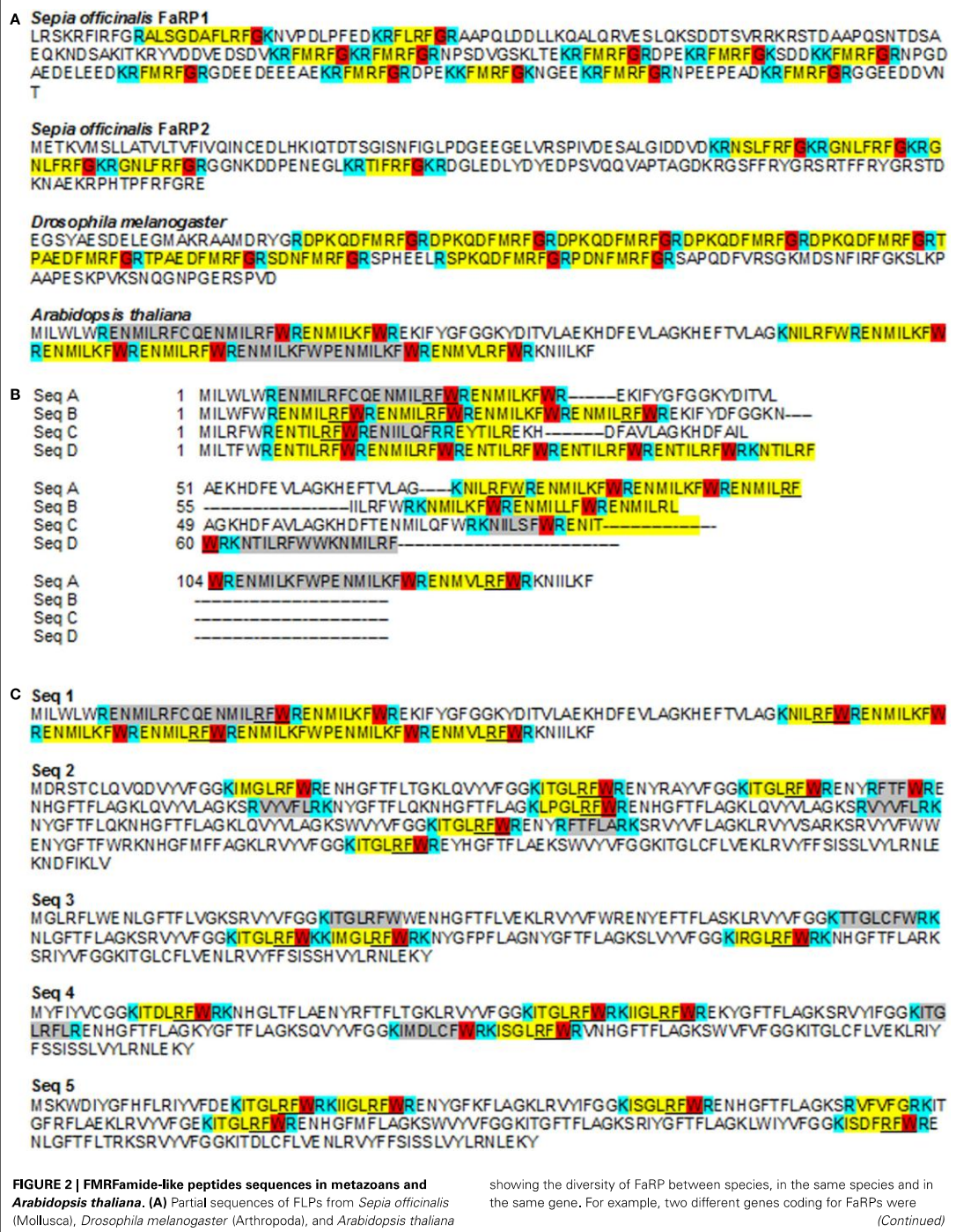
PUTATIVE FLPs FROM *A. THALIANA* COULD REGULATE SORBITOL-INDUCED PCD AND ROS GENERATION

As in animal cells, plant cell hyperosmotic stress may result in the induction of signaling events that leads to programmed cell death (PCD) (2, 43–47), an active cellular process that facilitates the removal of unwanted or damaged cells and is essential for cellular differentiation and tissue homeostasis. We recently showed that hyperosmotic stresses-induced ion channel regulations participate in pathways leading to PCD in plant cultured cells (2). Using synthetic peptides, we tested the effect of putative plant FLPs (ILRF and ILKF, 10 μ M each) on sorbitol-induced PCD in *A. thaliana*

suspension cells. The shifts in osmolality induced by addition of 400 mM sorbitol (from 128 to 524 mOsm) led to the death of about half of the cell population after 6 h (Figures 3A,B) when FLPs did not induce a significant increase in cell death (Figure 3A). Pretreatments of *A. thaliana* cells with 10 μ M ILRF or ILKF 15 min before addition of 400 mM sorbitol decreased the extent of the sorbitol-induced cell death (Figure 3A). Due to the lack of terminal G, which was systematically replaced by a W in plant sequence, the putative plant FLP could not be amidated (cf data from Figure 2). Thus, we further investigated the putative role of the terminal W by testing the same peptides augmented with a terminal W (ILKFW and ILRFW, 10 μ M each). These treatments decreased the extent of the sorbitol-induced cell death in the same range (Figure 3A), suggesting no specific role for the terminal W in this response. It is noteworthy that the terminal amidation did not increase the bioactivity, since the decrease in sorbitol-induced cell death by pretreatment with amidated peptides, ILRFa, ILKFa, and FLRFa (100 μ M each), were not drastically modified even with a 10 times higher concentration (Figure 3C).

A delayed O_2^- generation from NADPH-oxidase activity was also shown to play a central role in the hyperosmotic stress-induced PCD in plant cells (2). We thus evaluated the impact of putative plant FLPs (ILRF, ILKF, ILKFW, and ILRFW, 100 μ M each) on sorbitol-induced ROS generation. As observed for sorbitol-induced cell death, pretreatments of *A. thaliana* cells with these FLPs 15 min before addition of 400 mM sorbitol did

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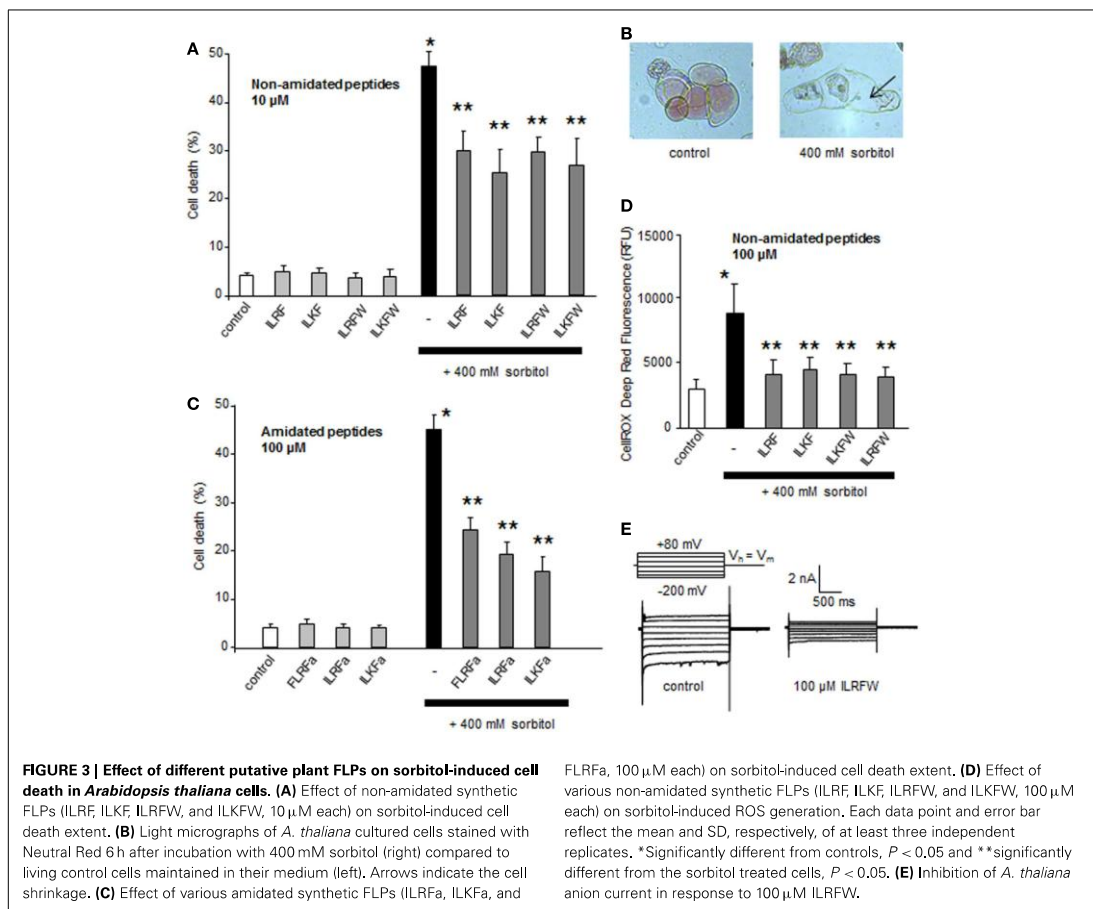


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FIGURE 2 | Continued

characterized in *Septia officinalis* with different composition and length. All peptides characterized in metazoans end with a G allowing amidation of the peptide after cleavage (38). The sequence of *A. thaliana* shows similar repetitions ending with RF but with a W instead of a G. Acc number: *S. officinalis* FaRP1: P91889; *S. officinalis* FaRP2: D8WXV2; *D. melanogaster*: AY070639; *A. thaliana*: P93742. **(B)** Different putative pro-peptides in *A. thaliana* genome detected using a poly-ILRF query. Sequences were manually aligned in Jalview. Acc. Numbers: SeqA At2g42050 (P93742_ARATH), 1-139; SeqB: BAB01828 (Q9LS63_ARATH)/1-86; SeqC: F26F24.19 (Q9LR27_ARATH_F26F24.19)/1-86; SeqD: F4N2.6 (Q9LQB1_ARATH)/1-77. **(C)** Examples of putative small peptides from *A. thaliana* including a RFW

end-sequence (underlined) or terminated by another sequence (highlighted in gray). These sequences are chosen to illustrate the presence of putative pro-peptide genes on each chromosome. Each sequence has been detected in mRNA sequencing (as shown by a tblastn against *A. thaliana* ESTs at the NCBI site) but are not recognized as ESTs in *A. thaliana* EnsemblPlants genomic automatic annotation due to their repeat structure. Instead, all are flagged as transposable elements. Acc. numbers: *A. thaliana*: seq1: P93742_ARATH; seq2: Q9LNR8_ARATH; seq3: Q9SVC3_ARATH; seq4: Q9SZF1_ARATH; seq5: Q9FGB7_ARATH. The putative cleavage sites (mono or dibasic) are indicated in blue, the “transitional peptide” in red, the putative functional peptide in yellow. The sequence highlighted in gray in *Arabidopsis* sequences is a putative peptide.



not cause sorbitol-induced ROS generation (Figure 3D). Moreover, the peptide ILRFW could reduce anion channel activity (Figure 3E) and induce a hyperpolarization of the cells of about -10 mV (not shown) as does FLRFa (Figure 1A). It is noteworthy that non-amidated putative plant FLPs were efficient in decreasing sorbitol-induced ROS generation and anion currents,

indicating that the peptide amidation was not necessary for their activities.

From these data it seems that, like in animal cells, FLPs could participate in osmoregulation through regulation of different events as ROS and PCD. The fact that the early FLP-induced ion current regulations in plant cells are the same as the one

observed in response to a hyperosmotic stress (Figures 1 and 3E) suggests that FLPs could participate to early induced process to maintain ion homeostasis and/or signalization, allowing to limit hyperosmotic stress-induced-PCD progress (Figure 3A).

PUTATIVE FLPs FROM *A. THALIANA* COULD REGULATE STOMATAL OPENING

Since putative plant FLPs could inhibit ROS generation (Figure 3D) and anion channel activity (Figures 1B and 3E) – both of which are known to regulate the stomatal aperture (48, 49) – we checked for the effect of a putative plant FLP (ILRF) on stomatal regulation. Stomata are pores in the plant epidermis that allow gas exchange between the intercellular spaces to the external environment. Two guard cells surround the stomatal pore, and changes in their turgor pressure regulate the size of the pore aperture allowing the CO₂ assimilation and limit excessive water loss by optimizing the aperture in response to the external environment. Epidermal strips from *A. thaliana* leaves were thus floated 2 h in two different conditions to either optimize the opening (opening buffer) or the closure of the stomata (closing buffer) (29) before addition of 100 μM ILRF. No effect could be observed on open stomata in response to addition of ILRF (Figure 4), while the same treatment induced an increase in the aperture of stomata from the epidermal strips placed in the closing buffer (Figure 4).

This ILRF-induced stomatal opening could appear counterintuitive, since upon drought stress, the stomatal closing is thought to be an important primary defense against tissue dehydration (48, 50). However, if such response is part of a fundamental response to severe drought stress, upon mild drought stress, prolonged closed stomata will stop growth by depriving the plant of CO₂

for photosynthesis. Adaptive plant growth in sporadic water availability will thus depend on the optimal tradeoff between stomatal closing and rapid re-opening capability. Thus, plants should adapt to mild soil water deficit by mechanisms that are distinct from those of severe dehydration. This was highlighted by a recent report in which acetylated 1,3-diaminopropane counteracted the canonical abscisic acid-induced stomatal closing (48) upon mild drought stress, but not upon severe drought stress (51).

CONCLUSION

Numerous cellular features are conserved in eukaryotic cells (18, 19), and many discoveries with direct relevance to animal biology and even human health and disease have been elaborated using plants (17). In this context, conservation of mechanisms of response to hyperosmotic stress, a stress that most of the living organisms have to face, appears logical. In a recent paper, Aalen (10) mentioned that plant peptide research is coming of age and that plant peptide signaling is of crucial importance for all aspects of plant growth and development. Recent works effectively implicate several families of small signaling peptides in various developmental processes in plants (11). However, the families of characterized peptides in plants represent <10% of the estimated number of secreted peptide ligands. Our preliminary results showing that some putative FLPs genes are present in *A. thaliana* genome and that putative plant FLPs could induce physiological responses involved in hyperosmotic stress responses warrant further studies on this topic. Furthermore, we cannot dismiss the possibility that other genes could be responsible for synthesis of others putative FLPs in plants. Drought frequency may increase by more than 20% in some regions of the globe by the end of the twenty-first century, with reductions in crop yields due to decreased water availability. Thus, understanding the putative role of FLPs in plants as regulators that mediate environmental influences on plant development and fitness is particularly relevant for plant biology. Moreover, this topic could also be relevant for metazoan biology since it could bring new insight in FLPs structures, functions, and evolution.

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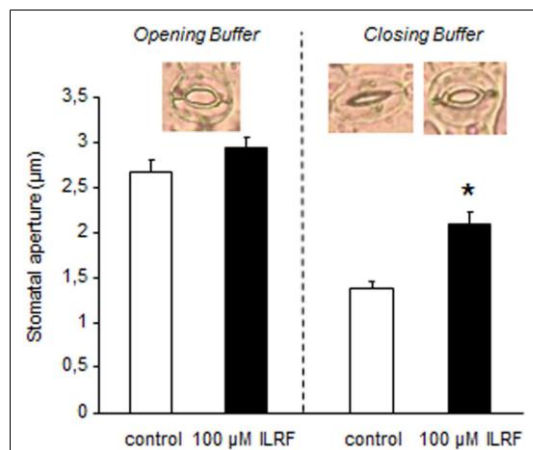


FIGURE 4 | Changes in *Arabidopsis* stomatal apertures of epidermal strips maintained in opening buffer or closing buffer upon treatment with 100 μM ILRF. Means ± SE ($n = 65-75$ stomata for each treatment). *Significant difference ($P < 0.05$) in stomatal aperture when compared to the control was found after treatment with ILRF in closing buffer, but not opening buffer.

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Chapter 5

Conclusions and perspectives

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The results described in this thesis enable discussion about the role of plasma membrane ion channels in the events leading to hyperosmosis-induced PCD. It is known that ion channels are critical components of apoptosis in animal cells; for example, the activation of Cl⁻ channel is an early prerequisite to apoptotic events, including cell shrinkage (termed AVD for apoptotic volume decrease). The volume changes are involved in numerous cellular process including cell death. (Lang *et al.*, 2007; Yu and Choi, 2000). The aim of my project was to investigate the role of ion channels in response to ionic and non-ionic hyperosmotic stress involved in the events leading to PCD. The BY2-tobacco suspension cell system was used to follow ion channels activity and other early cell responses under ionic- (NaCl) and non-ionic (sorbitol) hyperosmotic stress (chapter 2). We found that (ionic and non-ionic) stresses are accompanied by ROS generation and [Ca²⁺]_{cyt} increase. The early generation of ¹O₂ was detected after NaCl and sorbitol exposure. This early ROS generation seems not to be involved in the events leading to PCD in both case (ionic and non-ionic stress) since treatment with DABCO (scavenger of ¹O₂) failed to decrease sorbitol- and NaCl-induced cell death. Moreover in this model [Ca²⁺]_{cyt} increase appears to have no role in inducing cell death. This study also highlights linked responses such as ¹O₂ generation and cytosolic Ca²⁺ increase are not involved in PCD. A difference observed between sorbitol- and NaCl-induced PCD was the role of ion channels. In the case of NaCl treatment, a rapid depolarization was observed due to flux of Na⁺ ions into the cells through NSCCs, and this depolarization was involved in cell death. In contrast to this, sorbitol treatment caused a hyperpolarization due to the decrease of anion efflux. This early anion efflux inhibition could be involved in adaptative processes, as confirmed by the use of bromotretamisole, an activator of anion channels which failed to limit sorbitol-induced PCD, in contrast to its previously observed effect upon treatment with the HR-inducing elicitor HrpN_{ea} (Reboutier *et al.*, 2005). Another important difference found between sorbitol and NaCl treatment was the role of mitochondria. Only upon NaCl treatment we observed a mitochondrial depolarization involved in cell death; sorbitol did not appear to affect mitochondrial polarization. Collectively, these findings highlight the importance of ion channels in response to hyperosmotic stresses, suggesting they may play an important role in the events that lead to cell adaptation or PCD. In chapter 3 we better clarified the role of anion channels in response to sorbitol. By using *A. thaliana* cells we tested if a delayed anion current activation was involved in PCD. Sorbitol treatment induced a hyperpolarization due to a decrease in anion currents during the first 20 minutes. After 2h of sorbitol treatment we

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observed two responses due to the presence of two cell populations. One cell population (about 40% cells) repolarized to levels comparable to the control polarization (before hyperosmotic stress). The rest of the population displayed strong depolarization. A possible explanation for this is that the first cell population displays anion flux reduction which could be involved in the mechanisms of adaptation. The second population displayed an increase in anion activity which seems to be involved in the events leading to PCD. Our model study sheds light on the role of anion channels in non-ionic hyperosmotic stress-induced cell death. In particular a delayed increase in anion currents could participate in non-ionic hyperosmotic-induced cell death. In the hypothesis paper in chapter 4, we investigated the physiological effects in sorbitol- hyperosmotic stress responses of synthetic peptides belonging to the FMRF amide-like peptides (FLPs) family that have been shown to participate in osmoregulation in metazoans (López-Vera *et al.*, 2008) through regulation of ion channels. By using synthetic peptides, we conducted a set of experiments to verify a possible involvement of these peptides in osmoregulation in plants. These preliminary experiments indicate that such peptides could have a role in ion channel regulation in *A.thaliana* suspension cells. Indeed, pre-treatments with FLRF induced a cell plasma membrane hyperpolarization, correlated with a decrease in inward currents (anion currents). We also checked a possible involvement of these peptides in the regulation of the events leading to PCD. In Fig 20 a scheme is shown of the main findings reported in chapter 2 and chapter 3.

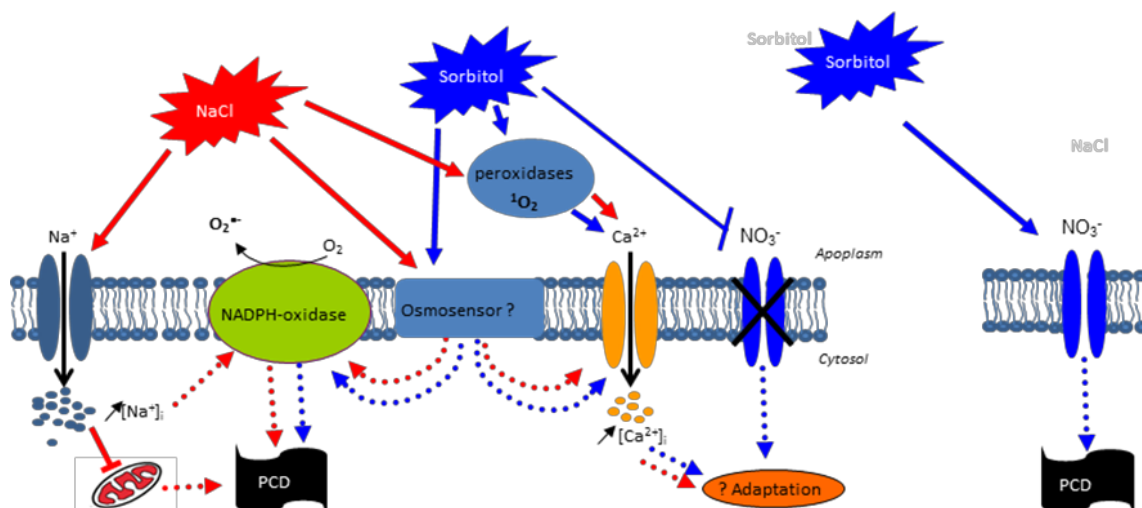


Figure 20: Cartoon showing the possible pathways induced by NaCl and sorbitol leading to cell death. The right side of figure shows the role of delayed anion channel activity in sorbitol induced cell death.

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In terms of future perspectives it is necessary to better understand the molecular nature of the role of anion channels modulation in the events involved in non-ionic hyperosmotic induced cell death. How the anion channels are integrated with other cell responses such as apoplast alkalization, is however an open question that require further research. Another important aspect to clarify is the involvement of caspase-like enzymes (Van Doorn *et al.*, 2011) in response to the hyperosmotic response and their overlapping effect with ion channel activity.

In chapter 4 is provided a hypothesis about the presence of FLPs in plants and their involvement in osmoregulation. These preliminary results show that some putative genes are present in the *A. thaliana* genome and could be involved in osmoregulation trough regulation of different events such as PCD and ROS generation. These FLPs were able to induce a decrease in sorbitol-induced ROS generation and anion currents involved in PCD. Further investigations on the related molecular aspects will be necessary to understand the involvement of these peptides in ROS production and anion channel regulation, especially the result of cross-talk of signaling pathways of these peptides and others. Another aspect to study is the possible involvement of these peptides in the regulation of caspase-like protein activity.

To better understand the biological role of these peptides it will be also of interest to determine the receptor of these peptides by homology with other organisms such as *Drosophila melanogaster* in which the FMRF amide receptor was identified (Meeusen *et al.*, 2002).

Another important aspect to study is the involvement of these peptides in regulation of ionic hyperosmotic stress, it could be interesting goal or task if there is a regulation of NSCCs, that as previously reported in chapter 2 are involved in the early response to ionic-hyperosmotic stress.

Therefore, investigating the peptide-dependent regulation of POXs at the molecular level could shed light on their involvement in ROS production.

Moreover, it the role of FLPs in stomatal opening regulation was also studied, showing that these peptides inhibit anion channel activity which is known to regulate stomatal opening (Negi *et al.*, 2008; Sirichandra *et al.*, 2009). The role of FLPs could be part of a sophisticated mechanism involved in stomatal closure regulation in response to drought as recently report for acetylated 1,3-diaminoprapane (DAP) which counteracted the canonical abscissic acid-induced stomatal closing (Sirichandra *et al.*, 2009) upon mild stress, but not upon severe stress (Jammes *et al.*, 2014). Such regulators which could allow plant to cope with osmotic

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stress could be of particular interest since, as mentioned above, drought frequency may increase by more than 20% in some regions of the globe by the end of the twenty-first century.

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