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 $^1O_2$ generation and subsequent $[Ca^{2+}]$cyt increase were not involved in PCD, while a delayed $O_2^{\cdot -}$ 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 hyperopolarization 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 hyperomotic-induced cell death. Becouse 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).

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

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

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 hypepolarization 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 ($O_2^{\bullet-}$) 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 pattway and a death pathway could be induced by the same hyperomotic conditions.

Key words: anion channels, *Arabidopis 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, $[Ca^{2+}]_{cyt}$, 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.

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

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 it 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 adjustement 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

role of anion channel regulation since (i) H_2O_2 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 hyperomoctic 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.

Materials and Methods

Cell culture conditions

Arabidopis 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 $\mu E.m^{-2}.s^{-1}$) 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 3.10^4 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).

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) • 100 ± 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).

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 MΩ 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 MΩ). 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 $O_2^{\bullet-}$ and $^1O_2$ 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 $[Ca^{2+}]_{cyt}$ variations were recorded with *A. thaliana* cell suspension expressing the aequorin gene (Brault *et al.*, 2004). Aequorin was reconstituted by overnight

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: pCa = 0.332588(-logk)+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 ± 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.

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

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 *arabidopis* 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.

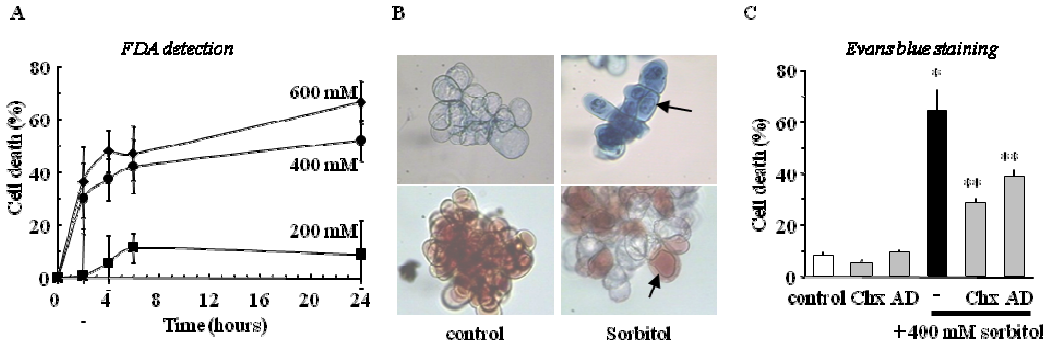

Figure 1: Sorbitol-induced cell death *A.thaliana* cells (A) Time course of the dose dependent cell death induced by sorbitol treatment. (B) Light micrographs of *A. thaliana* cells treated with 400 mM sorbitol for 6 hours and stained with Evans blue (upper panel) or neutral red (lower panel). (C) Effect 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, $P < 0.05$.

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 depolarizition 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 depolarizied (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 -

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).

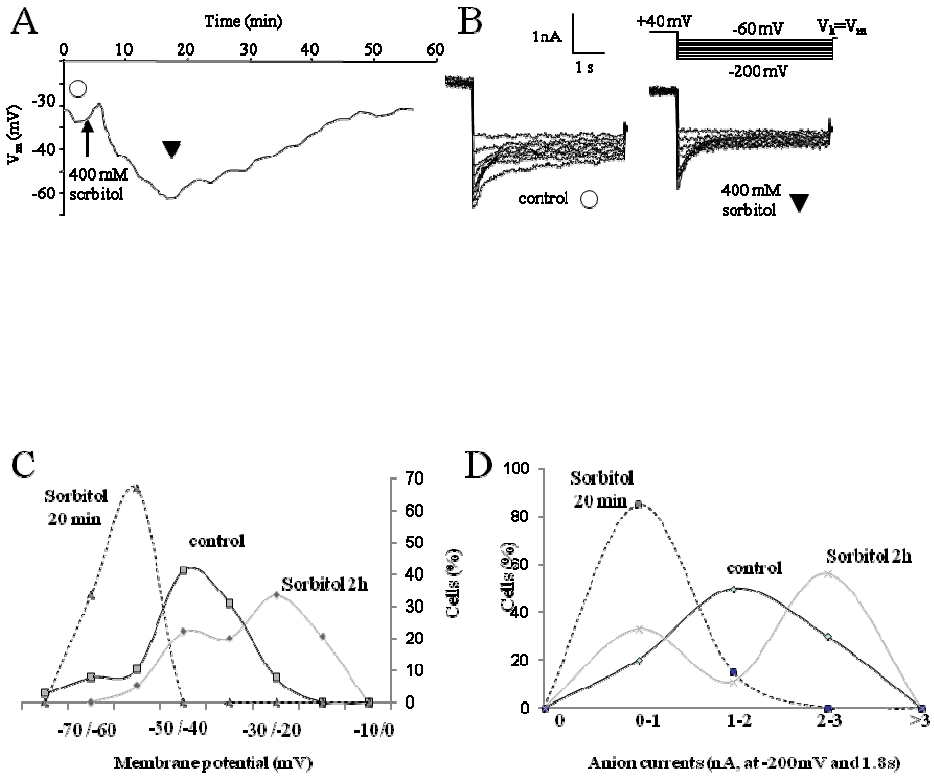

Figure 2: Biphasic regulation of *A. thaliana* cell polarization by 400 mM sorbitol (A) Typical modulation of cell plasma membrane (PM) potential variations observed in response to sorbitol. (B) Typical changes in whole cell current profiles after treatments with 400 mM sorbitol. The protocol was as 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 µM).

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 µM 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

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.

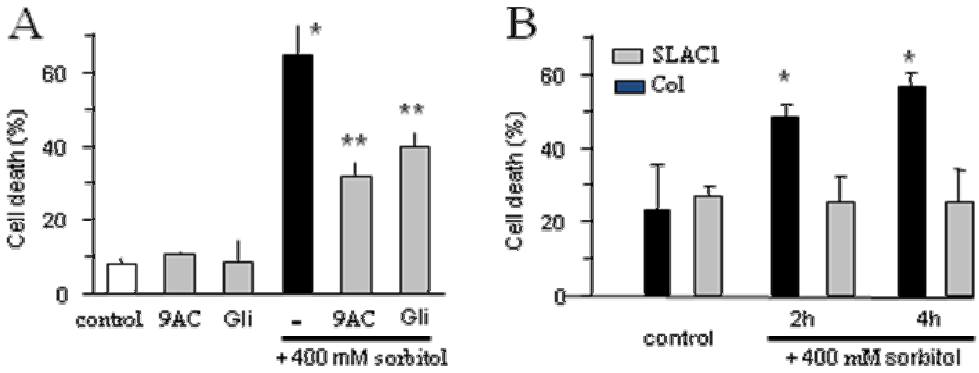

Figure 3: Anion channels are involved in sorbitol-induced PCD. (A) Effect of the anion channel blockers 9-AC and glibenclamide (200 µM each) on cell death induced by 400 mM sorbitol after 4h treatment. (B) Effect of 400 mM sorbitol on cell death extent in freshly prepared cultured cells from the wild 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 rinced 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.

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+} ($[Ca^{2+}]_{cyt}$), 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 .

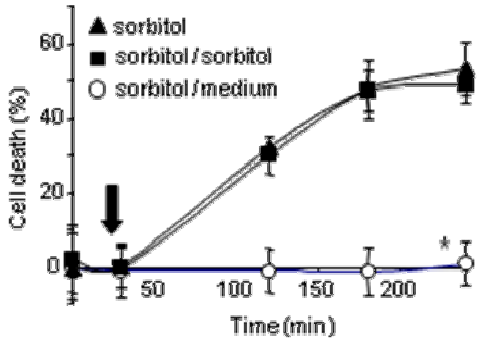

Figure 4. **Impact of sorbitol (400 mM) removal after 30 min on the *A. thaliana* cell death extent.** Cells were rinced 30 min after treatment with sorbitol with Gamborg medium free of sorbitol (Sorbitol/medium) or with Gamborg medium with 400 mM sorbitol (Sorbitol/sorbitol) and the cell death progression was compared with sorbitol treated cells without rincage 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 $[Ca^{2+}]_{cyt}$ are not involved in the sorbitol-induced cell death

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 arabidopis cells with 400 mM sorbitol resulted in a rapid transient increase in aequorin luminescence (Fig. 5A) reflecting an increase in $[Ca^{2+}]_{cyt}$ of about 0.6 µM. 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 $[Ca^{2+}]_{cyt}$ was mostly due to influx of Ca^{2+} across the plasma membrane through Ca^{2+} channels. In order to check wheter 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

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.

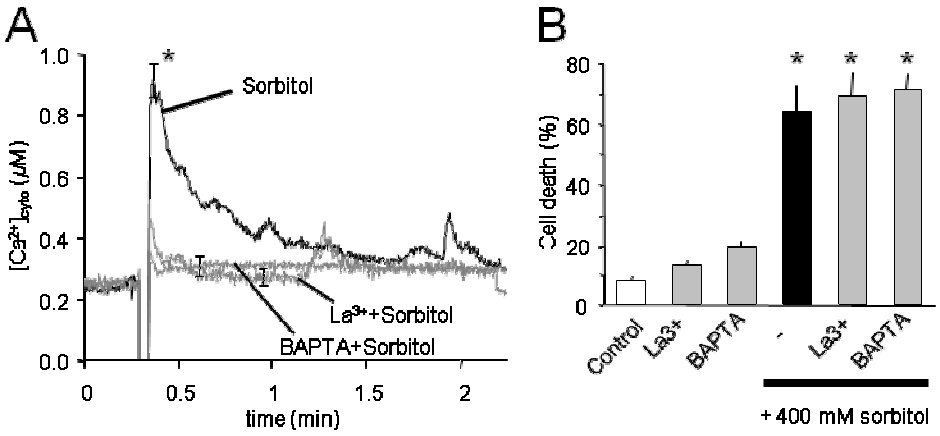

Figure 5: **Induction of $[Ca^{2+}]_{cyt}$ increase in aequorin expressing-*A. thaliana* cells by 400 mM sorbitol**. (A) Typical kinetic of sorbitol-induced increase in $[Ca^{2+}]_{cyt}$ and inhibition by the calcium channel blocker $LaCl_3$ (500 µM), and the calcium chelator BAPTA (1 mM). Each data 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 arabidopis cell culture we used the chemiluminescence of CLA which indicates the generation of $O_2^{\bullet-}$ and $^1O_2$. 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 $^1O_2$ scavenger, but not Tiron, a $O_2^{\bullet-}$ scavenger (Fig. 6C,D). Thus, the early increase in CLA-chemiluminescence seemed to be dependent on $^1O_2$ generation but not on $O_2^{\bullet-}$ generation. We further checked the impact of the DABCO on sorbitol-induced PCD. This scavenger of $^1O_2$ failed to decrease sorbitol-induced cell death (Fig. 6E), but 10 µM diphenyleneiodonium chloride (DPI), an inhibitor of the NADPH-oxidase, decreased the sorbitol-

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 µM 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.

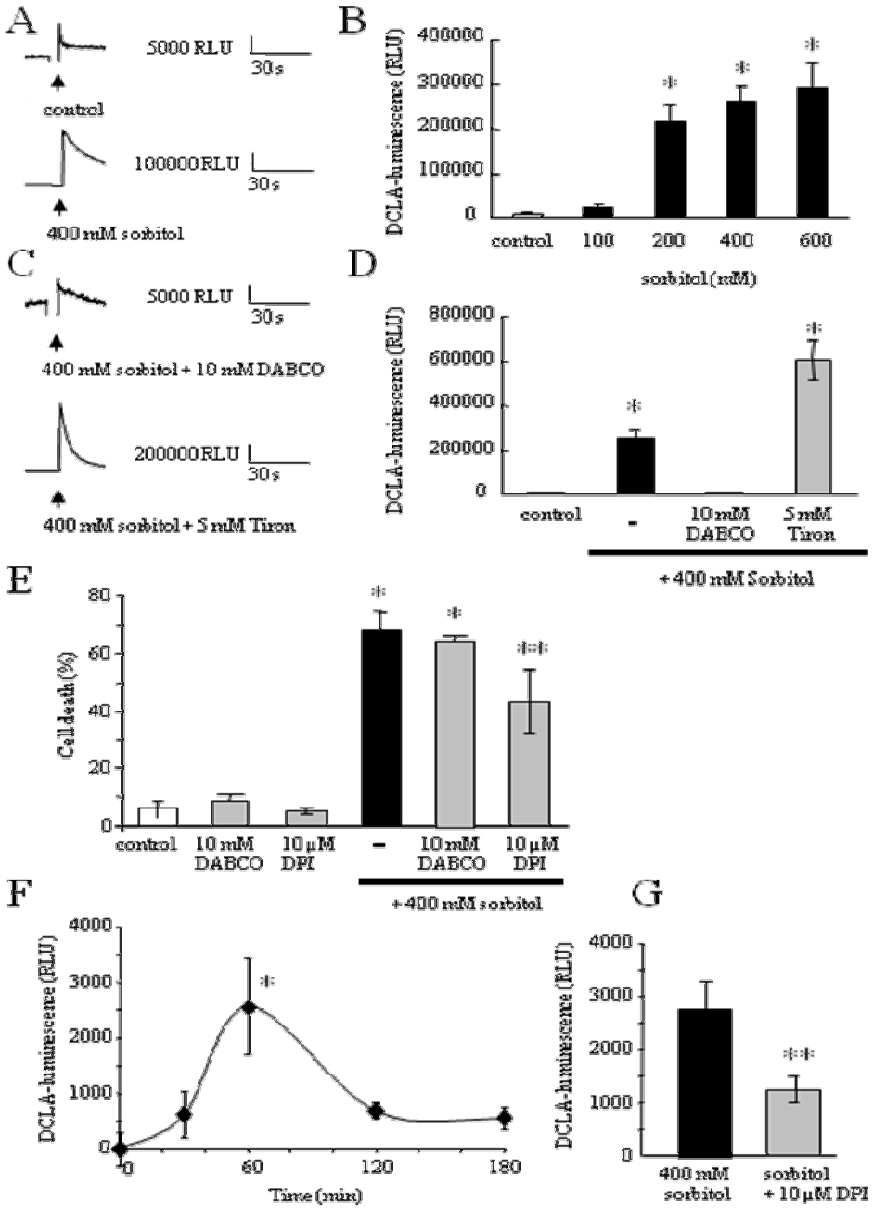

Figure 6. **Induction of ROS generation in *A. thaliana* cells by sorbitol.** (A) Typical kinetic of sorbitol-induced early increase in CLA-chemiluminescence reflecting the production ROS. (B) Effect of concentration of sorbitol on early ROS generation. (C) Modulation of sorbitol-induced ROS generation 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, $P < 0.05$.

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

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 adjustement since it allows maintaining anion in the cells and it is noteworthy that this regulation is observed in most of the cells. Morover 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.

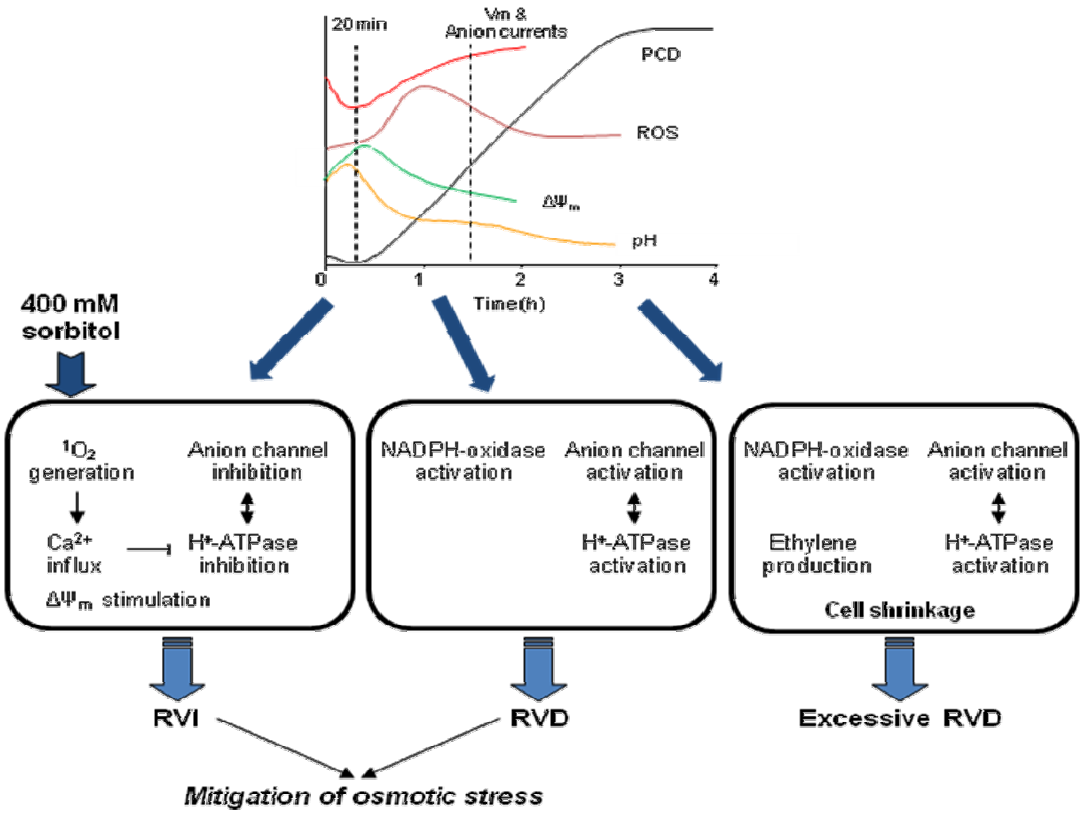

Figure 7: Events involved in sorbitol induced cell death

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 adjustement. 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

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

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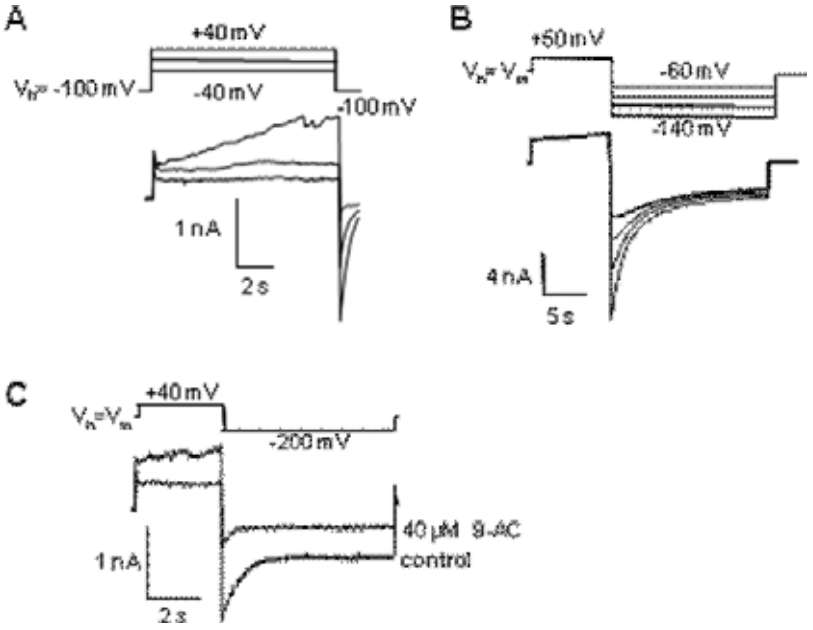
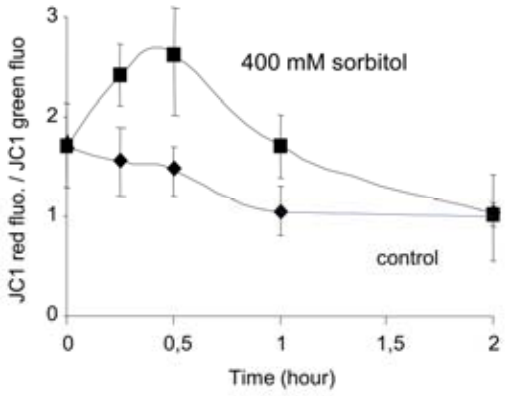
Supplementary matherials



Supplemental 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-anthacen carboxilic acid (C).



Supplemental 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

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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.

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

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

Chapter 4: FaRP-like peptides as osmoregulator in plant

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HYPOTHESIS AND THEORY ARTICLE
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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.

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

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

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 3.10^4 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 NO_3^- (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 $CaCl_2$ (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 $CaCl_2$ (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 Metreo 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

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)$_n$RFG[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,

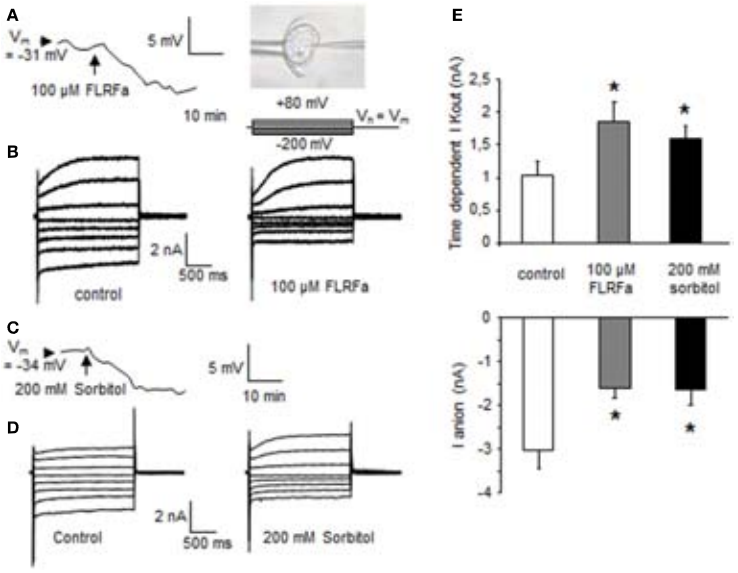

FIGURE 1 | FLRFa-induced hyperpolarization of a cultured cell of *Arabidopsis thaliana* maintained by a microfunnel and impaled by a microelectrode (A). Modulation of *A. thaliana* whole cell currents in response to 100 µM FLRFa **(B).** Sorbitol induced hyperpolarization **(C)**. Modulation of *A. thaliana* whole cell currents in response to 200 mM sorbitol **(D).** The protocol was as illustrated, holding potential (V_h) was V_m. Mean values of anion currents recorded at −200 mV after 1.8 s and of time dependent K^+ outward currents at +80 mV after 1.8 s **(E).** The data correspond to means of four independent experiments and error bars correspond to SD. *Significantly different from controls, $P < 0.05$.

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 programed 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 µM 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 µM 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 µM 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 µM each), were not drastically modified even with a 10 times higher concentration (**Figure 3C**).

A delayed $O_2^{\cdot-}$ 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 µM 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

Chapter 4: FaRP-like peptides as osmoregulator in plant

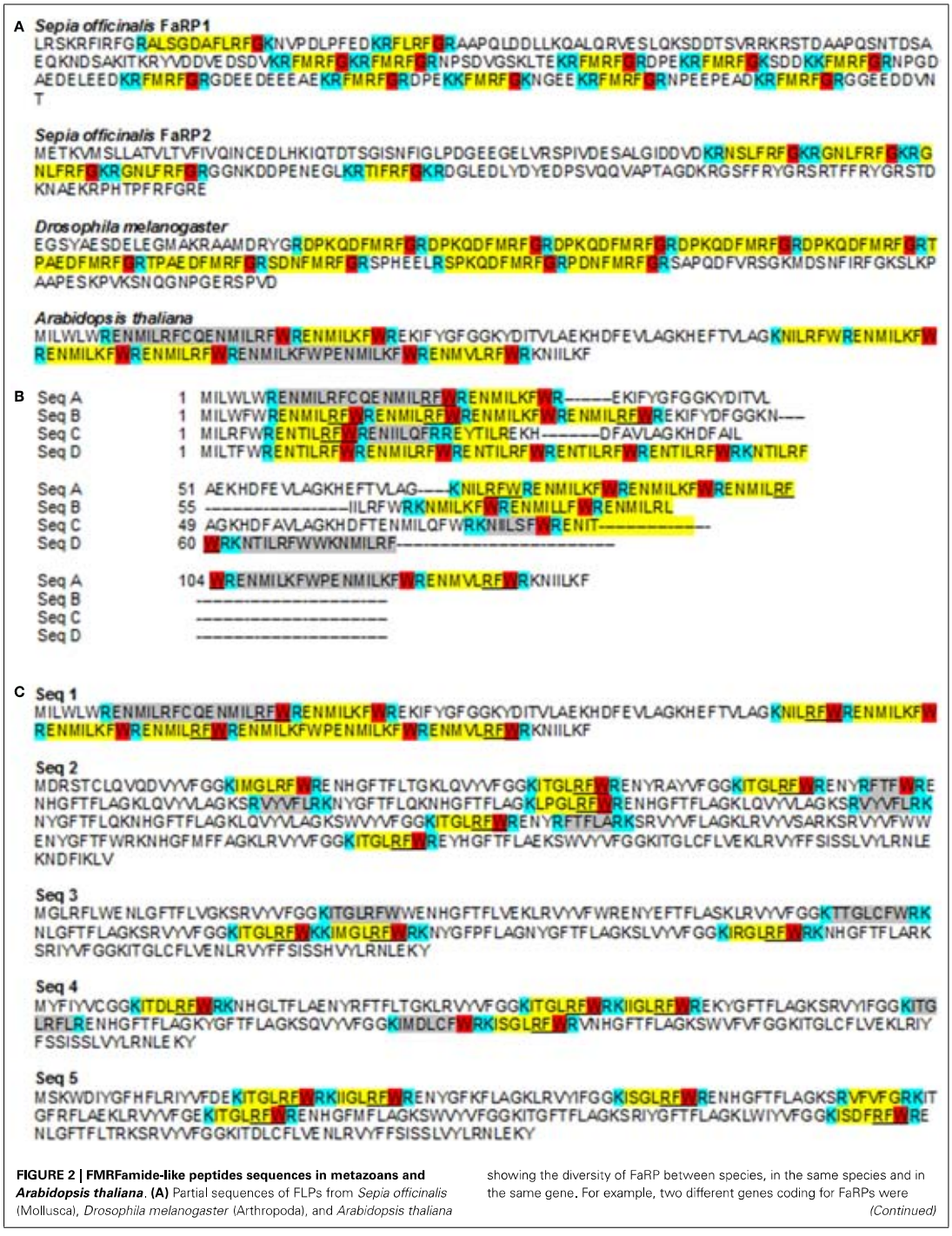

FIGURE 2 | FMRFamide-like peptides sequences in metazoans and *Arabidopsis thaliana*. (A) Partial sequences of FLPs from *Sepia officinalis* (Mollusca), *Drosophila melanogaster* (Arthropoda), and *Arabidopsis thaliana* showing the diversity of FaRP between species, in the same species and in the same gene. For example, two different genes coding for FaRPs were *(Continued)*

FIGURE 2 | Continued
characterized in *Sepia 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; seq.3: Q9SVC3_ARATH; seq.4: Q9SZF1_ARATH; seq.5: 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.

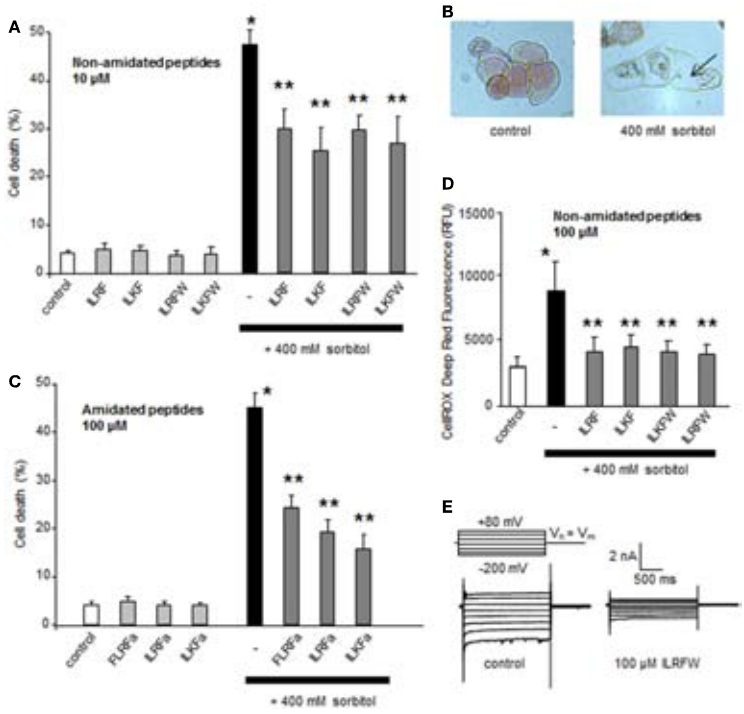

FIGURE 3 | Effect of different putative plant FLPs on sorbitol-induced cell death in *Arabidopsis thaliana* cells. (A) Effect of non-amidated synthetic FLPs (ILRF, ILKF, ILRFW, and ILKFW, 10 µM each) on sorbitol-induced cell death extent. **(B)** Light micrographs of *A. thaliana* cultured cells stained with Neutral Red 6 h after incubation with 400 mM sorbitol (right) compared to living control cells maintained in their medium (left). Arrows indicate the cell shrinkage. **(C)** Effect of various amidated synthetic FLPs (ILRFa, ILKFa, and FLRFa, 100 µM each) on sorbitol-induced cell death extent. **(D)** Effect of various non-amidated synthetic FLPs (ILRF, ILKF, ILRFW, and ILKFW, 100 µM each) on sorbitol-induced ROS generation. Each data point and error bar reflect the mean and SD, respectively, of at least three independent replicates. *Significantly different from controls, $P < 0.05$ and **significantly different from the sorbitol treated cells, $P < 0.05$. **(E)** Inhibition of *A. thaliana* anion current in response to 100 µM ILRFW.

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 CO_2 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 CO_2 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).

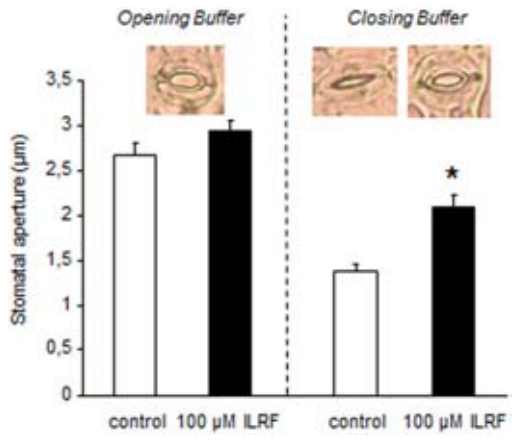

FIGURE 4 | Changes in *Arabidopsis* stomatal apertures of epidermal strips maintained in opening buffer or closing buffer upon treatment with 100 µM ILRF. Means ± SE ($n = 65$–75 stomata for each treatment). *Significant difference ($P < 0.05$) in stomatal aperture when compared to the control was found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

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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Chapter 5

Conclusions and perspectives

Chapter 5: Conclusions and perspectives

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 channes 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 $[Ca^{2+}]_{cyt}$ increase. The early generation of $^1O_2$ 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 $^1O_2$) failed to decrease sorbitol- and NaCl-induced cell death. Moreover in this model $[Ca^{2+}]_{cyt}$ increase appears to have no role in inducing cell death. This study also highlights linked responses such as $^1O_2$ generation and cytosolic Ca^{2+} 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 bromotretramisole, 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 inanion currents during the first 20 minutes. After 2h of sorbitol treatment we

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 hyperomotic-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.

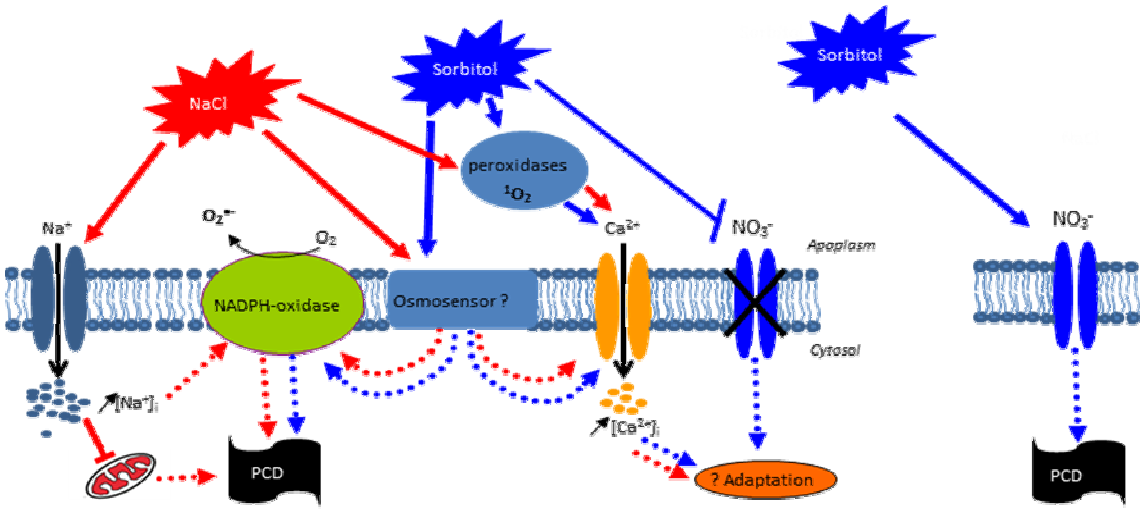

Figure 20: Cartoon showing the possible pathways induced by NaCl and sorbitol leading to cell death. The right side of figure shows the role of delayed anion channel activity in sorbitol induced cell death.

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 alkalinization, 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

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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