# Activation of plasma membrane H+−ATPases participates in dormancy alleviation in sunflower seeds

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# Activation of plasma membrane $H^+$-ATPases participates in dormancy alleviation in sunflower seeds

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ABSTRACT

Using various inhibitors and scavengers we took advantage of the size of sunflower (*Helianthus annuus*) seeds to investigate *in vivo* the effects of hormones, namely abscisic acid (ABA) and ethylene (ET), and reactive oxygen species (ROS) on the polarization of dormant (D) and non-dormant (ND) embryonic seed cells using micro-electrodes. Our data show that D and ND seed cells present different polarization likely due to the regulation of plasma membrane (PM) $H^+$-ATPase activity. The data obtained after addition of hormones or ROS scavengers further suggest that ABA dependent inhibition of PM $H^+$-ATPases could participate in dormancy maintenance and that ET-and ROS-dependent PM $H^+$-ATPase stimulation could participate in dormancy release in sunflower seeds.

## 1. Introduction

Although successful germination of seeds is a critical step in agriculture, some of the underlying molecular mechanisms of seed germination and dormancy regulation are still unknown. Seed dormancy allows some species to persist, blocking the germination of mature seed until conditions become favorable for seedling establishment. Seeds become capable of germination after a dry storage period called after-ripening, a process which reduces dormancy. The duration of this period depends on environmental factors such as temperature, light, oxygen and moisture. Germination begins with the absorption of water by the seed and ends when the embryonic axis begins to lengthen. Reactive oxygen species (ROS) and hormones have emerged as key regulatory actors in the regulation of seed germination and dormancy [1–3]. Abscisic acid (ABA) is a positive regulator of dormancy and a negative regulator of seed germination while other hormones such as gibberellins (GA) or ethylene (ET) promote germination completion by antagonising ABA effects [4,2,5,6]. In sunflower, ET is the major hormone acting as an antagonist of ABA and a positive regulator of seed germination [7–9]. In seeds, ROS and ABA have antagonistic effects [10,11], and ROS produced during soybean seed imbibition could promote ET production [12] and conversely, ET induced ROS accumulation in sunflower seeds [1]. Interaction of ROS with either hormone metabolism or signaling pathways seems important in this process [13,14] as it has been documented in other plant process such as stomatal opening regulation [15,16], plant microbe interactions [17,18] and cell death [19]. It is also noteworthy that ion transport systems are systematically involved and cross linked with ROS and hormones in these processes [20,21]. Although ABA is known to play a critical role in plant stress responses through transcriptional induction of defense genes in different organs, ABA does have non-transcriptional mechanisms modulating ion homeostasis resulting in plasma membrane (PM) $H^+$-ATPase inhibition during stomatal closure [22]. The effect of ABA on the root PM $H^+$-ATPase is sometimes controversial [23], but these varied responses could also be linked to ROS generation as observed in cultured cells of *Arabidopsis thaliana* where ROS are a central messenger of ABA in the signaling pathway leading to PM $H^+$-ATPase inhibition [24]. On the contrary, stimulation of PM $H^+$-ATPases by ET precursors have been suggested in various plant and cell types [25–27].

The PM $H^+$-ATPase is an important ion pump in the plant cell plasma membrane. By extruding protons from the cell and generating a membrane potential, this pump energizes the PM [28], a prerequisite to the activity of nutrient transporters ($K^+$, sugars, amino-acids) coupled to the electrochemical proton gradient and important for growth [29].

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Therefore, a mechanism related to cell polarization through PM $H^+$-ATPase activity regulation could be operating in seeds to allow for the participation of secondary ion transport in the energization of cells [28] and thus, dormancy release. Previous studies could not validate this hypothesis in the small seeds of *A. thaliana* and instead used roots because biochemical and electrophysiological measurements in roots correlated with growth phenotypes in germinating seeds. In the present work, we took advantage of the large size of sunflower seeds and used pharmacological agents to investigate *in vivo* the effects of hormones, namely ABA and ET, and ROS on the seed cell polarization as a marker of PM $H^+$-ATPase activity. We found that these hormones and ROS pharmacology act on seed cell polarization, suggesting that dormancy could be based on ABA dependent inhibition of PM $H^+$-ATPase and that ET- and ROS-dependent PM $H^+$-ATPase stimulation could be involved in dormancy release in sunflower.

## 2. Material and methods

### 2.1. Sunflower seeds and germination assays

Sunflower seeds (*Helianthus annuus* L.) of the cultivar LG5665 were grown and harvested by the agricultural cooperative Valgrain in an open field in the South of France in 2015. Mature seeds were stored at −20 °C to preserve dormancy, or at 20 °C for 2 months to release dormancy [30,31]. Germination assays were carried out by placing in the dark four replicates of 25 sunflower embryos (*i.e.* seeds without pericarp) on a layer of cotton wool moistened with water, with or without various pharmacological agents (abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), sodium orthovanadate (VAN), fusicoccin (FC), 1,4-diazabicyclo[2.2.2]octane (DABCO), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), diphenyleneiodonium chloride (DPI), salicylhydroxamic acid (SHAM), glucose (Glc), L-[β8-D-glucopyranoxyloxy-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-L-propanone (phoridzin)), in 9 cm Petri dishes in the darkness at 10 °C. Methyl viologen (MV) treatment was carried out by placing embryos on cotton wool moistened with a solution of 100 µM MV for 3 h in darkness at 10 °C, rinsed with water, and then placed on cotton wool moistened with water only for the remainder of the germination trial. Germination was counted daily, and an embryo was considered as having germinated when its radicle elongated by 2 mm. The final germination percentage (%) was expressed as G (%) = (A/B) × 100, where A is the total number of seeds germinated at the end of experiment and B is the total number of seeds tested. The time to reach 50% germination (T50) was calculated according to the formula of Coolbear et al. [32] modified by Farooq et al. [33]: T50 = ti + [(N / 2 - ni) (ti - tj)] / ni - nj (N is the final number of emergence, and ni and nj, the cumulative number of seeds germinated by adjacent counts at times ti and tj, respectively when ni < N / 2 < nj).

### 2.2. Electrophysiology

Intracellular voltage measurements were carried out on a vibration-stabilized bench in a Faraday cage. Microelectrodes were made from borosilicate capillary glass (Clark GC 150 F, Clark Electromedical, Pangbourne Reading, UK) pulled on a vertical puller (Narishige PEII, Japan), filled with 600 mM KCl and connected by a reversible Ag/AgCl half-cell to the measuring set-up consisting of an electrometer (Axoclamp 2 A, Molecular Devices, USA) and an oscilloscope (Gould 1425, Gould Instruments Ltd, UK). Microelectrodes were placed with micromanipulators (WR6-1, Narishige, Japan) and optically controlled with a microscope (Leitz, Germany). A pellet (E205, Phymep, France) was used as a reference electrode in the recording chamber. Impalements of sunflower embryo axis cells (Fig. 1A) were carried out in 5 cm Petri dishes filled with 5 ml of bathing medium (BM: 5 mM MES, 1 mM $CaCl_2$ and 1 mM KCl buffered at pH 5.8 with 5 mM Tris) at room temperature (20–22 °C) under light. Due to the thickness of the embryonic axis of the sunflower seeds, only blind impalement could be performed. We systematically check that the insertion of the microelectrode was accompanied by a rapid and steep rise in electrical resistance representing a time constant compatible with those of a plasma membrane (PM), indicating that the electrode has passed through the PM. Upon microelectrode entry into the sunflower embryo axis cells, the recorded potential drops within a few ms to an initial value ($V_p$) that could be maintained (Fig.1B) or decays to a depolarized potential in few seconds (Fig.1C). When an initial fast peak transient was measured upon microelectrode entry, it indicates that the microelectrode probably introduces a leakage into the PM, causing a change in PM potential ($V_m$) from its real value before impalement to a depolarized value. In this last case, the membrane could thereafter develop a slow hyperpolarization reaching a sustained membrane potential close to the recorded $V_p$ (Fig. 1C), as has already been observed in various animal and plant cells [34–37]. The initial fast peak transient Vp is considered as a good estimate of the true $V_m$ and that the slow hyperpolarizations after impalement should be regarded as transient repolarizations back to the original $V_m$ [35,35,36,37]. Thus, analysis of the initial fast impalement voltage ($V_p$) is considered as a valuable aid in the estimation of $V_m$ of cells by microelectrodes. Pharmacological studies were performed by direct application of the pharmacological agents in the Petri dishes after stabilization of the running potential or by pretreating the embryos in the BM with the pharmacological agent 30 min before voltage recordings.

### 2.3. ATP measurements

Adenosine-triphosphate (ATP) contents were determined using the bioluminescent method and following the protocol provided with an ATP Detection Kit (FLAA, Sigma-Aldrich, St. Louis, USA). Hulled seeds were placed on water-soaked cotton for imbibition at 10 °C for 3, 15 and 24 h. Dry and imbibed axes of D and ND seeds were collected and ground immediately on ice with 1 mL of acid extract from the ATP Detection Kit. Samples were centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was transferred to a new 1.5 mL tube and the same volume of alkaline extract was added. After mixing and again centrifuging for 10 min at 4 °C and 8000 × *g*, the supernatant was placed in a new tube on ice for the ATP assay. The luminescence was assayed from a 30 µL sample using a luminometer (FB12, Berthold, Germany) with 100 µL of the ATP detection buffer provided in the ATP detection Kit. All of the experiments were repeated in triplicate and the mean values were determined based on the three replicates ± standard deviation.

### 2.4. Seed water content determination

For water content measurements, hulled seeds were placed on water-soaked cotton for imbibition at 10 °C for 3, 15 and 24 h. Dry and imbibed sunflower seed axes (embryos without cotyledons) were put in coded, pre-weighed eppendorf tubes and weighed again. Tubes with open caps were put in a drying oven at 105 °C for 24 h. They were then placed in a desiccator for 10 min and weighed again. Values are means of three technical and three biological replicates ± standard deviation. WC (%) was determined using the following formula: (FW-DW)/DW*100); FW = weight of axes before drying; DW weight of axes after drying.

### 2.5. Statistical analysis

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

## 3. Results

Due to their hardness, dry seeds could not be impaled with glass

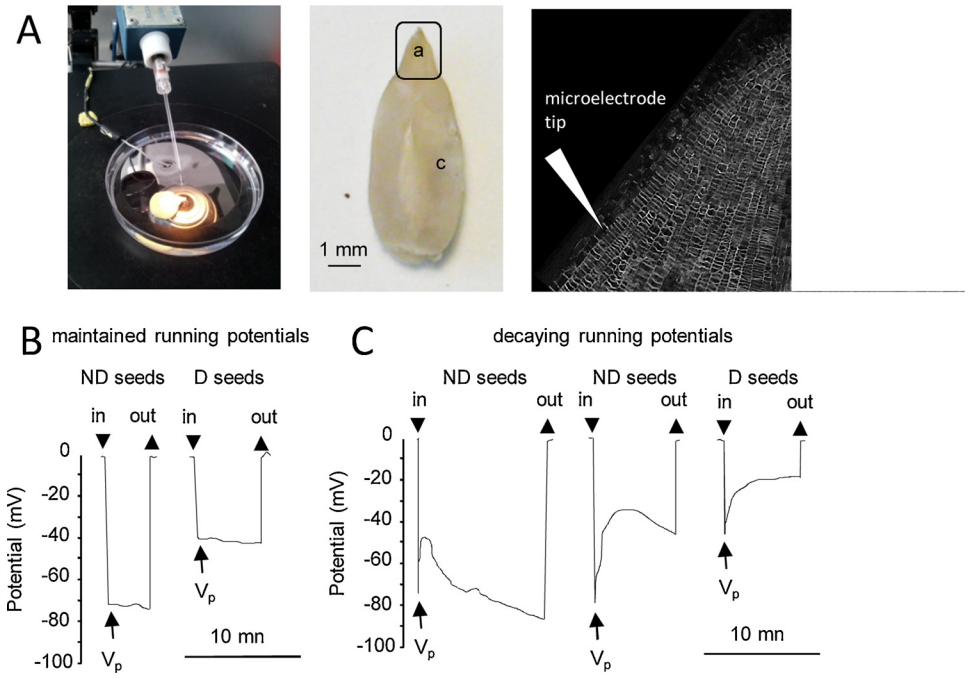


**Fig. 1. (A)** From left to right: impalements of sunflower embryo axis cells were carried out in 5 cm Petri dishes filled with 5 ml of bathing medium (BM: 5 mM MES, 1 mM CaCl2 and 1 mM KCl buffered at pH 5.8 with 5 mM Tris); sunflower embryo showing axe (a) and cotyledon (c) and the apex of the axe with an example of putative position of the microlelectrode tip into an epidermal cells after blind impalement. **(B)** Examples of maintained running potentials for cells of D and ND seeds recorded after blind impalements of the seed axis. **(C)** Examples of decaying running potentials for cells of D and ND seeds recorded after blind impalements of the seed axis (in = impalement of the axis with microelectrode, out = exit of the axis).

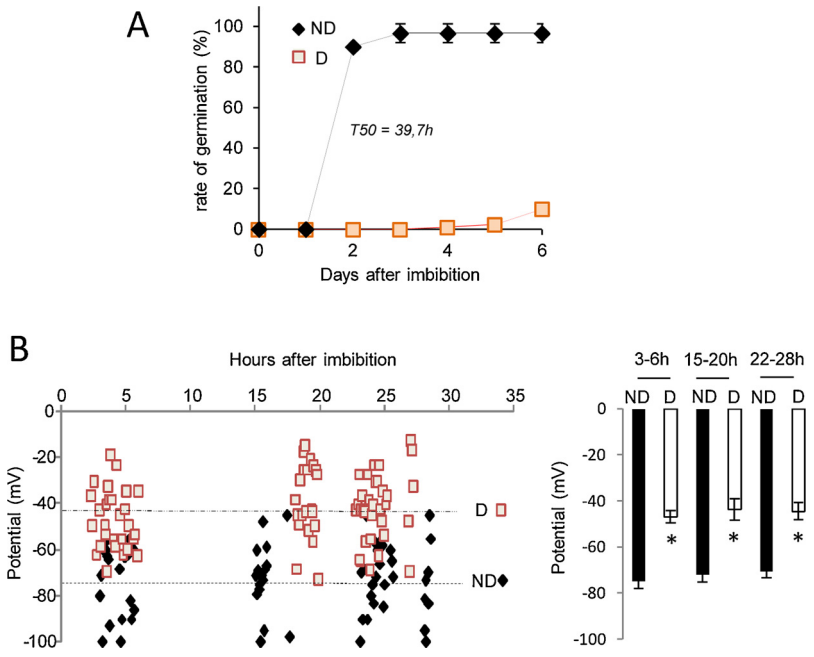


**Fig. 2. (A)** Typical rate of germination for dormant (D) and non-dormant (ND) seeds after imbibition. Half of the ND seeds are germinated after 39. 7 h **(B)** Values of cell polarizations after impalement for dormant (D) and non-dormant (ND) seed axes bathed in BM from 3 to 30 h after imbibition of the seeds. **(C)** Mean values of $V_p$ from ND and D cells for different time spans 3 to 6 h, 15 to 20 h and 22 to 28 h. Dashed lines with points and without points refer to the means values for $V_p$ of D cells and ND cells respectively. *Significantly different from ND.

microelectrodes, and thus impalements were achieved only 3 h after imbibition of the seeds in the bathing medium (Fig. 1A). Upon impalement (*i.e.* microelectrode entry into the sunflower embryo axis) the recorded potential drops within a few ms to an initial value ($V_p$) that could either be maintained (Fig. 1B) or decays to a depolarized potential in a few seconds (Fig. 1C). The same behaviors were observed in both D seeds and ND seeds but the initial values of $V_p$ were generally more hyperpolarized for ND when compared to D seeds (Fig. 1B, C). Fig. 2A presents the typical rate of germination for dormant (D) and non-dormant (ND) seeds after imbibition with deionized water. Half of the ND seeds germinated after 39.7 h. The distribution of $V_p$ values recorded for cells impaled in the axis of D and ND seeds from 3 to 28 h after the beginning of seed imbibition are shown in Fig. 2B. For that entire period, D seed cells presented more depolarized values with a mean $V_p$ of -42 ± 15 mV (n = 71) when non-dormant seed cells display more negative $V_p$ with a mean of -75 ± 16 mV (n = 82). The effects of various pharmacological agents were evaluated (see below), using $V_p$ as a valuable estimation of $V_m$ of cells by microelectrodes [35–37]; $V_p$ were thereafter considered as $V_m$.

### 3.1. Role of H+-ATPases in the polarization of cells from D and ND seed axes

Since the PM $H^+$-ATPase is an important ion pump in the plant cell membrane that participates in PM polarization by extruding protons from the cell [29], we checked if $H^+$-ATPase activity could be involved in polarization of cells from D and ND seed axes. Fig. 3A (left) illustrates one of the profiles recorded in a cell from a ND seed axis upon addition of 400 µM vanadate (VAN), an inhibitor of $H^+$-ATPases [25,29]. Vanadate rapidly depolarizes the PM of this cell while a cell of a D seed

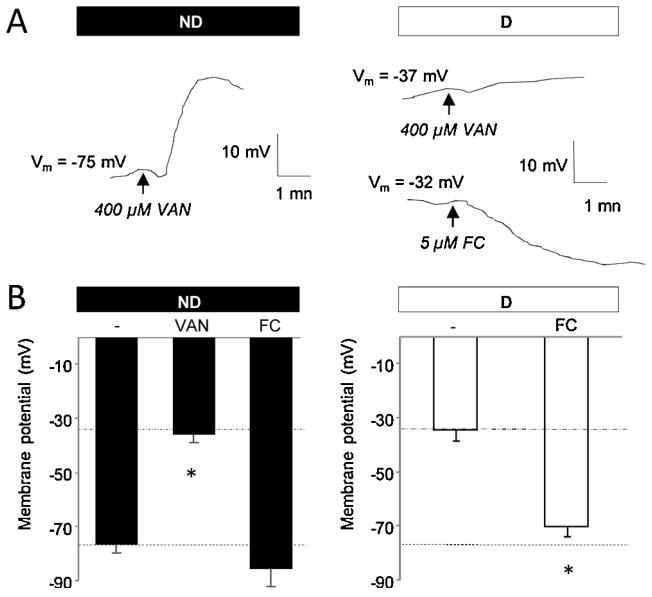


**Fig. 3. Role of $H^+$-ATPases in polarization of D and ND seed cells. (A)** Hyperpolarized ND seed cells could be depolarized upon addition of 400 µM vanadate (VAN), an inhibitor of $H^+$-ATPases while depolarized D seed cells could be hyperpolarized by addition of 5 µM fusicoccin (FC) an activator of $H^+$-ATPase, but not depolarized by vanadate. **(B)** When imbibed in the presence of 400 µM vanadate, the mean value of ND seed cell polarization was increased whereas the mean values of D seed cell polarization imbibed in the presence of 5 µM fusicoccin was decreased. Data represent mean values of at least ten cells and bars represent the standard error. Dashed lines with points and without points refer to the means values from Fig. 1B for instantaneous polarization for D cells and ND cells, respectively. *Significantly different from non-treated seeds.

axis was not depolarized upon addition of vanadate (Fig. 3A, right). On the contrary, the addition of 5 µM fusicoccin (FC), an activator of $H^+$-ATPase [29], to a cell of a D seed axis induces a hyperpolarization of the PM (Fig. 3A right). The average values of $V_m$ recorded on cells from ND and D seed axes pretreated 30 min before voltage recordings with 400 µM VAN (Fig. 3B, left) show that VAN depolarized the cells from ND seed axes to a value close to the $V_m$ recorded from D seed axis cells. On the contrary, 5 µM FC hyperpolarized D seed axis cells to a value close to that of ND seed axis cells (Fig. 3B, right). Addition of 5 µM FC to ND seeds only slightly hyperpolarized the axis cells (Fig. 3B, left). These data suggest that $H^+$-ATPases are not active in cells from a D seed axis though they can be stimulated by FC, and that they are responsible for a part of the polarization of cells from an ND seed axis. We further checked for the impact of VAN on the rate of germination of ND seeds. As shown in Fig. 4A (left), imbibition in presence of 400 µM VAN slows the germination of ND seeds, with the T50 being delayed for more than 8 h. On the contrary, imbibition in the presence of 5 µM FC accelerates the germination of D seeds (Fig. 4A, right). We further checked the availability of ATP, which is necessary for PM $H^+$-ATPase functioning, in D and ND seed axes. No difference in ATP contents could be detected between D and ND seeds (Fig. 4B). The increase in ATP contents follows precisely the seed imbibition curve recorded for D and ND seeds (Fig. 4C), strongly suggesting that ATP is not the limiting factor for the activation of PM $H^+$-ATPases in D seeds.

### 3.2. *The roles of hormones in the polarization of cells from D and ND seed axes*

Hormones, especially ABA and ET, are important actors in seed dormancy [38,5,1]. They are also known to regulate ion transport systems, notably PM $H^+$-ATPase [23,21], thus impacting cell polarization. We checked if ABA, known to maintain the dormant state of seeds, and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene known to alleviate dormancy [39], could regulate the polarization of D and ND seed axis cells. The average values of $V_m$ recorded on cells from ND seed axes pretreated 30 min before voltage recordings with 40 µM ABA (Fig. 5A) show that ABA depolarizes the cells of ND seed axes to a value close that cells of D seed axes. Addition of 40 µM ABA to D seeds did not modified the axis cell polarization (Fig. 5B). The average values of $V_m$ recorded on cells from D seed axes

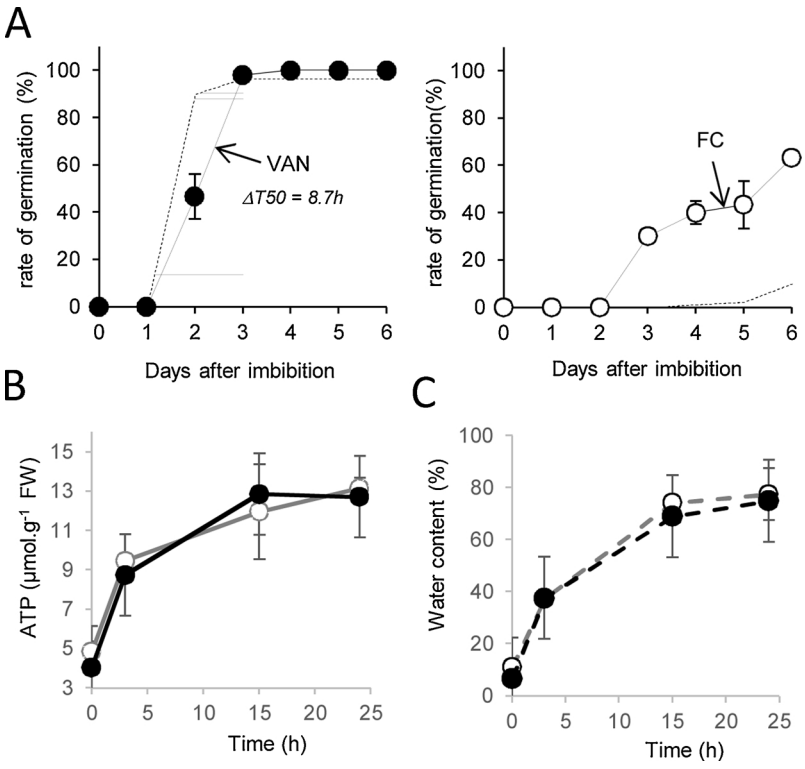


**Fig. 4.** (A) Imbibition in the presence of 400 µM vanadate slows the germination of ND seeds, while imbibition in the presence of 5 µM fusiccocin accelerates the germination of D seeds. Dashed lines refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf Fig. 1A). All these data were recorded in the same set of experiments. (B) ATP contents of D (○) and ND (●) seed axes during imbibition. (C) Water content of D (○) and ND (●) seed axes during imbibition.

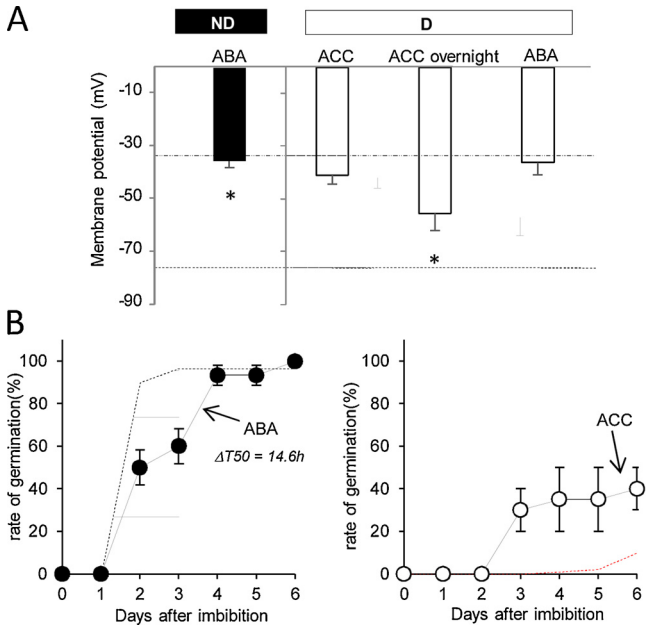


**Fig. 5. Role of hormones in the polarization of D and ND seed cells. (A)** The ND seed cells appeared depolarized when imbibed in the presence of 20 µM µM abscisic acid (ABA), a plant hormone known to maintain dormant state, while D seed cells are hyperpolarized as compared to non-treated D seed cells, especially after overnight treatment with 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene known to alleviate dormancy. Mean values are of at least ten cells, and bars represent the standard error. Dashed lines with points and without points refer to the means values from Fig. 1B for instantaneous polarization for D cells and ND cells respectively. **(B)** Imbibition in the presence of ABA slows the germination of ND seeds, whereas imbibition in the presence of ACC accelerates the germination of D seeds. Dashed lines refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf Fig. 1A). All these data were recorded in the same set of experiments. *Significantly different from non-treated seeds.

pretreated 30 min before voltage recordings with 1 mM ACC show only a weak effect on the polarization of cells, but overnight pretreatments with 1 mM ACC allow for the hyperpolarization of these cells (Fig. 5A). These data show that ABA could maintain a low cell polarization, while in contrast, ET could participate in the hyperpolarization of these cells, further suggesting that hormones could participate in the dormancy process by regulating the polarization of seed axis cells. We further confirmed the known impact of ABA on the rate of germination of ND seeds. As shown in Fig. 4B (left), imbibition in presence of 20 µM ABA slows the germination of ND seeds, the T50 being delayed for more than 14 h. On the contrary, as expected from previous data [39], imbibition in presence of 1 mM ACC accelerates the germination of D seeds (Fig. 5B, right).

### 3.3. Roles of reactive oxygen species (ROS) in the polarization of D and ND seed axis cells

Reactive oxygen species have also been described as important actors in seed dormancy [1,30], possibly through the activation of NADPH-oxidases or peroxidases [40,41]. They are also known to regulate ion transport systems and interact with hormone pathways [42,43]. Thus we checked if ROS scavengers namely TIRON, a scavenger of anion superoxide, and DABCO, a scavenger of singlet oxygen, and inhibitors of NADPH-oxidases and peroxidases, diphenyleneiodonium (DPI) and salicylhydroxamic acid (SHAM), respectively, could regulate the polarization of D and ND seed axis cells. The average values of $V_m$ recorded on cells from ND seed axes pretreated 30 min before voltage recordings with 5 mM DABCO show that it does not depolarize these cells, while TIRON (5 mM), DPI (50 µM) and SHAM (10 µM) depolarize the cells from ND seed axis to a value close to that of cells from D seed axes (Fig. 6A). We also checked for the putative role of methylviologen (MV), known to produce ROS and alleviate dormancy [1,30], on the polarization of cells from D seed axes. The average values of $V_m$ recorded on these cells pretreated 30 min before voltage recordings with 100 µM MV indicate the hyperpolarization of these cells (Fig. 6A). These data show that down regulation of ROS levels could maintain a low cell polarization while inversely, ROS generation could participate in the hyperpolarization of these cells. We further confirmed

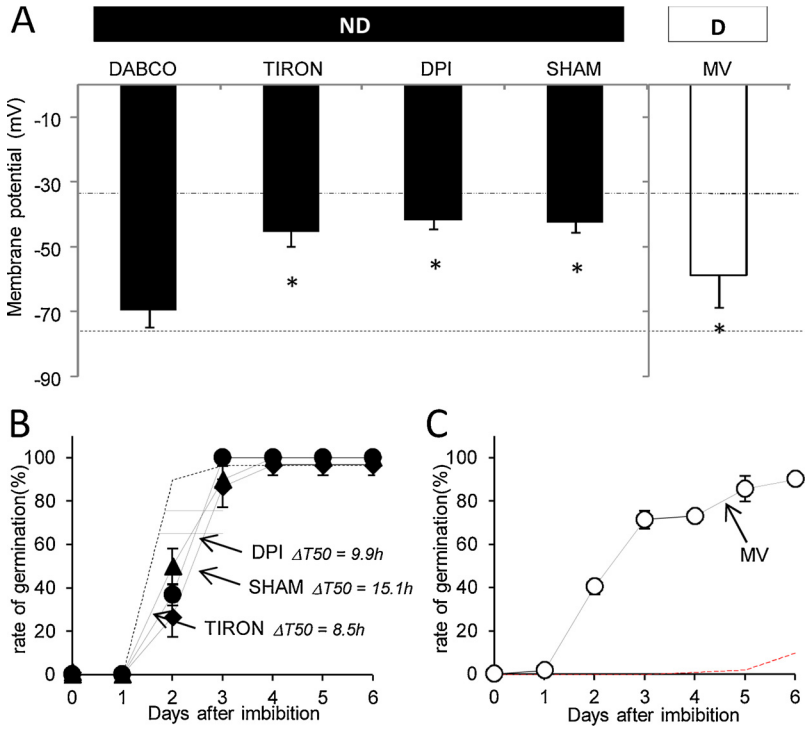


**Fig. 6. Role of reactive oxygen species (ROS) in the polarization of D and ND seed cells. (A)** The ND seed cells appeared depolarized when imbibed in the presence of 5 mM Tiron, a scavenger of anion superoxide, but also in the presence of 50 µM diphenyleneiodonium (DPI) and 10 µM salicylhydroxamic acid (SHAM), inhibitors of NADPH-oxidase and peroxidase, respectively, while 5 mM DABCO (a scavenger of singlet oxygen) did not depolarize these cells. D seed cells were hyperpolarized when compared to non-treated D seed cells after overnight treatment with 100 µM methylviologen (MV), known to produce ROS and alleviate dormancy. Mean values are of at least ten cells and bars represent the standard error. Dashed lines with points and without points refer to the means values from Fig. 1B for instantaneous polarization for D cells and ND cells respectively. **(B)** Imbibition in the presence of Tiron, DPI and SHAM slow the germination of ND seeds, while imbibition in the presence of MV **(C)** accelerates the germination of D seeds. Dashed lines refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf Fig. 1A). All these data were recorded in the same set of experiments. *Significantly different from non-treated seeds.

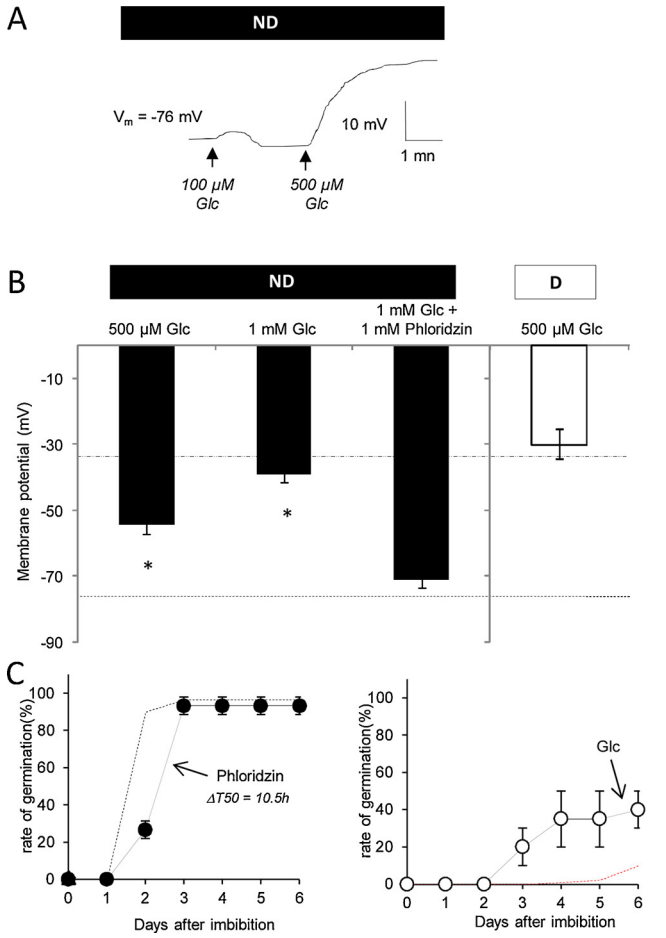


**Fig. 7. ND cell hyperpolarization allows for sugar influx through $H^+$/sugar symporter. (A)** Addition of 500 µM glucose (Glc) induced the depolarization of ND seed cells. **(B)** Imbibition in presence of 500 µM or 1 mM glucose allowed the depolarization of ND seed cells when compared to untreated ND seed cells, while co-imbibition with glucose and phlorizin, a sugar $H^+$/Glc inhibitor, prevented sugar-induced depolarization. Imbibition in the presence of 500 µM glucose did not permit the depolarization of D seed cells when compared to untreated D seed cells. Mean values are of at least ten cells and bars represent the standard errors. Dashed lines with points and without points refer to the means values from Fig. 1D for instantaneous polarization for D cells and ND cells, respectively. **(C)** Imbibition in the presence of 1 mM phlorizin slows the germination of ND seeds, while imbibition in the presence of 500 µM glucose accelerates the germination of D seeds. Dashed lines refer to ND seed germination rate for the left panel and D seed germination rate for the right panel (cf Fig. 1A). All these data were recorded in the same set of experiments. *Significantly different from non-treated seeds.

the known impact of ROS treatments on the rate of germination of ND seeds. As shown in Fig. 5B, imbibition in presence of 5 mM TIRON, 50 µM DPI or 10 µM SHAM slow the germination of ND seeds, the T50 being delayed for more than 8, 9 or 15 h, respectively. In contrast, as expected from previous data, imbibition in presence of 100 µM MV accelerates the germination of D seeds (Fig. 6C). This further suggests that the regulation of seed axis cell polarization by ROS levels could participate in the dormancy process.

### *3.4. A role for seed axis cell polarization in sugar influx?*

The alleviation of dormancy is thought to require an active metabolism fueled by sugar. The PM $H^+$-ATPase energizes the PM by extruding protons from the cell and generating a membrane potential [28,44], which is a prerequisite for secondary transport of various solutes comprising hexoses [45,29]. Hexoses are thus able to cross the PM through $H^+$/hexose symporters due to the $H^+$ gradient, the entry of $H^+$ with sugar inducing a depolarization of the PM [25,45]. We recently showed in a metabolomic study [9] that the amount of glucose and fructose was increase in ND as compared to D sunflower seed axis. We thus checked for the effect of addition of glucose on polarization of cells from D and ND seed axes. Fig. 7A illustrates a profile recorded in a cell from a ND seed axis upon addition of 500 µM glucose (Glc) which rapidly depolarizes the PM. The averages values of $V_m$ recorded on cells of ND seed axis pretreated 30 min before voltage recordings with 500 µM or 1 mM Glc (Fig. 7B) show that Glc could depolarize the cells from ND seed axis in a dose dependent manner. The addition of 1 mM phlorizin, a competitive inhibitor of $Na^+$/Glc cotransporter in animal cells [46] and $H^+$/Glc symporter in plants [25], during the pretreatment with 1 mM Glc prevents the depolarization of the cells from ND seed axis (Fig. 7B). D seed axis cells pretreated with 500 µM Glc were not depolarized (Fig. 7B). These data strongly suggest that in cells from ND seed axis Glc could be loaded through $H^+$/Glc symporter. Thus we checked for the impact of phlorizin on the rate of germination of ND seeds. As shown in Fig. 7C (left), 1 mM phlorizin slows the germination of ND seeds, the T50 being delayed for more than 10 h. On the contrary, imbibition in the presence of 500 µM Glc accelerates the germination of D seeds (Fig. 7C, right).

## 4. Discussion

In the present work, we took advantage of the relatively large size of sunflower seeds to perform electrophysiological recordings with microelectrodes and investigate cell polarization of D and ND seeds as an *in vivo* marker of $H^+$-ATPase activity, a well-known enzyme responsible for cell energization [28,29]. In accordance with our hypothesis that the energization of the PM could be required in dormancy alleviation and subsequent germination we found that the embryonic axis cell polarizations were different between D and ND seeds, with ND seeds showing more hyperpolarized cells. Using an inhibitor of PM $H^+$-ATPase (VAN), we could further show that ND seed cell polarization was reduced as previously observed in other plant cells [25,28,47]. Vandate was ineffective in D seeds, supporting the argument for an absence of active PM $H^+$-ATPases. The addition of FC, a toxin known to activate the PM $H^+$-ATPase by promoting its binding with 14-3-3 protein [28,29], allowed for observations of D seed cell hyperpolarization. This toxin is known to activate PM $H^+$-ATPase at the post-translational level [48] suggesting that PM $H^+$-ATPase proteins were present in the PM of D seeds but that their activity was down regulated. ATP availability was not responsible for the lack of PM $H^+$-ATPase activity in D seeds since no difference in ATP levels was found between D and ND seed axes. As a whole, these data suggest that the regulation of PM $H^+$-ATPase activity could be responsible for the ND cell polarization in accordance with the known role of PM $H^+$-ATPase in hyperpolarization of the PM through the extrusion of protons from the cell leading to their energization [28,29,44]. The fact that VAN could delay the germination of ND seeds and that FC activates the germination of D seeds further argues for the involvement of PM $H^+$-ATPase activity in dormancy alleviation. These data correlate with the decrease in $H^+$-ATPase protein concentration in PM during seed aging, which is related to slow germination in maize [49].

We also showed that the energization of the PM likely allows the influx of hexoses through the functioning of symporters using the $H^+$ gradient (proton motive force). Effectively, the Glc-induced depolarization of ND seed cells could not be due to the uncharged sugar influx but could be caused by the coupled influx of $H^+$ with Glc through a symporter [25,45]. This data is supported by the absence of depolarization in the presence of phlorizin, a compound known to block $H^+$/Glc symporters [25] and the absence of depolarization in D seed cells upon addition of Glc, with no favorable $H^+$ gradient being available for the symporter functioning due to the inactivity of PM $H^+$-ATPase in D seeds. Phlorizin could delay the germination of ND seeds suggesting a

role for sugar uptake in dormancy alleviation. These data are also in line with the increase in Glc and fructose contents that we have recently reported for sunflower ND embryonic axes relative to D axes [9]. It's worth noting that among the 86 metabolites detected in sunflower seeds, only the amounts of Glc, fructose and xylose were increased in ND axes [9]. The proton motive force generated by PM $H^+$-ATPase activity could allow influx not only of sugars but also of various solutes such as $K^+$ that are necessary for the cell especially during growth processes such as germination. We have previously shown that putative sugar and $K^+$ transporters transcripts are present in D and ND seeds [9].

The rapid stimulation of PM $H^+$-ATPases by FC on D seed cells suggests that the difference of polarization of the D and ND seed cells could be due to post-transcriptional or post-translational regulation of PM $H^+$-ATPase rather than transcriptional regulation. These data are in accordance with our recent transcriptomic study [9] that showed, the same transcript levels for AHA5-like gene, a homolog of *A. thaliana* AHA genes coding PM $H^+$-ATPases [29], and the absence of effect of ABA on AHA5-like transcript level in both ND and D sunflower seed axes. However, the delayed effect of ACC that was observed in D seed cell polarization suggests an additional transcriptional regulation of the gene coding PM $H^+$-ATPases in accordance with the ET-induced increase in AHA5-like gene transcription by > 4 fold in D sunflower seeds axis [9]. ABA, known to favor dormancy, demonstrates a non-transcriptional mechanism resulting in inhibition of PM $H^+$-ATPase in the guard cell model [22]. Interestingly, in ND sunflower seed cells we observed a depolarization after pretreatment with ABA that suggests that ABA could participates in dormancy by blocking PM $H^+$-ATPases in accordance with what was described by Planes et al. [23] during root cell growth inhibition. The opposite effect of ET that allows alleviation of dormancy and hyperpolarization of D seed cells, probably through the stimulation of PM $H^+$-ATPase, as observed in other plant cells [25,27], further suggests that the control of PM $H^+$-ATPase could have a central role in dormancy regulation. This hypothesis is reinforced by our data on ROS, also known to participate in dormancy regulation. Effectively, MV allows D seed cell hyperpolarization suggesting a stimulation of PM $H^+$-ATPase as observed with FC. Inversely, the decrease of ROS levels by superoxide scavengers like TIRON, or by inhibitors of NADPH-oxidases and peroxidases known to generate superoxides, such as DPI or SHAM, depolarized the ND seed cells possibly through a decrease in PM $H^+$-ATPase activity. The effect of ROS on PM $H^+$-ATPases is not well known but may not be transcriptional in our model,as no change in the level of AHA5-like gene transcript was observed in MV treated D sunflower seeds [1]. However, the $H^+$ transport activity of AHAs could be regulated by the cytoplasmic redox state which is linked to ROS [50]. The effects of the ROS pharmacology on dormancy demonstrated MV-stimulated dormancy alleviation and germination delay by inhibitors of superoxide generation, confirming the link between the seed cell polarization and its ability to germinate reinforcing the hypothesis of a role in regulation of PM $H^+$-ATPase during dormancy alleviation.

The exact pathways involved in the regulation of the PM $H^+$-ATPases in seeds remain to be determined and none of the inhibitors used in our physiological studies could be claimed to be highly specific for PM $H^+$-ATPases. However, our *in vivo* electrophysiological data and germination tests on D and ND sunflower seeds with PM $H^+$-ATPases pharmacology are consistent with known roles of ABA, ET and ROS in dormancy control. They further highlight that a mechanism related to cell polarization though PM $H^+$-ATPase activity regulation could be operative and participate in energization of the cells, and thus lead to dormancy release in sunflower seeds.

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