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Activation and activity of plasma membrane H^+-ATPase: key events in germinating *Vicia faba* seeds

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Research Paper

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Abstract

The regulation of plasma membrane H^+-ATPase was considered in imbibing *Vicia faba* seeds, a distinctive feature of which is germination by cell elongation, whereas the mitotic activity starts later. The enzyme activation is known to precede germination because it provides H^+ ion efflux from the cytoplasm to cell walls which favours their modification and loosening, being the prerequisites of cell elongation commencement. The presence of an enzyme in imbibing embryo axes was confirmed immunochemically. H^+ ion efflux was recorded with a pH-meter as acidification of ambient solution by the embryonic axes for 5 min. The activation of the enzyme and its subsequent activity are regulated in different ways. Enzyme activation is hydration-driven, it starts when water content increases up to the threshold level of 55% (fresh weight basis). This value was confirmed by imbibition in the presence of the osmoticum polyethylene glycol 6000, at various osmotic potentials. The activation does not depend on indolylacetic or abscisic acid treatment. Hydration-triggered activation of the enzyme favours rapid seed germination and its correspondence to the soil water potential. Enzyme activity after its activation is inhibited by 60–70% by 10^{-5}–10^{-7} M abscisic acid, whereas indolylacetic acid exerted no effect. The regulation of plasma membrane H^+-ATPase activity is presumably accomplished by the interaction of the enzyme with 14-3-3 proteins and endogenous fusicoccin, present in imbibing axes.

Introduction

The implementation of the seed germination programme takes place in mature non-dormant seeds during imbibition. For successful reproduction, the germination tends to occur as rapidly as possible to agree with optimal environmental conditions, sufficient water supply among them. It is often the soil water content in spring that determines the rate of seed imbibition and, ultimately, the beginning of germination. Radicle emergence is the first visible manifestation of germination, resulting from elongation of embryonic axis cells.

According to our hypothesis, rapid germination is favoured by activation of H^+-ATPase, the enzyme located in the plasma membrane (PM) of embryonic axis cells and accomplishing H^+ ion efflux from the cytoplasm to the cell walls at the expense of ATP energy (Obroucheva, 2017). The mechanism of H^+ transfer has been studied in detail by Buch-Pedersen et al. (2009). The physiological role of this enzyme is at the very centre of plant physiology (Falhof et al., 2016), but its application to growth initiation in germinating seeds has largely escaped the attention of seed physiologists, perhaps because this high-molecular weight enzyme (almost 100 kDa) turned out to be beyond the standard size of proteomic plates.

A close relationship between enzyme activation and germination has been confirmed by experiments with *o*-vanadate, an inhibitor of the enzyme, and fusicoccin, a specific enzyme activator and stabilizer (Camoni et al., 2013), which inhibited or activated both H^+ ion efflux and radicle emergence, respectively (Obroucheva, 2017). The close correlation between H^+ efflux and germination was found in both orthodox and recalcitrant non-dormant seeds, as well as in freshly harvested and ageing seeds.

Extrusion of H^+ ions from imbibing embryonic axes occurs prior to radicle emergence (Obroucheva et al., 2013; Obroucheva, 2017). H^+ ions accumulated in cell walls induce their acidification and, hence, activation, not only of xyloglucan-endotransglycosylase/hydrolase, inducing partial degradation of xyloglucans, but also of expansins. Expansins facilitate the mobility of xyloglucan chains relative to cellulose microfibrils. Such acidification of cell walls provides the loosening of their structure and results in higher extensibility (Park and Cosgrove, 2015). Under the pressure of inflowing water, such cell walls expand and cell elongation in embryonic axes commences. This pathway is similar to the initiation of so-called acid-induced growth, a well-known growth mechanism operating in protruding coleoptiles or growing hypocotyls (Hager, 2003; Arsuffi and Braybrook, 2018).

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The aim of this paper was to analyse the activation of PM H^+-ATPase and its subsequent activity prior to the initiation of germination in imbibing *Vicia faba* seeds. These seeds were chosen because they start radicle protrusion only by elongation of embryo axis cells, whereas cell division begins later. This makes *Vicia faba* seeds a suitable model for studying the start of germination.

Materials and methods

Seed germination

Vicia faba seeds cv. Russkie chernye were purchased from Aelita (Moscow, Russia). Seeds were stored at room temperature and characterized by 80% germination. Seeds were sterilized with 5% sodium hypochlorite for 1 h, carefully washed for 3 h and dried. Seeds were germinated either in Petri dishes on filter paper wetted with distilled water, or in enamelled cuvettes covered with glasses, in which seeds were uniformly distributed within pleated filter paper, with their micropyle downward. Cuvettes contained 350 ml of distilled water or other solutions. Petri dishes and cuvettes with seeds were incubated in the dark at 27°C. For the estimation of radicle emergence rate, the number of emerged seeds was recorded every 2 h.

Measurement of water content

Embryonic axes were excised and weighed before drying, then dried in an oven for 1 h at 105°C and then at 80°C to constant weight. Water content (WC) on a fresh weight basis was estimated in triplicate, 20 embryo axes per sample, and expressed as % fr.wt.

Treatment with polyethylene glycol

Seeds were incubated in PEG 6000 (Panreac, Spain) solutions of various concentrations in order to fix the WC of the embryonic axes, at which PM H^+-ATPase starts functioning. Three concentrations of PEG were used, namely 20, 25 and 30%. The experiments with PEG were done in triplicate.

Microscopic observations

Initiation of cell division in embryonic axes was evaluated on longitudinal sections after fixation of the imbibing seeds in Brodsky fixative (ethanol:formalin:glacial acetic acid = 3:10:1) and a standard protocol of impregnation of axes by paraffin. Location of the first mitotic figures was observed under a light microscope in cortical cells of radicle tips after the preparations were dyed with Schiff solution, a reagent for DNA staining. Eight mitotic figures in the field of vision clearly indicated the initiation of cell proliferation.

Measurement of *PM* H^+-ATPase activity

PM H^+-ATPase activity was measured by H^+-ion efflux from embryonic axes. A sample of 40 excised embryonic axes was incubated at first 5 min in 10^{-4} M $CaCl_2$ for membrane stabilization, transferred to 10^{-3} M KCl for saturation with K^+ ions and placed into the cell of a pH-meter (pH-410 Akvilon, Russia) containing 10^{-4} M KCl. The pH inside the cell of the pH-meter was about 6.2–6.3 which corresponded to the enzyme activity optimum. The pH decrease was recorded for 10 min. The H^+ ion efflux was expressed as $-\Delta$pH/min/40 embryonic axes.

Protein content

Protein content was determined with the Bicinchoninic acid protein assay kit (Sigma-Aldrich, USA).

Isolation of microsomes

Excised embryonic axes (5 g) were homogenized in 300 ml of 300 mM sucrose, 10 mM EDTA, 5 mM potassium *m*-bisulfite, 5 mM dithiothreitol, 5 mM PMSF, 0.6% polyvinylpyrrolidone and 100 mM Tris-HCl, pH 8.0. The homogenate was filtered through two layers of Miracloth (Calbiochem, USA) and centrifuged at 10,000*g* for 15 min. The supernatant was then centrifuged at 100,000*g* for 1 h. The resulting microsomal pellet was resuspended in 300 mM sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM bisTris propane-Mes pH 7.2 and stored at −70°C.

Measurement of PM H^+-ATPase activity

PM H^+-ATPase activity was measured by ATP hydrolysis. The activity of *o*-vanadate-sensitive PM H^+-ATPase was measured by the increase of inorganic phosphate in microsomal fractions from embryonic axes (Janicka-Russak et al., 2012). The reaction mixture contained 50 µg of protein, 33 mM Tris-Mes buffer pH 7.5, 3 mM ATP, 2.5 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 0.1 mM Na_2MoO_4, 50 mM $NaNO_3$, 200 µM Na_3VO_4 and 0.02% Triton X-100. Reaction time was 20 min. Enzyme activity was measured as the difference between −vanadate and +vanadate tests and expressed as µmol P_i/mg protein/h. P_i was measured according to the standard procedure.

Immunodetection of PM H^+-ATPase and 14-3-3 proteins

Microsomal proteins were separated by SDS-PAGE electrophoresis in a 9% gel for identification of the enzyme and in a 12.5% gel for 14-3-3 proteins. Then, proteins were transferred to nitrocellulose membrane Hybond C-extra (Sigma, USA) for western-blot analysis according to Towbin et al. (1979) with the addition of 0.05% sodium dodecyl sulphate and 10% methanol to the buffer. The membrane was incubated in phosphate-buffered saline supplemented with 3% gelatin and 0.05% Tween 20 for blocking non-specific binding, then overnight with antibodies against plasmalemma H^+-ATPase (Agrisera, Sweden) or against 14-3-3 protein (K-19, Santa Cruz Biotechnology, USA). Protein bands were probed with horse-radish peroxidase-labelled anti-rabbit antibodies (Promega, USA) in phosphate-buffered saline. Cross-reactivity was visualized by peroxidase colour reaction with 1-chloro-4-naphthol and hydrogen peroxide.

Statistical analysis

Data are expressed as means ± SE, which were calculated in all treatments. The main measurements of acidifying capacity of embryonic axes were carried out 20 times or more, whereas other experiments were done in triplicate.

Results and discussion

Vicia faba beans belong to the class of orthodox seeds, that is, can be stored dry and rapidly absorb available water to complete germination. Figure 1 shows the time course of the main physiological events in imbibing seeds as related to WC of the

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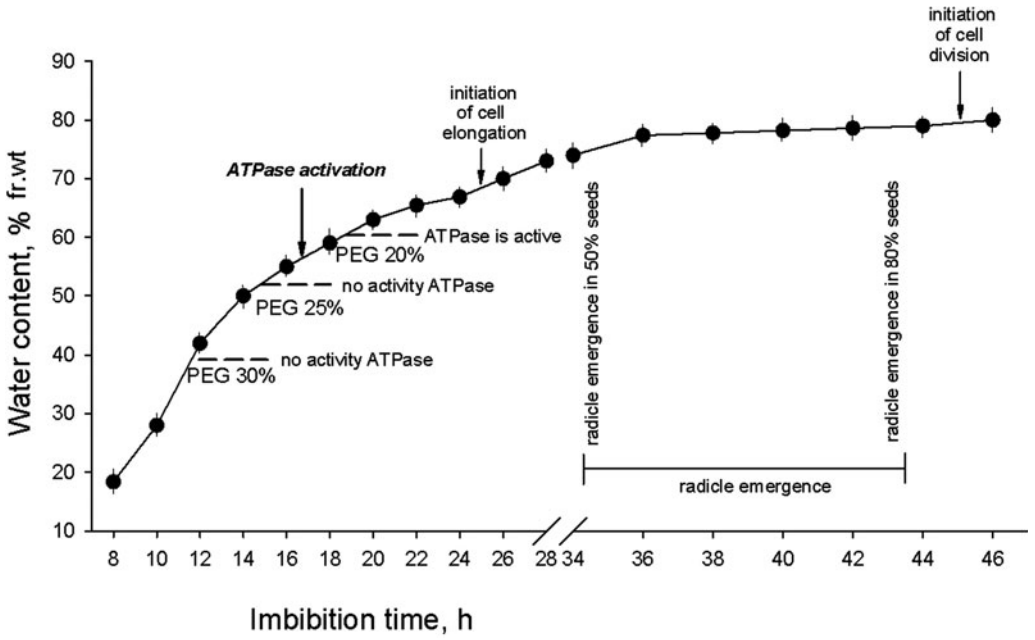

Fig. 1. Activation of PM H^+-ATPase in embryo axes of imbibing *Vicia faba* seeds. Dependence of enzyme activity in embryonic axes on their water content, as related to growth initiation. Activation of the enzyme occurred prior to growth commencement, at a water content between 50 and 60%, on average at 55% (arrow). Three concentrations of PEG were applied to control this value. Points of water uptake after incubation in PEGs are shown by dashed lines, and activation of enzyme or its absence is indicated. Cell elongation was evaluated by the rate of radicle emergence, which started at 25 h after imbibition (long arrow), and ended at 43–44 h, when all viable seeds showed radicle protrusion. Initiation of cell division (small arrows) was recorded by the appearance of mitotic figures after DNA staining of embryo axis sections with Schiff reagent.

embryonic axes. The WC curve displays the three typical phases: slow water uptake (Phase I for 19 h, WC range of 10–62%), more rapid water inflow (Phase II, 19–25 h, WC range of 62–70%) and Phase III characterized by active water uptake due to the cell elongation initiated in embryonic axes after 25 h of imbibition. Cell elongation starts in embryonic axes much earlier than cell division, which only begins after radicle protrusion.

The WC dynamics was compared with the capacity of embryonic axes to acidify the ambient solution, that is, with the activity of PM H^+-ATPase (Fig. 2A). PM H^+-ATPase activity sharply appeared in the embryo axes imbibed for 18 h. We tested also the response of PM H^+-ATPase in embryonic axes to fusicoccin (Fig. 2B), a specific activator and stabilizer of this enzyme (Camoni et al., 2013). Fusicoccin exerted a pronounced stimulating effect on H^+ efflux. Thus, embryonic axes contain typical PM H^+-ATPase. This enzyme belongs to the P-type ATPases as its activity is inhibited by *o*-vanadate, imitating phosphate analogue in the ATP hydrolysis cycle (Fig. 3).

For convenience, we indicated on the WC curve the threshold of enzyme activation by an arrow (Fig. 1) at 18 h imbibition and WC of 55%. To be sure that activation occurred at this WC, a series of treatments with the osmoticum PEG-6000 at various concentrations was performed. 30% PEG permitted the axes to imbibe to 39 ± 1%, in 25% PEG embryonic axes had a WC of 50 ± 1.5%, and at 20% PEG, WC was held at of 60 ± 2%. After incubation in 30% PEG, WC in embryonic axes was only 38%, and no PM H^+-ATPase activity was recorded (Fig. 1). The same situation was observed with 25% PEG, which confined 50% WC in embryonic axes without any effect on H^+ extrusion. Only at 20% PEG maintaining 60% WC in embryonic axes, the activation of PM H^+-ATPase was demonstrated. These data confirm that PM H^+-ATPase is activated by inflowing water when WC in imbibing axes reached 55%. The activation events occurred prior to cell elongation commencement.

PM H^+-ATPase has been identified in developing Arabidopsis and wheat seeds (Harper et al., 1994; Rober-Kleber et al., 2003). Apparently, the enzyme is stored during seed maturation because, in our experiments, embryonic axes incubated in an α-amanitin solution (1 μg ml^{-1}) are capable to germinate and extrude H^+ ions; this means that H^+ ion efflux is related to stored mRNAs of PM H^+-ATPase. In maize embryos (Sanchez-Nieto et al., 2011), it was shown that after 5 h of imbibition, the enzyme differs from that in dry seeds by a number of biochemical properties, including the sensitivity to *o*-vanadate, trypsin and denaturing temperatures. These observations are in agreement with the conclusion about hydration-driven activation of the enzyme, but 5 h of imbibition provides a too low hydration for activation of PM H^+-ATPase.

In imbibing embryonic axes from bean seeds, the presence of PM H^+-ATPase protein was confirmed immunochemically with a specific antibody. Figure 4 shows the bands of this enzyme during imbibition. Concurrently, the presence of 14-3-3 protein was demonstrated. These data showed the presence of the enzyme in embryonic axes capable to be activated at 55% FW during imbibition.

The following explanation of the role of high water content in PM H^+-ATPase activation can be put forward. This high-molecular weight intrinsic protein has a tertiary structure characterized by the interaction of functional domains and conformational changes within them leading to protein functioning. For successful H^+ transport from the cytoplasm to the apoplast, the movement across the hydrophobic plasma membrane is a critical event (Buch-Pedersen et al., 2009). H^+ enters the PM as a hydrated molecule and moves along a 'protein wire', a chain of

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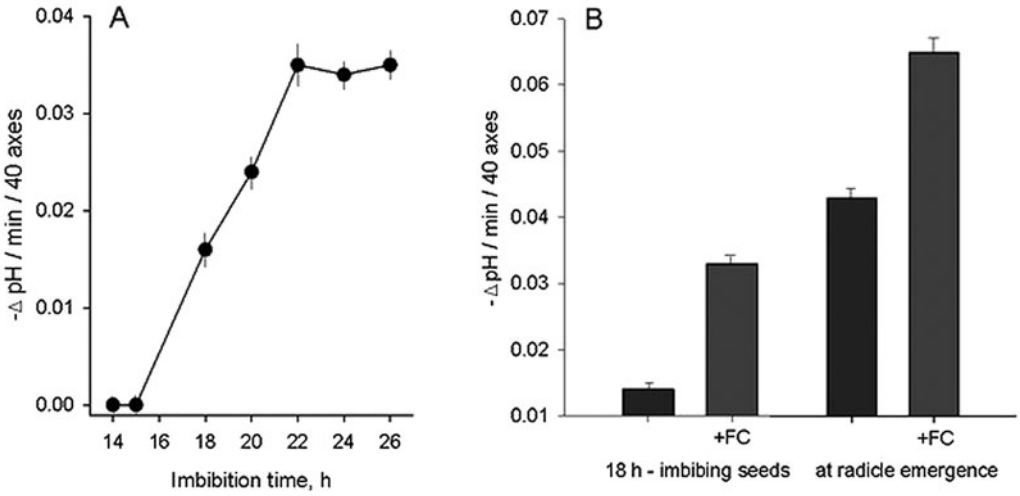

Fig. 2. (A) Activity of PM H^+-ATPase in embryonic axes of imbibing *Vicia faba* seeds. (B) Effect of 10^{-6} M fusicoccin on PM H^+-ATPase activity.

charged water molecules, jumping from one molecule to another towards Asp684, its acceptor/donor (Buch-Pedersen and Palmgren, 2003; Guerra and Bondar, 2015). Asp684 is located near the central water cavity of 80 A^3 volume filled with 10–16 water molecules; it represents a unique structure specific for H^+-ATPase absent in other ATPases of the P-type (Buch-Pedersen et al., 2009). Due to conformational changes of Asp486 and Arg655 positioned at the opposite sites of water cavity, H^+ enters the water cavity, which then changes its configuration and opens at the extracellular site of the PM. Such H^+ pathway from hydrophilic cytoplasmic domains through a hydrophobic transmembrane domain needs high water content and conformational transitions coupled to ATP hydrolysis.

To check the role of phosphorylation, we measured its rate by Pi accumulation (Fig. 5). It increased at enzyme activation at 18 h and is even more active later, when the activity of PM H^+-ATPase rises. Therefore, the enzyme hydration-driven activation is accompanied by activation of ATP hydrolysis. This conclusion was confirmed by the action of *o*-vanadate on the capacity of embryonic axes to extrude H^+ ions (Fig. 3) because this inhibitor interferes with the hydrolysis of ATP by substitution for phosphate.

Hydration-activated enzyme acquires its proper confirmation and is not in short of substrate delivery. After enzyme activation, H^+ efflux continues, supported by phosphorylation of penultimate threonine or some other amino acid residues located in the regulatory domain of the enzyme molecule (Falhof et al., 2016). 14-3-3 protein is an activator of phosphorylation and fusicoccin is its stabilizer (Camoni et al., 2013). Along with this regulatory mechanism, a phosphorylation-independent pathway can operate via 14-3-3 binding to the phosphoprotein (Borch et al., 2002; Fuglsang et al., 2003). In the case of *Vicia faba*, the regulation of enzyme activity after its hydration-driven activation is performed in the regulatory domain via its interaction with 14-3-3 protein and endogenous fusicoccin. We have shown that 14-3-3 proteins (Fig. 4) are concurrently present with the enzyme in imbibing embryonic axes of *Vicia faba*; furthermore, the endogenous fusicoccin ligands were previously discovered in *Vicia faba minor* embryonic axes (Antipova et al., 2003).

Rapid activation of PM H^+-ATPase is one of a series of water-triggered metabolic systems in imbibing embryonic axes. Previously, threshold WC levels were demonstrated for activation of glycolysis, TCA cycle and amino acid metabolism (20–23%), for activation of respiration rate due to completion of mitochondriogenesis (45%), for proteolysis in protein-storing vacuoles (51–55%) and amylolysis in amyloplasts (45%), for initiation of ribosomal protein synthesis (52–55%) and gene transcription (42–47%) (Obroucheva, 2012). As a result, successive activation of main metabolic systems abundant in dry seeds occurs in embryonic axes of orthodox seeds due to the imbibition up to threshold WC levels. The importance of such activation of protein synthesis has been confirmed by Galland et al. (2014) with imbibing Arabidopsis seeds incubated in labelled methionine solutions. They identified a bulk of non-labelled (not *de novo* synthesized) proteins mainly during the first 8 h of imbibition. Apparently, these proteins were synthesized from amino acids produced by amino acid metabolism activated at low WC.

It is worth noting some specific features of such hydration-driven activation. In orthodox seeds, it occurs automatically, because a level of 60% WC is achieved even in dead seeds, which absorb water as any capillary-porous body containing a high number of hydrophilic sites (Obroucheva et al., 2013). In viable seeds, *de novo* gene transcription can start actually at WC exceeding 60% and is supported by activated translation. Hydration-driven activation of existing main metabolic systems in combination with gene transcription at high WC can be considered as a tool providing rapid seed germination and its correlation with available water.

Investigation of enzyme activation raises the question of discrimination between the terms 'enzyme activation' and 'enzyme activity'. After hydration-driven activation, PM H^+-ATPase continues to be active up to radicle emergence and including it, remains high after radicle emergence, during further cell elongation growth. Its function is the maintenance of acid growth in seedling organs. The importance of PM H^+-ATPase is not limited to growth initiation at radicle emergence, that is, to germination, but it continues to maintain acid growth in the growing seedling. It can be exemplified by hypocotyl elongation in Arabidopsis (Takahashi et al., 2012; Hayashi et al., 2014), by coleoptile growth in maize seedlings (Rudashevskaya et al., 2005) and by roots of Arabidopsis (Planes et al., 2015).

As the activity of PM H^+-ATPase can be stimulated by auxin (Takahashi et al., 2012; Arsuffi and Braybrook, 2018) and

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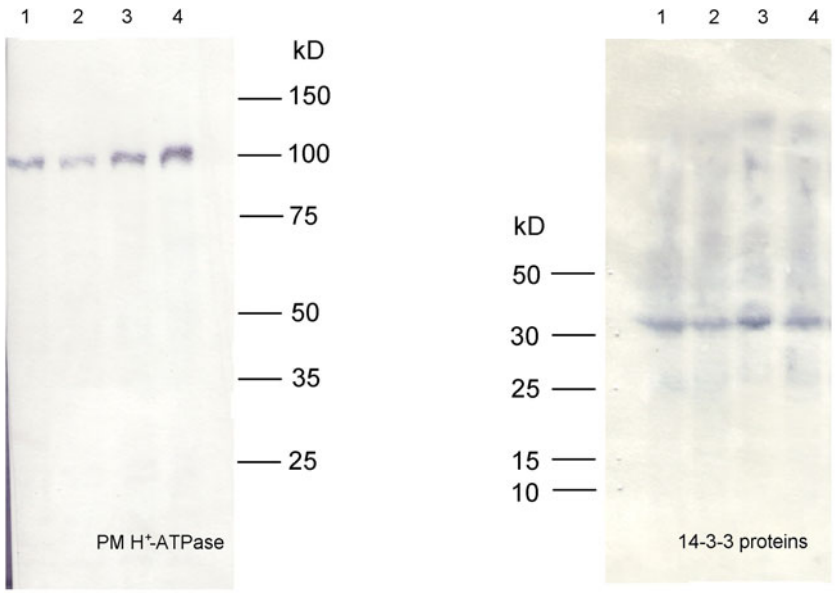

Fig. 3. Identification of PM H^+-ATPase and 14-3-3 proteins in embryonic axes of *Vicia faba* seeds. 1: before 18 h imbibition; 2: after 18 h imbibition; 3: at radicle emergence; 4: after radicle emergence.

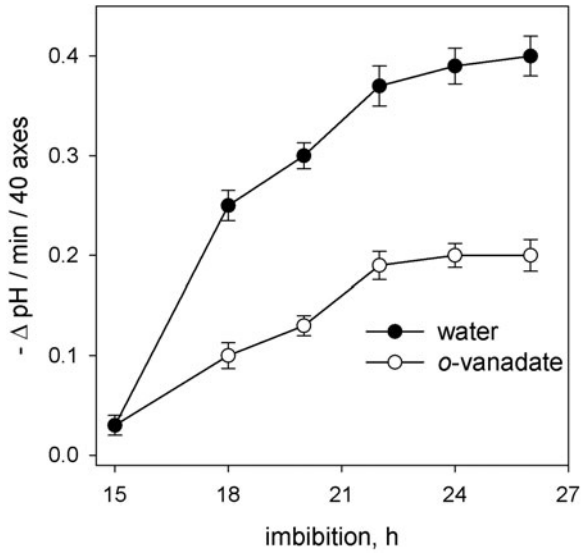

Fig. 4. Effect of *o*-vanadate on H^+ ion efflux from embryonic axes of *Vicia faba* seeds.

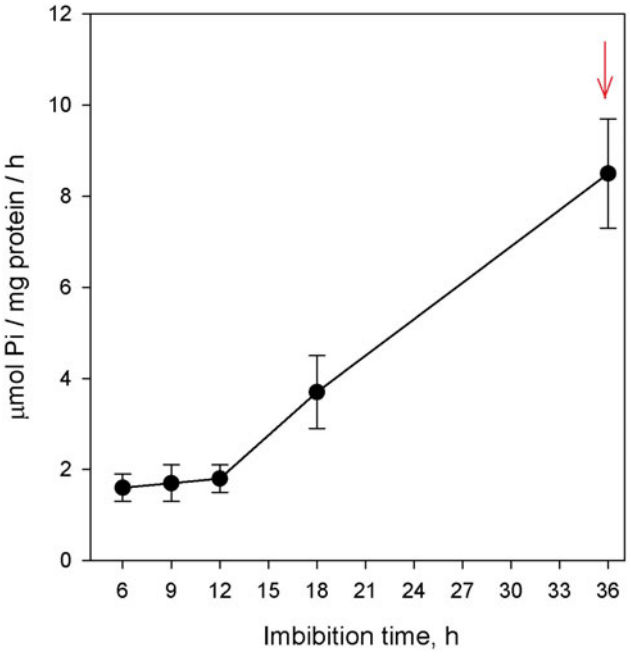

Fig. 5. H^+-ATPase activity measured by ATP hydrolysis in imbibing embryonic axes of *Vicia faba*. The experiments were done in triplicates. Activation time 18 h, time of radicle protrusion 36 h. Arrow indicates germination.

inhibited by abscisic acid (Planes et al., 2015), the next question was to clarify whether the target of ABA and auxin is the activation of the enzyme or the activity of the enzyme after its activation. For this purpose, in imbibing *Vicia faba* embryonic axes (Fig. 6A,C) the effects of IAA and ABA were estimated at a WC of 52–54%, that is, slightly below the WC threshold for enzyme activation, and at a WC of 63–65%, when the enzyme is already active. Auxin did not significantly stimulate either the activation or activity of PM H^+-ATPase within the concentration range of 10^{-5}–10^{-7} M, which corresponds to the auxin-induced doubling of acid growth in elongating hypocotyls (Takahashi et al., 2012). In our experiments, IAA did not promote the elongation of embryonic axes as followed from their fresh weight data. Germination of *Vicia faba* seeds and activity of PM H^+-ATPase just after germination appear to be auxin-independent which may be due to the inability of embryonic axes to accept exogenous auxin because mature *Vicia faba* seeds lack endogenous auxins and do not synthesize them after radicle emergence (Plesse et al., 1984; Sztein et al., 2002).

Similar experiments were carried out with ABA (Duchefa Biochemie, The Netherlands) at concentrations of 10^{-5}–10^{-7} M (Fig. 6). At a WC below the threshold level, enzyme activation and growth were inhibited at 10^{-5} M ABA, but no inhibition was recorded at lower concentrations (Fig. 6A). At early imbibition, we must take into account the active accumulation of transcripts of 8′-hydroxylase ABA, the enzyme responsible for ABA degradation (Kimura and Numbara et al., 2010). As a result, the level of endogenous ABA drops to 10^{-8}–10^{-9} M and remains low. In the presence of toxic 10^{-5} M exogenous ABA, synthesized

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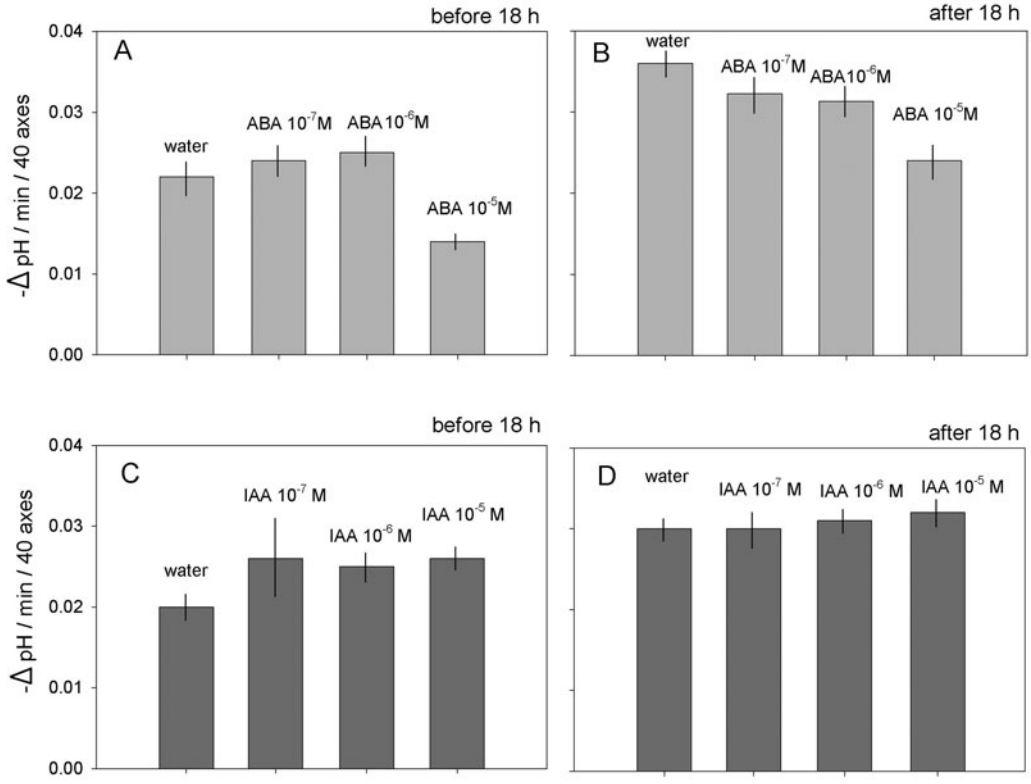

Fig. 6. Effect of ABA and IAA on activation and activity of PM H^+-ATPase in embryo axes of imbibing *Vicia faba* seeds. (A,C) Before activation of PM H^+-ATPase and (B,D) after activation of PM H^+-ATPase. (A,B) Effect of ABA and (C,D) effect of IAA.

8′-hydroxylase ABA apparently cannot minimize the inhibitory action of ABA on growth and activation of PM H^+-ATPase. At lower 10^{-6}–10^{-7} M ABA, 8′-hydroxylase ABA could destroy it, counteracting the ABA inhibitory effect on H^+ efflux. Such an approach permits to conclude that 10^{-6} and 10^{-7} M ABA did not inhibit the activation of PM H^+-ATPase.

Already activated enzyme (Fig. 6B) was partially inhibited at all ABA concentrations. Such inhibition occurs against a background of residual ABA 8′-hydrolase synthesis, which could not counteract the inhibitory action of ABA on the activity of H^+-ATPase and germination. These data correspond to ABA-induced growth inhibition (Hayashi et al., 2014; Planes et al., 2015) observed in seedling organs at high WC.

To conclude, auxin did not stimulate activation and activity after activation of PM H^+-ATPase, whereas ABA inhibited only the enzyme activity after its activation, which seems to be the target of hormonal regulation of this enzyme.

It is quite possible that the regulation of PM H^+-ATPase in dormant seeds is more complicated. In horse chestnut seeds, which are characterized by a long deep physiological dormancy during winter, the enzyme activity was recorded during the whole dormant period and dormancy release. These seeds, like other recalcitrant seeds, have high WC, embryonic axes in dormant seeds have a WC of 62%, and germination needs a WC of 72% (Obroucheva et al., 2016). Apparently, the enzyme is already activated. However, the activity changed along the course of dormancy: the first 5 weeks, that is, during embryo dormancy, the activity is weak and not sensitive to fusicoccin, but during the following 10 weeks (coat-imposed dormancy) it increased and was stimulated by fusicoccin (Obroucheva et al., 2018). It is tempting to speculate that in dormant seeds the enzyme is at first in an autoinhibited state (Portillo, 2000). In sunflower embryos at low WC (10%), the measurement of membrane potential in the radicle epidermis of dormant and non-dormant seeds has shown that in dormant seeds, the enzyme was not active although it was stimulated by fusicoccin (De Bont et al., 2019). The authors assumed that in dormant seeds, the enzyme was inhibited by ABA and that dormancy release is related to ethylene and ROS compounds. Therefore, the state of the enzyme and its regulation in dormant seeds needs further investigation.

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