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ORIGINAL ARTICLE

Congruence between PM H^+-ATPase and NADPH oxidase during root growth: a necessary probability

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Abstract

Plasma membrane (PM) H^+-ATPase and NADPH oxidase (NOX) are two key enzymes responsible for cell wall relaxation during elongation growth through apoplastic acidification and production of $^{\bullet}OH$ radical via $O_2^{\bullet-}$, respectively. Our experiments revealed a putative feed-forward loop between these enzymes in growing roots of *Vigna radiata* (L.) Wilczek seedlings. Thus, NOX activity was found to be dependent on proton gradient generated across PM by H^+-ATPase as evident from pharmacological experiments using carbonyl cyanide m-chlorophenylhydrazone (CCCP; protonophore) and sodium ortho-vanadate (PM H^+-ATPase inhibitor). Conversely, H^+-ATPase activity retarded in response to different ROS scavengers [$CuCl_2$, N, N' –dimethylthiourea (DMTU) and catalase] and NOX inhibitors [$ZnCl_2$ and diphenyleneiodonium (DPI)], while H_2O_2 promoted PM H^+-ATPase activity at lower concentrations. Repressing effects of Ca^{+2} antagonists (La^{+3} and EGTA) on the activity of both the enzymes indicate its possible mediation. Since, unlike animal NOX, the plant versions do not possess proton channel activity, harmonized functioning of PM H^+-ATPase and NOX appears to be justified. Plasma membrane NADPH oxidase and H^+-ATPase are functionally synchronized and they work cooperatively to maintain the membrane electrical balance while mediating plant cell growth through wall relaxation.

Keywords Calcium · NADPH oxidase (NOX) · Plasma membrane (PM) · H^+-ATPase · Proton gradient · Root growth · ROS

Introduction

Growth, as required for successful thriving of plants, particularly for roots that explore for water and nutrients to support incessant growth (Nibau et al. 2008; Baluška et al. 2010), depends on rapid cell division and cell elongation. Major enlargement process is constituted by cell expansion which dwells on the delicate balance between relaxation/loosening of the wall polysaccharides and maintenance of turgor (Cosgrove 2000a, 2000b). While wall acidification induced enzymatic and expansin mediated cleavage of the bonds are known for long (McQueen-Mason and Cosgrove 1994), reactive oxygen species (ROS), specifically hydroxyl radical ($^{\bullet}OH$), have emerged later as a key agent for mediating non-enzymatic scission of the polysaccharides (Schopfer 2001; Liszkay et al. 2003).

The importance of ROS metabolism in plants becomes evident from the presence of a strikingly large number of potent sources for ROS generation throughout the plant body functioning in accordance to their sites of action (Kar 2015). Besides cell wall peroxidase, plant NADPH oxidases (NOXs) or respiratory burst oxidase homologs (RBOHs; homologs of gp91^{phox} subunit of mammalian phagocyte NOX complex (Torres et al. 1998)) are one of the candidate enzymes responsible for production of apoplastic ROS (Sagi and Fluhr 2006). The $O_2^{\bullet-}$ produced by NOX from reduction of O_2 with electrons coming from cytosolic NADPH oxidation (Fluhr 2009) are converted to suitable forms of ROS and take part in growth and development processes, e.g., H_2O_2 in cell wall stiffening (Schopfer 1996) or $^{\bullet}OH$ in

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cell wall loosening (Schopfer 2001; Liszkay et al. 2003; Airianah et al. 2016). On the other hand, in addition to other essential functions such as energization of the membrane, maintenance of cellular pH balance, promotion of cell wall relaxation by activating wall-loosening enzymes (in coordination with auxin) is also one of the primary activities of plasma membrane (PM) H^+-ATPase enzyme (Janicka-Russak 2011; Janicka-Russak et al. 2013). Upon receiving signals from different agents (i.e., auxin, blue light) PM H^+-ATPase is rescued from its low-activity state (incurred by C-terminal autoinhibitory domain (Speth et al. 2010)) by binding of 14-3-3 proteins preceded by phosphorylation of Thr947 (Hager 2003).

Nonetheless, cytoplasmic acidification and membrane depolarization would result from NOX activity if the charge imbalance (caused by electron transfer) is not stabilized readily (Ramsey et al. 2009). Although distinct mechanisms are reported in animal system for this purpose, e.g., built-in or separate voltage-gated proton channels (Maturana et al. 2002; Ramsey et al. 2009), no such machinery is known in plants. PM H^+-ATPase seems to be suitable in this regard which would compensate the charge by extruding excess protons from cytosol as well as provide them to the apoplastic enzymes for dismutation/conversion of $O_2^{\bullet-}$ to H_2O_2. Since both apoplastic $^{\bullet}OH$ production (in a series starting from $O_2^{\bullet-}$ production by NOX) and wall acidification (mediated by PM H^+-ATPase) are necessary for cell wall loosening leading to elongation growth, presence of a functional harmony between PM H^+-ATPase and NOX seems reasonable. However, reports available in this field are scanty and inconclusive leaving the question unanswered. Functional relationship between these two enzymes, if any, should include several linking factors and Ca^{+2}, an important second messenger (Batistič and Kudla 2012) seems to fit well. Over the last decade, importance of Ca^{+2} for NOX activity has been well documented (Sagi and Fluhr 2006; Ogasawara et al. 2008; Gilroy et al. 2014; Mendrinna and Persson 2015). However, the role of calcium (Ca^{+2}) in PM H^+-ATPase activity is still unresolved because of dispersed reports. Thus, cytosolic Ca^{+2} has been demonstrated to inhibit PM H^+-ATPase in both guard and mesophyll cells of *Vicia faba* (Kinoshita et al. 1995) and roots of *Zea mays* (De Nisi et al. 1999). On the contrary, increased $[Ca^{+2}]_{cyt}$ and H^+ extrusion was found to precede stomatal opening in *Paphiopedilum tonsum* (Irving et al. 1992) and IAA-induced membrane hyperpolarization was found to be inhibited by EGTA (Ca^{+2} chelator) and Verapamil (Ca^{+2} channel blocker) in *Petunia hybrida* (Voronkov et al. 2010). Reports on differential effects of Ca^{+2}-mediated phosphorylation on PM H^+-ATPase, i.e., enhancement or diminution of activity further complicates the situation (Janicka-Russak 2011; Morth et al. 2011).

In the present investigation, attempts have been made to recognize and comprehend the plausible functional synchronization between PM H^+-ATPase and NOX in growing roots of *Vigna radiata* (L.) Wilczek seedlings following tissue staining experiments, spectrophotometric analyses and *in gel* native polyacrylamide gel electrophoresis (PAGE) assays. The potent role of Ca^{+2} in mediating the harmony has also been examined by pharmacological experiments. A working model representing the probable relationship between PM H^+-ATPase and NOX has been proposed.

Materials and methods

Plant material Surface sterilized seeds of *V. radiata* (L.) Wilczek var. B1 were germinated for 12 h on moistened (with distilled water) Whatman No. 1 filter papers in Petri dishes. Germinated seeds were transferred to and incubated in test solutions for 48 h under darkness. Temperature for both germination and incubation was maintained at 30 ± 2 °C in a seed germinator. Root portions of the 48-h grown seedlings were used for experiments.

Superoxide ($O_2^{\bullet-}$) localization studies In vivo production of superoxide ($O_2^{\bullet-}$) in both control and treated samples were determined following Singh et al. (2014) with little modification. Intact roots were incubated in $O_2^{\bullet-}$-specific staining medium (consisting of Nitro blue tetrazolium chloride (NBT, 0.5 mM) dissolved in Na phosphate buffer (50 mM, pH 6.8)) for 30 min at 30 °C. Photographs were taken (with Canon Power Shot A640) after discontinuing the reaction by washing the samples thoroughly with distilled water.

Spectrophotometric estimation of apoplastic superoxide Apoplastic superoxide production was estimated by XTT reduction assay following Schopfer et al. (Schopfer et al. 2001) and Liszkay et al. (Liszkay et al. 2004) with some modifications. Excised roots (300 mg) were incubated in 1 mL K-phosphate buffer (20 mM, pH 6.0) containing XTT (500 μM). After dark incubation on a shaker for 45 min at room temperature, absorbance of the bathing medium was measured at 470 nm using a UV-Vis spectrophotometer (Systronics, India). The molar concentration of $O_2^{\bullet-}$ (calculated from A_{470} by using molar extinction co-efficient of 2.16×10^4 L mol^{-1} cm^{-1}) was estimated for individual treatments. Wound-induced superoxide was avoided by keeping the excised roots, at first, in distilled water for 10 min.

Spectrophotometric assay of NOX Spectrophotometric assay of NOX was carried out following Frahry and Schopfer (2001) with some modifications. Root tissue (300 mg) was homogenized in extraction medium comprising of Na-phosphate buffer (50 mM, pH 6.8) with 0.5% Triton X-100 and subjected to centrifugation at 10000 rpm for 15 min at 4 °C. Reaction mixture, comprised of 250 μL enzyme sample (20 μg protein), NADPH (final concentration 250 μM), and

XTT (final concentration 500 μM), was incubated at 37 °C for 15 min. Absorbance (at 470 nm) was taken at 0 and 15 min of incubation. Enzyme activity was calculated from the mean difference of absorbance at 15 and 0 min.

Spectrophotometric assay of PM H^+-ATPase Activity of PM H^+-ATPase was determined following the method of Hejl and Koster (2004) with some modifications. Root tissue (300 mg) was homogenized in 1.5 mL Tris-MES buffer (12.5 mM, pH 7.8) containing 250 mM sucrose, 1.25 mM DTT, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 5000 rpm for 15 min at 4 °C followed by centrifugation of the supernatant at 16000 rpm for 45 min at 4 °C. The pellet was resuspended in 100 μL Tris-MES buffer (1 mM, pH 7.6) containing 250 mM sucrose, 1 mM DTT, and 1 mM PMSF. Subsequently 50 μL enzyme sample was added to 200 μL Tris-MES buffer (37.5 mM, pH 6.5) containing 62.5 mM KCl, 7.6 mM $MgSO_4$, 100 mM KNO_3, 1.25 mM Na_2MoO_4, 1.25 mM NaN_3, and 3 mM ATP and incubated at 37 °C for 20 min. The reaction was stopped by the addition of 160 μL SDS (10%) followed by incubation for 10 min at 37 °C. Reaction mixture was further reacted with 550 μL 0.905% Na_2MoO_4 (in 1.45 N HCl) and 40 μL 0.05% ANSA. Inorganic phosphate (P*i*) production was quantified by measuring the absorbance at 700 nm, which was correlated with the relative activity of PM H^+-ATPase. Presence of potent inhibitors of tonoplast ATPase (KNO_3), acid phosphatases (Na_2MoO_4), and mitochondrial ATPase (NaN_3) confirmed the source of P*i* to be PM H^+-ATPase only.

Native PAGE assay of NOX In gel assay for NOX activity was performed following the methods of Carter et al. (2007) and Singh et al. (2014) with some modifications. Root tissue (300 mg) was homogenized in extraction medium comprising of 50 mM Na-phosphate buffer (pH 6.8) and 0.5% Triton X-100 and centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was used as enzyme stock and 40 μg protein was run in native PAGE (10% polyacrylamide gel with 5% stacking gel). The gel slabs were immersed in superoxide-specific staining medium, i.e., Tris-HCl buffer (10 mM, pH 7.4) containing 100 μM $MgCl_2$, 1 mM $CaCl_2$, 1 mM NaN_3, 200 μM NBT, and 134 μM NADPH and incubated for 30 min. Violet bands appeared indicating NOX activity.

Native PAGE assay of PM H^+-ATPase Root tissue (300 mg) was homogenized in the same extraction buffer used in spectrophotometric assay of PM H^+-ATPase with 0.5% Triton X-100 being added in the buffer. The homogenate was centrifuged at 10000 rpm for 15 min at 4 °C and the supernatant was used for in gel assay of PM H^+-ATPase activity (10% polyacrylamide gel with 5% stacking gel; native, non-denaturing). The gel was stained according to Suhai et al. (2009) with modifications. The gel slabs were immersed in Tris-MES buffer (37.5 mM, pH 6.5) containing 62.5 mM KCl, 7.6 mM $MgSO_4$, 3 mM ATP (freshly prepared), and 0.1% $Pb(NO_3)_2$, incubated for 4 h on a gel rocker and kept unaltered overnight. White bands of lead phosphate appeared corresponding to the released P*i*. These bands were transformed into definite brownish-black bands of lead sulfide by immersing the gel in 10 mM sodium sulfide solution for 5 min after thorough washing in distilled water.

Statistical analysis Data have been presented with standard error (SE) of the mean as vertical bar in the figures. Data were analyzed by appropriate single-factor ANOVA and post hoc comparisons were done with Tukey's honest significant difference (HSD) to determine statistically significant differences among individual treatments at $P < 0.05$ level following Singh et al. (Singh et al. 2015).

Results

Effects of vanadate and CCCP on NOX Significant reduction in superoxide ($O_2^{\bullet -}$) generation (linked to NOX activity) in the roots of *V. radiata* was noted under the treatments with protonophore (carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 50 μM) and P-type ATPase inhibitor (sodium ortho-vanadate, 100 μM) (Fig. 1). Thus, localization of apoplastic superoxide using NBT stain demonstrated sharp decline in the amount of accumulated superoxide (in terms of intensity of the resulting deep blue/violet coloring of the root tissue) during growth (Fig. 1a). Further assessment of apoplastic superoxide production was carried out with the help of XTT reduction assay of the bathing medium, which confirmed the lowering of apoplastic superoxide production in both CCCP and vanadate treatments (Fig. 1b). Spectrophotometric assay for NOX activity using XTT clearly revealed an inhibition of NOX activity under CCCP and ortho-vanadate treatments (Fig. 1c). Finally, in gel native PAGE assay for NOX activity also demonstrated the drop in NOX activity under the treatments as indicated by thinner bands than that of control (Fig. 1d).

Effects of ROS and ROS scavengers on H^+-ATPase Involvement of ROS in the regulation of PM H^+-ATPase was evident from the treatments with different ROS scavengers or ROS enzyme inhibitors and H_2O_2 (Fig. 2). Spectrophotometric assay showed significant inhibition of PM H^+-ATPase under individual treatments with NOX inhibitors (diphenyleneiodonium (DPI), 20 μM; $ZnCl_2$, 500 μM); superoxide scavenger ($CuCl_2$, 100 μM); and H_2O_2 scavengers (catalase, 500 U/mL; dimethylthiourea (DMTU), 100 μM) (Fig. 2a). Further substantiation was achieved from in gel native PAGE assay for PM H^+-ATPase, which exhibited distinctly weaker bands under DPI, $ZnCl_2$, and $CuCl_2$ (Fig. 2b) as

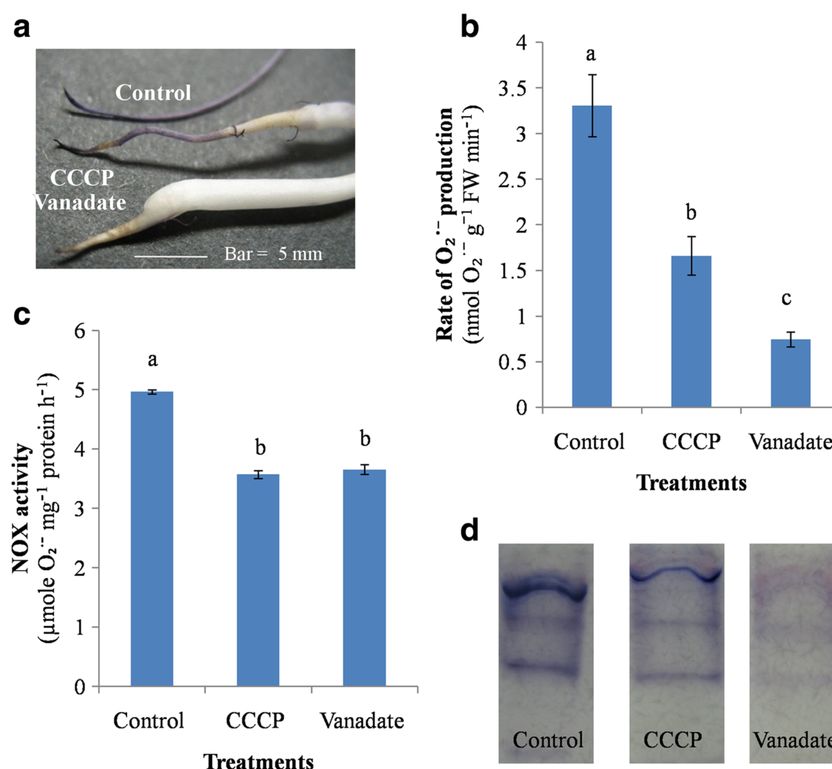

Fig. 1 Effects of PM H^+-ATPase inhibitor (sodium ortho-vanadate) and cross-PM proton gradient dissipater (CCCP) on apoplastic superoxide production and NADPH oxidase (NOX) activity in *Vigna radiata* (L.) Wilczek root. **a** Localization of superoxide using NBT staining under treatments of vanadate (100 μM) and CCCP (50 μM) along with control (distilled water). **b** Spectrophotometric assay of rate of superoxide production under treatments of vanadate (100 μM) and CCCP (50 μM) along with control ($n = 3$; $F = 30.34$; $p < 0.001$). **c** Spectrophotometric assay of NOX activity under treatments of vanadate (100 μM) and CCCP (50 μM) along with control ($n = 3$; $F = 144.87$; $p < 0.001$). **d** In gel assay of NOX activity under treatments of vanadate and CCCP along with control (cropped lanes are shown; full-length gels are presented in Supplementary Fig. S1). **b**, **c** Data are mean of three replicates and ± SE are shown as vertical bars. Data were subjected to ANOVA and post hoc comparison was carried out with Tukey's HSD to determine statistical significance among treatments. Different letters indicate significant difference among various treatments

well as catalase and DMTU (Fig. 2c) treatments than control. $CuCl_2$ was found to have the strongest negative influence on PM H^+-ATPase in both spectrophotometric as well as native PAGE assays. On the other hand, application of different concentrations of exogenous H_2O_2 (in the seedling growth medium) showed dose-dependent effects on PM H^+-ATPase (Fig. 2d). Thus, an enhanced activity of PM H^+-ATPase was noted at low concentrations, e.g., 1 and 50 μM, although higher concentrations (at mM range) were inhibitory.

Ca^{+2} homeostasis and NOX and PM H^+-ATPase activity In order to assess any possible role of Ca^{+2} in the regulation of activity of NOX and PM H^+-ATPase, 12-h germinated seeds of *V. radiata* were treated with $LaCl_3$ (plasma membrane Ca^{+2} channel inhibitor, 100 μM), EGTA (Ca^{+2} chelator, 500 μM), and LiCl (endosomal Ca^{+2} release blocker, 5 mM). Spectrophotometric analysis of NOX activity using XTT reduction assay, showed significant but weak inhibition of activity upon treatment with $LaCl_3$ and EGTA (Fig. 3a). This was further corroborated by the native PAGE assay where $LaCl_3$ and EGTA were of significant negative influence on the activity of NOX (Fig. 3b). LiCl also showed similar inhibition of NOX activity, although the effect was not significant in case of spectrophotometric assay (Fig. 3a, b). On the other hand, regulation of PM H^+-ATPase also seemed to be Ca^{+2}-dependent. Thus, spectrophotometric analysis revealed lower PM H^+-ATPase activity in the samples treated with $LaCl_3$ and EGTA than in control set (Fig. 3c). In gel assay (native PAGE), data also reflected the same, i.e., downregulation of PM H^+-ATPase activity under less-available cytosolic Ca^{+2} incurred by $LaCl_3$ and EGTA (Fig. 3d). Treatment with LiCl, though effectively decreased activity as shown in spectrophotometric analysis, did not show any significant effect in native PAGE assay of PM H^+-ATPase (data not shown).

Discussion

That ROS regulates a number of plant processes—short-term responses like stomatal opening/closure, chloroplast

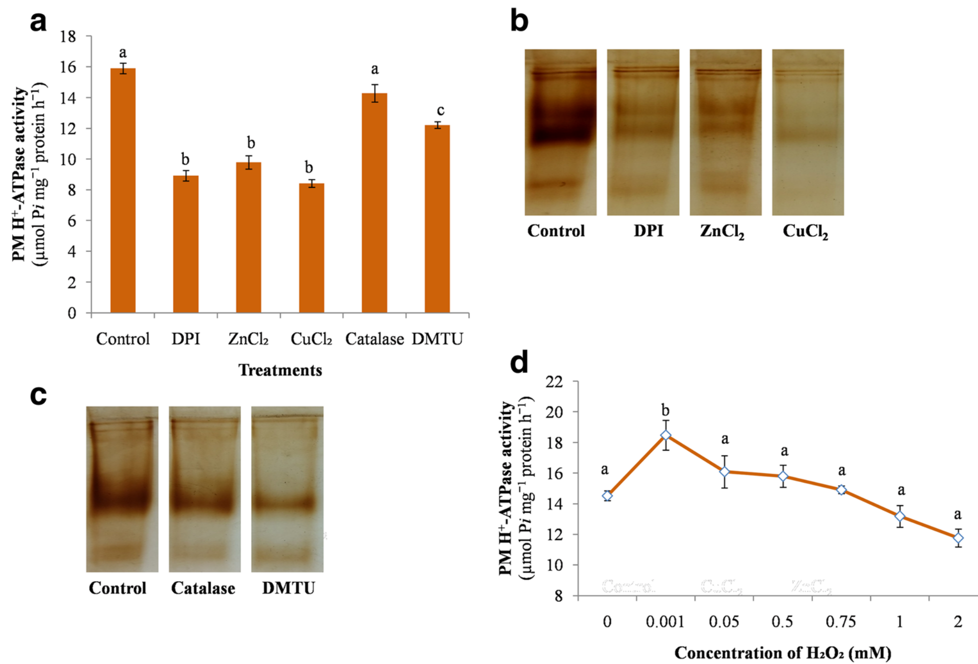

Fig. 2 Effects of ROS scavengers ($CuCl_2$, catalase, and DMTU) and NOX inhibitors (DPI and $ZnCl_2$) on PM H^+-ATPase activity in *Vigna radiata* (L.) Wilczek root. **a** Spectrophotometric assay of PM H^+-ATPase activity under treatments of DPI (20 µM), $ZnCl_2$ (500 µM), $CuCl_2$ (100 µM), catalase (500 U/mL), and DMTU (100 µM) along with control ($n = 3$; $F = 75.28$; $p < 0.001$). **b** In gel assay of PM H^+-ATPase under DPI, $ZnCl_2$, and $CuCl_2$ treatments along with control (cropped lanes are shown; full-length gels are presented in Supplementary Fig. S2). **c** In gel assay of PM H^+-ATPase under catalase and DMTU treatments with control (cropped lanes are shown; full-length gels are presented in Supplementary Fig. S3). **d** Spectrophotometric assay of PM H^+-ATPase activity under treatment of different concentrations of H_2O_2 (0.001, 0.05, 0.5, 0.75, 1, and 2 mM) with control ($n = 3$; $F = 9.1$; $p < 0.001$). **a**, **d** Data are mean of three replicates and ± SE are shown as vertical bars. Data were subjected to ANOVA and post hoc comparison was carried out with Tukey's HSD to determine statistical significance among treatments. Different letters indicate significant difference among various treatments

movements to long-term plastic developmental events (e.g., seed germination, root growth) has been revealed by several researches in recent times (Liszkay et al. 2004; Dunand et al. 2007; Singh et al. 2014; Majumdar and Kar 2015; Tsukagoshi 2016). Such regulatory role of ROS is mostly being played in the apoplastic space where plasma membrane-located NADPH oxidase is one of the major sources of apoplastic superoxide (Sagi and Fluhr 2006). However, activity of NADPH oxidase results in membrane depolarization because of electron transport across PM. In animal phagocytes, the VSOP/Hv1 proton channels are reported to diminish these effects by conducting pH-sensitive proton currents (Ramsey et al. 2006; Sasaki et al. 2006). However, no such counteracting mechanism has been reported so far for plant NOX or RBOH (NADPH oxidase). PM H^+-ATPase, which is responsible for majority of the charge-dependent plant processes like solute uptake and phloem loading, may be apprehended to possibly counteract NOX-dependent depolarization by proton efflux. It is quite likely that NOX and H^+-ATPase, both located at PM, are cooperatively regulated. In the present study, experiments on superoxide localization by NBT staining, apoploastic superoxide production, and analysis of NOX activity (both spectrophotometric and in gel assay) with root tissues of growing seedlings clearly indicated that either inhibition of PM H^+-ATPase using vanadate or dissipation of trans-PM proton gradient by treating with CCCP leads to simultaneous downregulation of NOX activity (Fig. 1). It corroborates the observation that NOX activity is membrane potential-dependent (Babior 1999; Liszkay et al. 2004). It also helps to explain the observation that fusicoccin can increase $^{\bullet}OH$ production by roots (Marrè et al. 1974) where fusicoccin-induced H^+-ATPase activity might, in turn, lead to ROS formation possibly by influencing NOX activity. It can be postulated that PM H^+-ATPase extrudes protons, on one hand, to stabilize the membrane electrical imbalance and simultaneously to provide them to produce H_2O_2 after combining with superoxide spontaneously or through superoxide dismutase (SOD).

However, the role of H_2O_2 or ROS on PM H^+-ATPase activity is highly debated and appears to be site-specific. While it is reported that ABA–H_2O_2–Ca^{+2} system inhibits PM H^+-ATPase in the guard cells (Taiz et al. 2015), Voronkov et al. (2010) have observed DPI-mediated obliteration and H_2O_2-induced restoration of membrane hyperpolarization, sensitive to vanadate, in germinating male gametophytes of *P. hybrida*. Based on the results that

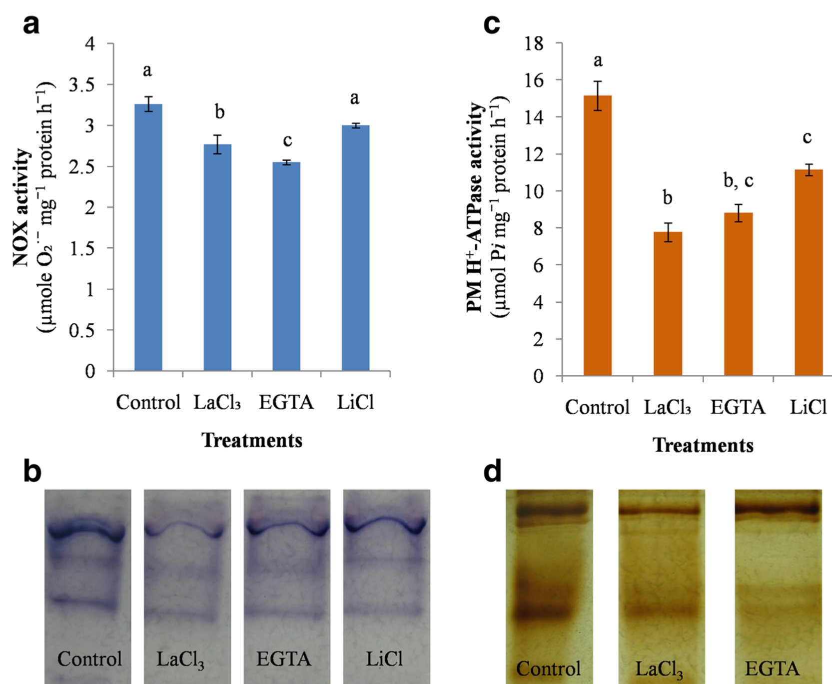

Fig. 3 Effects of plasma membrane Ca^{+2} channel blocker ($LaCl_3$), Ca^{+2} chelator (EGTA), and endosomal Ca^{+2} release inhibitor (LiCl) on NOX and PM H^+-ATPase activity in *Vigna radiata* (L.) Wilczek root. **a** Spectrophotometric assay of NOX activity under treatments of $LaCl_3$ (100 µM), EGTA (500 µM), and LiCl (5 mM) along with control ($n = 3$; $F = 17.32$; $p < 0.001$). **b** In gel assay of NOX activity under treatments of $LaCl_3$, EGTA, and LiCl along with control (cropped lanes are shown; full-length gels are presented in Supplementary Fig. S1). **c** Spectrophotometric assay of PM H^+-ATPase activity under treatments of $LaCl_3$ (100 µM), EGTA (500 µM), and LiCl (5 mM) along with control ($n = 3$; $F = 36.6$; $p < 0.001$). **d** In gel assay of PM H^+-ATPase activity under treatments of $LaCl_3$ and EGTA along with control (cropped lanes are shown; full-length gels are presented in Supplementary Fig. S4). **a**, **c** Data are mean of three replicates and ± SE are shown as vertical bars. Data were subjected to ANOVA and post hoc comparison was carried out with Tukey's HSD to determine statistical significance among treatments. Different letters indicate significant difference among various treatments

ROS scavengers ($CuCl_2$, catalase, and DMTU) and NOX inhibitors (DPI and $ZnCl_2$) strongly inhibit PM H^+-ATPase (Fig. 2a–c), present study also supports the positive involvement of ROS in PM H^+-ATPase regulation and the prominent dose-dependent activation by H_2O_2 (Fig. 2d) confirms it further. Supportive evidences also come from Zhang et al. (2007), who reported that in *Populus euphratica* callus, direct augmentation of PM H^+-ATPase was brought about by H_2O_2 while NO induced it through NOX activity. Moreover, the salt stress (NaCl) induced high activity of PM H^+-ATPase was diminished by both DPI and NMMA (NOS inhibitor). Again in two different salt stressed poplars, i.e., *P. euphratica* and *Populus popularis*, extracellular ATP maintained cellular Na^+/K^+ homeostasis by enhancing H^+ efflux (by inducing PM H^+-ATPase) which was mediated by Ca^{+2} and H_2O_2 and the homeostasis was sensitive to $LaCl_3$ and DPI (Zhao et al. 2015). H_2O_2-induced PM H^+-ATPase upregulation has been reported by Li et al. (2011) in *Carex moorcroftii* callus. The stimulation was greater in combination with NaCl and H_2O_2 than the sole treatments of NaCl or H_2O_2. However, the salt stress-induced upregulation was counteracted by DPI indicating a potent orchestration between NOX and PM H^+-ATPase. Stimulation of PM H^+-ATPase by NOX derived H_2O_2 has also been reported in cucumber (*Cucumis sativus*) roots under both heat (Janicka-Russak and Kabała 2012) and salt (Janicka-Russak et al. 2013) stress. The stimulatory effect has been interpreted as a result of increased gene expression of PM H^+-ATPase induced by H_2O_2 (e.g., *CsHA4*, *CsHA8*, and *CsHA9* in heat stress and *CsHA10* in salt stress). Regarding the inhibitory effect of H_2O_2 on PM H^+-ATPase, the observation of Lee et al. (2004) seems appropriate that the excess production of H_2O_2 at different circumstances, e.g., low temperature in their study, raises the level higher than physiological level reaching to a stressful range (1 to 125 mM; Claeys et al. 2014) which may inactivate the enzyme, probably by oxidizing the thiol groups (Lee et al. 2004; Beffagna et al. 2005).

The mechanism of this presumed functional harmony between NOX and PM H^+-ATPase should include several linking factors and Ca^{+2}, one most important signaling molecule in plants (Batistič and Kudla 2012), appears to play crucial roles here. Role of Ca^{+2} in activation of NOX by direct binding to the N-terminal EF hand motif or by phosphorylating the enzyme through CDPKs or by inducing phosphatidic acid (PA) and Rho-type (ROP)-GTPase binding to NOX is

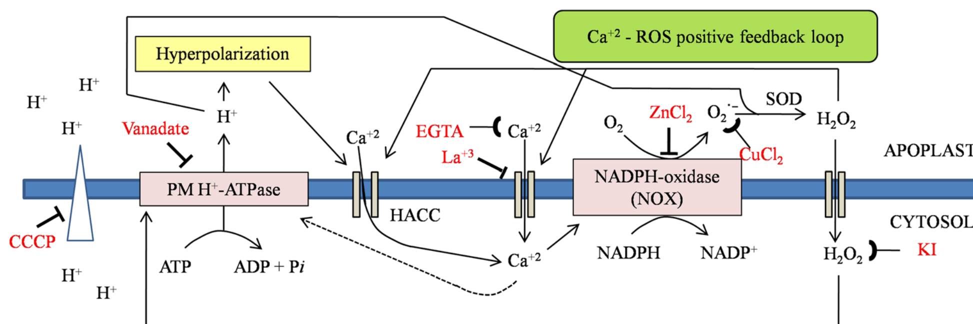

Fig. 4 Possible working model demonstrating the Ca^{+2}-mediated synchronization of PM H^+-ATPase and NADPH oxidase in *Vigna radiata* (L.) Wilczek root. PM NADPH oxidase (RBOH) mediated $O_2^{\bullet-}$ production is one of the prerequisites for cell elongation growth through wall relaxation. H_2O_2, being produced spontaneously or through SOD, either gets converted to $^{\bullet}OH$ radical and cleave wall polysaccharides or crosses the PM through aquaporins and serves as signaling molecule, Ca^{+2} channel activator, PM H^+-ATPase inducer (at low concentration), etc. The membrane depolarization resulting from the transfer of electron by NOX is stabilized by protons extruded out by PM H^+-ATPase, which simultaneously provides substrate (protons) for SOD. PM H^+-ATPase induced membrane hyperpolarization, besides being promontory for expansins and certain wall relaxation-related enzymes, activates HACC which, together with other H_2O_2 stimulated Ca^{+2} channels, builds up $[Ca^{+2}]_{cyt}$ through cross-PM Ca^{+2} influx. By binding with EF hand motif Ca^{+2} activates NOX whereas it modulates H^+-ATPase activity by phosphorylation at different sites. La^{+3}, a Ca^{+2} channel blocker and EGTA, a Ca^{+2} chelator inhibits the formation of $[Ca^{+2}]_{cyt}$ and repress both NOX and PM H^+-ATPase activity. The inhibition of both NOX and PM H^+-ATPase by sodium ortho-vanadate (PM H^+-ATPase inhibitor), CCCP (protonophore), $CuCl_2$ ($O_2^{\bullet-}$ scavenger), KI (H_2O_2 scavenger), and $ZnCl_2$ (NOX inhibitor) treatments indicates towards a Ca^{+2}-intervened functional harmony between the two enzymes

well known (Sagi and Fluhr 2006; Ogasawara et al. 2008; Gilroy et al. 2014; Kurusu et al. 2015). Our results, depicting the inhibitory effects of $LaCl_3$ and EGTA on NOX (Fig. 3a, b), corroborates the earlier findings. However, disputes persist regarding its role in PM H^+-ATPase activity specifically concerning the effect of phosphorylation of the enzyme (in a Ca^{+2}-induced manner) being negative or positive for its functioning (Janicka-Russak 2011). Interestingly, our experiments show that blocking Ca^{+2} channels (by $LaCl_3$) or chelating it (by EGTA) inhibited PM H^+-ATPase in both spectrophotometric and native PAGE assays (Fig. 3c, d) providing definite evidences in favor of the essentiality of a threshold $[Ca^{+2}]_{cyt}$ for activating PM H^+-ATPase and the critical Ca^{+2} concentration being built up from Ca^{+2} influx only. Some studies have also demonstrated positive roles of Ca^{+2} in PM H^+-ATPase activity. Thus, in contrast to the documented Ca^{+2}-ABA system inhibiting PM H^+-ATPase in guard cells (Taiz et al. 2015), Yu et al. (2006) have reported an ABA-stimulated calcium-dependent protein kinase (ACPK1) in grape berry mesocarp that enhances PM H^+-ATPase activity by phosphorylating the enzyme and acting on its C-terminal autoinhibitory domain in a completely Ca^{+2}-dependent manner, i.e., removal of Ca^{+2} eliminates the enhancement totally. However, considering several phosphorylation sites, e.g., Thr931, Thr947, Thr955, Ser931, Ser938, and many conflicting reports regarding the effect of phosphorylation (Yu et al. 2006; Janicka-Russak 2011), it may be postulated that Ca^{+2} regulates PM H^+-ATPase activity in a dose-dependent manner by phosphorylating different sites at different concentrations. Finally, considering the HACC channel properties, a subtle and dynamic co-regulation can be hypothesized between PM H^+-ATPase and Ca^{+2} where the $[Ca^{+2}]_{cyt}$ is extremely delicate and decisive for this pathwaym i.e., greater concentration of Ca^{+2} than threshold $[Ca^{+2}]_{cyt}$ will inhibit PM H^+-ATPase instead of stimulating it. Since both ROS and PM H^+-ATPase regulate HACC activity (Demidchik et al. 2002; Foreman et al. 2003), the proposed synchronization between PM H^+-ATPase and NOX seems to be Ca^{+2} mediated. However, apart from Ca^{+2}, the coordination between PM H^+-ATPase and NOX, may also involve many other factors such as NaRALF (*Nicotiana attenuata* Rapid alkalinisation factor) which, according to Wu et al. (2007), regulates root growth and apoplastic pH by modulating the activity of PM H^+-ATase and cytosolic ROS level.

Conclusion

It may be concluded that in plants, NOX and PM H^+-ATPase are functionally harmonized and work cooperatively to maintain the membrane electrical balance while mediating cell growth through cell wall relaxation. Ca^{+2} influx through PM builds up the $[Ca^{+2}]_{cyt}$ and links the two enzymes in a feed-forward loop. A working model demonstrating the Ca^{+2}-mediated integration of NOX and PM H^+-ATPase has been proposed (Fig. 4).

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Author contributions RKK envisaged the study. AM and RKK designed the work. AM performed the experiments. AM and RKK wrote the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Airianah OB, Vreeburg RAM, Fry SC (2016) Pectic polysaccharides are attacked by hydroxyl radicals in ripening fruit: evidence from a fluorescent fingerprinting method. Ann Bot 117(3):441–455. https://doi.org/10.1093/aob/mcv192

Babior BM (1999) NADPH oxidase: an update. Blood 93(5):1464–1476

Baluška F, Mancuso S, Volkmann D, Barlow PW (2010) Root apex transition zone: a signalling response nexus in the root. Trends Plant Sci 15(7):402–408. https://doi.org/10.1016/j.tplants.2010.04.007

Batistič O, Kudla J (2012) Analysis of calcium signaling pathways in plants. Biochim Biophys Acta 1820(8):1283–1293. https://doi.org/10.1016/j.bbagen.2011.10.012

Beffagna N, Buffoli B, Busi C (2005) Modulation of reactive oxygen species production during osmotic stress in *Arabidopsis thaliana* cultured cells: involvement of the plasma membrane Ca^{+2}-ATPase and H^+-ATPase. Plant Cell Physiol 46(8):1326–1339. https://doi.org/10.1093/pcp/pci142

Carter C, Healy R, O'Tool NM, Saqlan Naqvi SM, Ren G, Park S, Beattie GA, Horner HT, Thornburg RW (2007) Tobacco nectarines express a novel NADPH oxidase implicated in the defense of floral reproductive tissues against microorganisms. Plant Physiol 143(1):389–399. https://doi.org/10.1104/pp.106.089326

Claeys H, Van Landeghem S, Dubois M, Maleux K, Inzé D (2014) What is stress? Dose-response effects in commonly used *in vitro* stress assays. Plant Physiol 165:519–527

Cosgrove DJ (2000a) Expansive growth of plant cell walls. Plant Physiol Biochem 38(1/2):109–124. https://doi.org/10.1016/S0981-9428(00)00164-9

Cosgrove DJ (2000b) Loosening of plant cell walls by expansins. Nature 407(6802):321–326. https://doi.org/10.1038/35030000

De Nisi P, Dell'Orto M, Pirovano L, Zocchi G (1999) Calcium-dependent phosphorylation regulates the plasma-membrane H^+-ATPase activity of maize (*Zea mays*) roots. Planta 209(2):187–194. https://doi.org/10.1007/s004250050621

Demidchik V, Bowen HC, Maathuis FJ, Shabala SN, Tester MA, White PJ, Davies JM (2002) *Arabidopsis thaliana* root non-selective cation channels mediate calcium uptake and are involved in growth. Plant J 32(5):799–808. https://doi.org/10.1046/j.1365-313X.2002.01467.x

Dunand C, Crèvecoeur M, Penel C (2007) Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: possible interaction with peroxidises. New Phytol 174:332–341

Fluhr R (2009) Reactive oxygen-generating NADPH oxidases in plants. In: Rio LA, Puppo a (eds) reactive oxygen species in plant signalling. Springer-Verlag, Berlin, pp 1–23. https://doi.org/10.1007/978-3-642-00390-5_1

Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JDG, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422(6930):442–446. https://doi.org/10.1038/nature01485

Frahry G, Schopfer P (2001) NADH-stimulated, cyanide-resistant superoxide production in maize coleoptiles analyzed with a tetrazolium-based assay. Planta 212(2):175–183. https://doi.org/10.1007/s004250000376

Gilroy S, Suzuki N, Miller G, Choi W-G, Toyota M, Devireddy AR, Mittler R (2014) A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signalling. Trends Plant Sci 19(10):623–630. https://doi.org/10.1016/j.tplants.2014.06.013

Hager A (2003) Role of the plasma membrane H^+-ATPase in auxin induced elongation growth: historical and new aspects. J Plant Res 116(6):483–505. https://doi.org/10.1007/s10265-003-0110-x

Hejl AM, Koster KL (2004) Juglone disrupts root plasma membrane H^+-ATPase activity and impairs water uptake, root respiration, and growth in soybean (*Glycine max*) and corn (*Zea mays*). J Chem Ecol 30(2):453–471. https://doi.org/10.1023/B:JOEC.0000017988.20530.d5

Irving HR, Gehring CA, Parish RW (1992) Changes in cytosolic pH and calcium of guard cells precede stomatal movements. Proc Natl Sci Acad, USA 89(5):1790–1794. https://doi.org/10.1073/pnas.89.5.1790

Janicka-Russak M (2011) Plant plasma membrane H^+-ATPases in adaptation of plants to abiotic stresses. In: Shanker A (ed) Abiotic stress response in plants—physiological, biochemical and genetic perspectives. InTech, pp 197–218. https://doi.org/10.5772/24121

Janicka-Russak M, Kabała K (2012) Abscisic acid and hydrogen peroxide induce modification of plasma membrane H^+-ATPase from *Cucumis sativus* L. roots under heat shock. J Plant Physiol 169(16):1607–1614. https://doi.org/10.1016/j.jplph.2012.05.013

Janicka-Russak M, Kabała K, Wdowikowska A, Kłobus G (2013) Modification of plasma membrane proton pumps in cucumber roots as an adaptation mechanism to salt stress. J Plant Physiol 170(10):915–922. https://doi.org/10.1016/j.jplph.2013.02.002

Kar RK (2015) ROS signalling: relevance with site of production and metabolism of ROS. In: Gupta DK, Palma JM, Corpas FJ (eds) Reactive oxygen species and oxidative damage in plants under stress. Springer, Switzerland, pp 115–125

Kinoshita T, Nishimura M, Shimazaki K-i (1995) Cytosolic concentration of Ca^{+2} regulates the plasma membrane H^+-ATPase in guard cells of fava bean. Plant Cell 7:1333–1342

Kurusu T, Kuchitsu K, Tada Y (2015) Plant signalling networks involving Ca^{+2} and Rboh/Nox-mediated ROS production under salinity stress. Front Plant Sci. https://doi.org/10.3389/fpls.2015.00427

Lee SH, Singh AP, Chung GC (2004) Rapid accumulation of hydrogen peroxide in cucumber roots due to exposure to low temperature appears to mediate decreases in water transport. J Exp Bot 55(403):1733–1741. https://doi.org/10.1093/jxb/erh189

Li J, Chen G, Wang X, Zhang Y, Jia H, Bi Y (2011) Glucose-6-phosphate dehydrogenase-dependent hydrogen peroxide production is involved in the regulation of plasma membrane H^+-ATPase and Na^+/H^+ antiporter protein in salt-stressed callus from *Carex moorcroftii*. Physiol Plant 141(3):239–250. https://doi.org/10.1111/j.1399-3054.2010.01429.x

Liszkay A, Kenk B, Schopfer P (2003) Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. Planta 217:658–667

Liszkay A, Van der Zalm E, Schopfer P (2004) Production of reactive oxygen intermediates ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$) by maize roots and their role in wall loosening and elongation growth. Plant Physiol 136:3114–3123

Majumdar A, Kar RK (2015) Integrated role of ROS and Ca^{+2} in blue light-induced chloroplast avoidance movement in leaves of *Hydrilla verticillata* (L.f.) Royle. Protoplasma 253(6):1529–1539. https://doi.org/10.1007/s00709-015-0911-5

Marrè E, Lado P, Ferroni A, Ballarin Denti A (1974) Transmembrane potential increase induced by auxin, benzyladenine and fusicoccin. Correlation with proton extrusion and cell enlargement. Plant Sci Lett 2(5):257–265. https://doi.org/10.1016/0304-4211(74)90081-9

Maturana A, Krause K-H, Demaurex N (2002) NOX family NADPH oxidases: do they have built-in proton channels? J Gen Physiol 120:781–786. https://doi.org/10.1085/jgp.20028713

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce cell

extension. Proc Nat Acad Sci 91(14):6574–6578. https://doi.org/10.1073/pnas.91.14.6574

Mendrinna A, Persson S (2015) Root hair growth: it's a one way street. F1000Prime Rep 7:23. https://doi.org/10.12703/P7-23

Morth JP, Pedersen BP, Buch-Pedersen MJ, Andersen JP, Vilsen B, Palmgren MG, Nissen P (2011) A structural overview of the plasma membrane Na^+, K^+-ATPase and H^+-ATPase ion pumps. Nat Rev Mol Cell Biol 12(1):60–70. https://doi.org/10.1038/nrm3031

Nibau C, Gibbs DJ, Coates JC (2008) Branching out in new directions: the control of root architecture by lateral root formation. New Phytol 179(3):595–614. https://doi.org/10.1111/j.1469-8137.2008.02472.x

Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, Hishinuma H, Senzaki E, Yamagoe S, Nagata K, Nara M, Suzuki K, Tanokura M, Kuchitsu K (2008) Synergistic activation of *Arabidopsis* NADPH oxidase AtrbohD by ca^{+2} and phosphorylation. J Biol Chem 283(14):8885–8892. https://doi.org/10.1074/jbc.M708106200

Ramsey IS, Moran MM, Chong JA, Clapham DE (2006) A voltage-gated proton-selective channel lacking the pore domain. Nature 440:1213–1216

Ramsey IS, Ruchti E, Kaczmarek JS, Clapham DE (2009) Hv1 proton channels are required for high-level NADPH oxidase-dependent superoxide production during the phagocyte respiratory burst. Proc Natl Acad Sci 106(18):7642–7647. https://doi.org/10.1073/pnas.0902761106

Sagi M, Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol 141(2):336–340. https://doi.org/10.1104/pp.106.078089

Sasaki M, Takagi M, Okamura Y (2006) A voltage sensor-domain protein is a voltage-gated proton channel. Science 312:589–592.

Schopfer P (1996) Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. Planta 199:43–49

Schopfer P (2001) Hydroxyl radical-induced cell-wall loosening *in vitro* and *in vivo*: implications for the control of elongation growth. Plant J 28(6):679–688

Schopfer P, Plachy C, Frahry G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberllin and abscisic acid. Plant Physiol 125:1591–1602

Singh KL, Chaudhuri A, Kar RK (2014) Superoxide and its metabolism during germination and axis growth of Vigna radiata (L.) Wilczek seeds. Plant Signal Behav. https://doi.org/10.4161/psb.29278

Singh KL, Chaudhuri A, Kar RK (2015) Role of peroxidase activity and Ca^{+2} in axis growth during seed germination. Planta 242:997–1007. https://doi.org/10.1007/s00425-015-2338-9

Speth C, Jaspert N, Marcon C, Oecking C (2010) Regulation of the plant plasma membrane H^+-ATPase by its C-terminal domain: what we know for sure? Eur J Cell Biol 89(2-3):145–151. https://doi.org/10.1016/j.ejcb.2009.10.015

Suhai T, Heidrich NG, Dencher NA, Seelert H (2009) Highly sensitive detection of ATPase activity in native gels. Electrophoresis 30(20):3622–3625. https://doi.org/10.1002/elps.200900114

Taiz L, Zeiger E, Møller IM, Murphy A (2015) Plant physiology and development. Sinauer, Sunderland

Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG (1998) Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase ($gp91^{phox}$). Plant J 14(3):365–370. https://doi.org/10.1046/j.1365-313X.1998.00136.x

Tsukagoshi H (2016) Control of root growth and development by reactive oxygen species. Curr Opin Plant Biol 29:57–63. https://doi.org/10.1016/j.pbi.2015.09.008

Voronkov AS, Andreev IM, Timofeeva GV, Kovaleva LV (2010) Electrogenic activity of plasma membrane H^+-ATPase in germinating male gametophyte of *Petunia* and its stimulation by exogenous auxin: mediatory role of calcium and reactive oxygen species. Russ J Plant Physiol 57(3):401–407. https://doi.org/10.1134/S102144371003012X

Wu J, Kurten EL, Monshausen G, Hummel GM, Gilroy S, Baldwin IT (2007) NaRALF, a peptide signal essential for the root hair tip apoplastic pH in *Nicotiana attenuata*, is required for root hair development and plant growth in native soils. Plant J 52(5):877–890. https://doi.org/10.1111/j.1365-313X.2007.03289.x

Yu X-C, Li M-J, Gao G-F, Feng H-Z, Geng X-Q, Peng C-C, Zhu S-Y, Wang X-J, Shen Y-Y, Zhang D-P (2006) Abscisic acid stimulates a calcium-dependent protein kinase in grape berry. Plant Physiol 140(2):558–579. https://doi.org/10.1104/pp.105.074971

Zhang F, Wang Y, Yang Y, Wu H, Wang D, Liu J (2007) Involvement of hydrogen peroxide and nitric oxide in salt resistance in the calluses from *Populas euphratica*. Plant Cell Envo 30(7):775–785. https://doi.org/10.1111/j.1365-3040.2007.01667.x

Zhao N, Wang S, Ma X, Zhu H, Sa G, Sun J, Li N, Zhao C, Zhao R, Chen S (2015) Extracellular ATP mediates cellular K^+/Na^+ homeostasis in two contrasting poplar species under NaCl stress. Trees 30(3):825–837. https://doi.org/10.1007/s00468-015-1324-y