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Research article

Involvement of phospholipase D and NADPH-oxidase in salicylic acid signaling cascade

Tetiana Kalachova*, Oksana Iakovenko, Sergii Kretinin, Volodymyr Kravets

Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Science of Ukraine, 1 Murmanska, Kyiv 02094, Ukraine

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ABSTRACT

Salicylic acid is associated with the primary defense responses to biotic stress and formation of systemic acquired resistance. However, molecular mechanisms of early cell reactions to phytohormone application are currently undisclosed. The present study investigates the participation of phospholipase D and NADPH-oxidase in salicylic acid signal transduction cascade.

The activation of lipid signaling enzymes within 15 min of salicylic acid application was shown in *Arabidopsis thaliana* plants by measuring the phosphatidic acid accumulation. Adding of primary alcohol (1-butanol) to the incubation medium led to phosphatidylbutanol accumulation as a result of phospholipase D (PLD) action in wild-type and NADPH-oxidase RbohD deficient plants. Salicylic acid induced rapid increase in NADPH-oxidase activity in histochemical assay with nitroblue tetrazolium but the reaction was not observed in presence of 1-butanol and NADPH-oxidase inhibitor diphenylene iodide (DPI). The further physiological effect of salicylic acid and inhibitory analysis of the signaling cascade were made in the guard cell model. Stomatal closure induced by salicylic acid was inhibited by 1-butanol and DPI treatment. *rbohD* transgenic plants showed impaired stomatal reaction upon phytohormone effect, while the reaction to H_2O_2 did not differ from that of wild-type plants. Thus a key role of NADPH-oxidase D-isoform in the process of stomatal closure in response to salicylic acid has been postulated. It has enabled to predict a cascade implication of PLD and NADPH oxidase to salicylic acid signaling pathway.

1. Introduction

Plant growth and survival are the result of successful adaptation to environmental changes. Abiotic stresses and pathogen attack induce rapid changes in cell metabolism. To provide this, plants developed a wide signaling network regulated by small active molecules – phytohormones. Salicylic acid (SA) is one of stress-associated phytohormones, a signal molecule in the induction of defense mechanisms to biotrophic pathogen effects [1]. It plays a key role in the regulation of different aspects of plant growth and development, especially in adaptive reactions to environmental changes. On physiological and molecular level SA is involved to various processes including seed germination, dormancy, flowering [2], ion transport [3], photosynthetic intensity and transpiration regulation [4]. Among the molecular targets of salicylic acid are discussed reactive oxygen species (ROS) production enzymes, several kinases, proteins with lipase activity [5]. However, early stages of signaling cascades of this phytohormone and the participation of membrane components in them are currently undisclosed.

Phosphatidic acid (PA) is a universal lipid second messenger formed from structural lipids by phospholipase D (PLD) hydrolyzing activity as well as from diacylglycerol by diacylglycerolkinases. Several exogenous stimuli seem to trigger PA production, including abiotic stresses, hormone actions and pathogen infection. The specificity of PA targets and further signals depends on the fatty acid composition [6]. There are different groups of proteins discussed to be the possible targets for PA in signaling events. Among them are the enzymes of MAPK pathway, PDK1 pathway and OXI1 protein kinase especially, ABI1 as a step in abscisic acid signaling and ROS production enzymes [7,8]. It was previously reported that SA induces PLD activation in arabidopsis suspension cultures after 45 min of application. At the same time adding of 1-butanol affects expression of SA-associated genes that suggests involvement of PA production by PLD to SA signaling cascade [9].

Abbreviations: SA, salicylic acid; PA, phosphatidic acid; PLD, phospholipase D; ROS, reactive oxygen species; DPI, diphenylene iodide; TLC, thin-layer chromatography; NBT, nitroblue tetrazolium.

* Corresponding author. Tel.: +38 044 5732655; fax: +38 044 5732552.
E-mail address: kalachova@bpci.kiev.ua (T. Kalachova).

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The aim of the present work was to investigate the possible involvement of PLD signaling and further ROS generation to the salicylic acid cascades.

2. Results and discussion

2.1. Effect of exogenous salicylic acid application on lipid signaling

To study the effect of exogenous salicylic acid application on phospholipid metabolism plant leaves were labeled by ^{33}P and exposed to SA. Then the phospholipids were analyzed by TLC and the changes in phosphatidic acid content were measured. Significant increase in PA-levels in SA-treated leaves represents the evidence of lipid signaling enzymes activation (Fig. 1). Then we studied a possible source of PA accumulation. 1-butanol was previously added to the incubation medium and then after 2 h incubation SA was supplemented. Such conditions facilitated more than two fold increase in phosphatidylbutanol content after 30 min of salicylic acid action in plants suggesting the inclusion of PLD to the SA signaling cascade (Fig. 2).

Involvement of PLD-derived PA to ROS generation is now discussed into signaling cascade of different phytohormones, especially salicylic and abscisic acid. Decreasing of constitution expression of PLDα1 in *pldα1* mutant resulted in total decrease of ROS level. But then PA application restored ROS generation in mutant plants [10]. It was shown that ABA application caused NADPH-oxidase activation and ROS generation in wild-type plants, but not in pldα1 mutants [14]. Also PLDα1 and PLDδ1 are both necessary for the ABA-induced response with PA accumulation, ROS generation and stomatal closure, but their function is not fully overlapped and could be realized only in cooperation [11]. NADPH-oxidases (also called respiratory burst oxidase homologs – Rboh) catalyze production of superoxide radicals involved to the primary defense responses in plants and animals [12,13]. In plants NADPH-oxidases are a source of ROS generation under different stimuli effects including pathogen attack, root hairing etc [14,15]. Also plant NADPH-oxidase protein contained a special PA-binding region in primary sequence. *In vitro* studies showed a PA capability to interact physically with both recombinant isozymes of NADPH-oxidases RbohD and RbohF [15]. Plant NADPH-oxidase gene family consists of three main isozyme classes that differ in constituent expression due to tissue identity. Specifically, RbohD and RbohF are expressed uniformly through the whole plant; RbohA, RbohG and RbohI are mainly presented in roots and RbohH and J are more common during pollen development [16]. In one of the previous studies the role of two NADPH oxidase isoforms RbohD and RbohF in regulating defense-associated metabolism was compared during compatible and incompatible biotic interactions and modulated oxidative stress. Analysis provided that RbohF interacts closely with intracellular oxidative stress and appears to be a key player not only in cell death during hypersensitive reaction but also in regulating of metabolic responses and resistance [17].

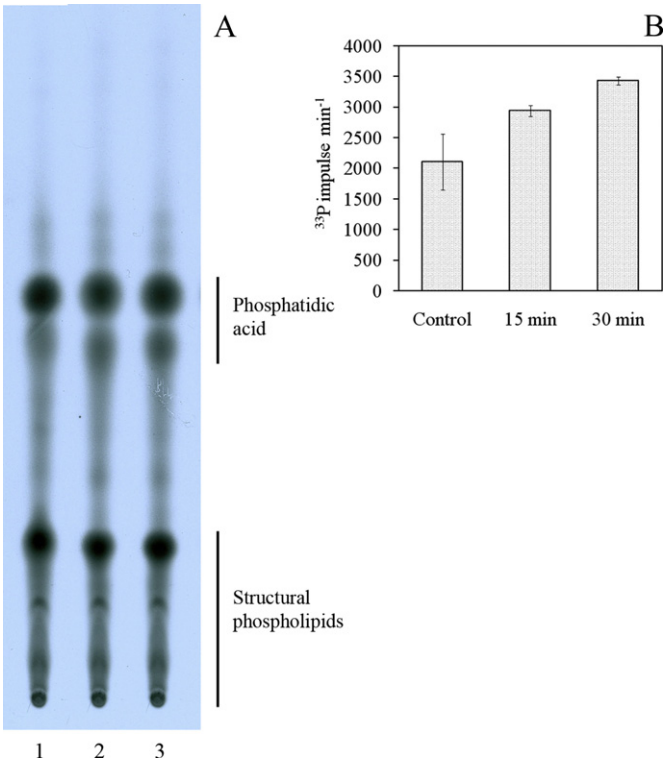

Fig. 1. Effect of salicylic acid on phosphatidic acid accumulation in *Arabidopsis thaliana* Col-1 tissues (A – autoradiography, B – scintillation counting) 1 – control; 2 – SA, 15 min; 3 – SA, 30 min.

In our experiments phosphatidylbutanol accumulation was detected both in wild type and defective *rbohD* plants, that is the evidence for the upstream localization of lipid signaling elements over the ROS production in SA cascade (Fig. 2). However, in the control conditions *rbohD* mutants showed a 30% decrease in phosphatidylbutanol content compared to WT, and in presence of SA phosphatidylbutanol accumulation was 50% higher in WT then in *rbohD*. Thus, RbohD activation could be also necessary for the amplification of the SA-induced PLD signaling.

2.2. Effect of salicylic acid treatment on rapid ROS generation in plant leaves

Reactive oxygen species generation is one of the most important signaling messengers in plant cells, mainly during abiotic and biotic stress responses. For example, exogenous avirulent elicitor application caused oxidative burst in *Solanum lycopersicum* plants [18]. ROS associated with cell-to-cell signaling are produced mainly near plasma membrane, substantially at the apoplast by NADPH-oxidases. In the present study rapid activation of superoxide anion producing NADPH-oxidase by salicylic acid was revealed in histochemical assay (Fig. 3). To detect the role of PLD in SA-induced superoxide production we used primary alcohol 1-butanol as an inhibitor of PLD-derived PA accumulation. It was shown that in presence of 1-butanol superoxide generation by SA was attenuated, while adding of 2-butanol did not affect the reaction. Also we used diphenylene iodide (DPI), specific inhibitor of NADPH-oxidases that inhibited superoxide generation under SA treatment and provided the reaction specificity. Thereby the upstream localization of PLD over NADPH-oxidase RbohD in the salicylic acid cascade was established.

Superoxide generation in epidermis is discussed to be a local signal messenger with a fast physiological effect on stomatal status. Stomata are pores on abaxial side of the leaf plate formed by two guard cells. Stomata play different important regulation roles in plant organism, especially gas and water exchange, pathogen defense. The maximum distance between guard cells membrane is called stomatal aperture and is regulated in response to environmental changes. The molecular mechanisms of stomatal movement regulation are extremely current subject of research interest. Guard cells have a dynamic regulation network and are able to react on different environmental stimuli including abiotic stress factors and pathogen infection [19]. The ability of stomatal cells to react on bacterial attack depends on coordination of bacterial virulence factors and plant resistance genes. In plant organism different signaling cascades are involved into pathogen induced stomatal

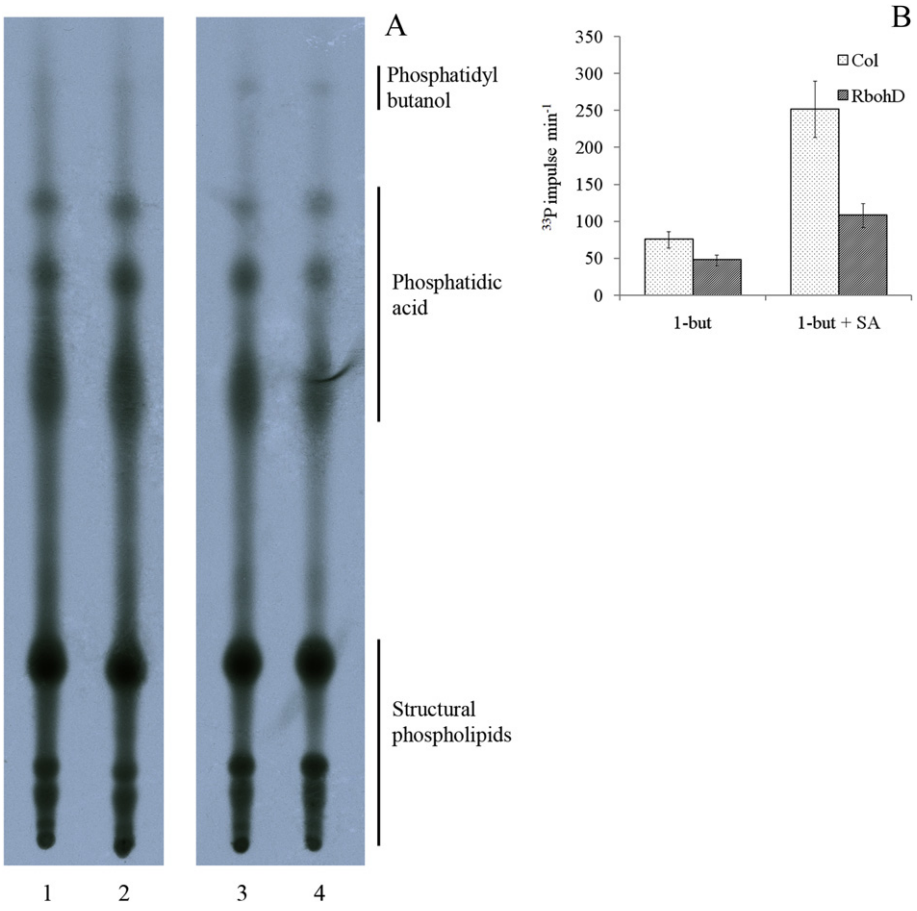

Fig. 2. Phospholipase D activation under salicylic acid action (30 min, 1 mM) in Col-1 and AtRbohD plants (phosphatidylbutanol formation), A – autoradiography, B – scintillation counting. 1 – Col,1-butanol; 2 – Col,1-butanol + SA; 3 – RbohD, 1-butanol; 4 – RbohD, 1-butanol + SA.

movement. Some of these cascades are the same as in abiotic responses, for example abscisic acid cascade including phospholipases action, guard cell-specific open-stomata 1 (OST1) kinase activation, the production of ROS by cell wall enzymes, the heterotrimeric G protein activation, and the regulation of ion channels [20]. However, there are potential pathogen response-specific chemicals required for stomatal closure, especially salicylic acid [21]. It was previously provided that some elements of SA-associated cascades could be an important part of stomatal regulation network. For example, involvement of downstream SA signaling effector – Non-expresser of PR 1 (NPR1), is discussed to be required for stomatal closure [22]. NPR1 is best known for controlling the expression of SA-responsive genes and systemic acquired resistance development, and a microbe associated molecular patterns signaling later leads to expression of SA response genes in arabidopsis leaves [23]. In present research we studied involvement of the elements of SA signaling on stomatal movement regulation.

2.3. The role of different NADPH-oxidase isoforms in stomatal movement regulation by salicylic acid

One of the key regulators of stomatal movement is ROS generation by apoplastic NADPH-oxidases [24]. In order to detect a role of different isozymes of NADPH-oxidases in plant reaction to SA effect we used *rbohD*, *rbohF* and *rbohDF* mutants (Figs. 4 and 5). It was shown that salicylic acid treatment caused stomatal closure in wild-type arabidopsis plants. After 30 min of SA treatment stomatal aperture showed 200% decrease but in *rbohD* transgenic plants it was only 10% of control level that suggests about a key role of the isozyme in salicylic acid effect realization. RbohF deficiency also caused stomatal movement inhibition, but aperture changes were about 30% less than in control. That indicates the involvement of both isoforms to the stomatal movement regulation but with the major role of RbohD. Interestingly, that double *rbohDF* mutant showed only 20% aperture decreasing in comparison to untreated control what could be explained as a compensatory effect of other isoforms in the conditions of RbohD and RbohF constitutive lack or activation of other signaling pathways.

2.4. The role of PLD and NADPH-oxidases in stomatal movement regulation by salicylic acid

As it was previously described by Zhang et al., PLD-derived phosphatidic acid is involved into NADPH-oxidase regulation, especially by physical PA–Rboh interaction. The PA binding site in RbohD was located in the cytosolic region between the two EF-hands and N terminus. The binding of Rbohs by the lipid messenger PA identified in this study may reveal production is induced by a wide spectrum of stimuli [18], this mechanism is likely to have a central role in the coupling of extracellular signals to Rboh activation. This novel regulatory mechanism for RbohD activation by PA is realized in abscisic acid signaling in guard cells. Activation of PLD by ABA results in PA production. Then PA binds to the cytosolic region in the N-terminus of RbohD, resulting in the stimulation of NADPH oxidase activity and ROS production in

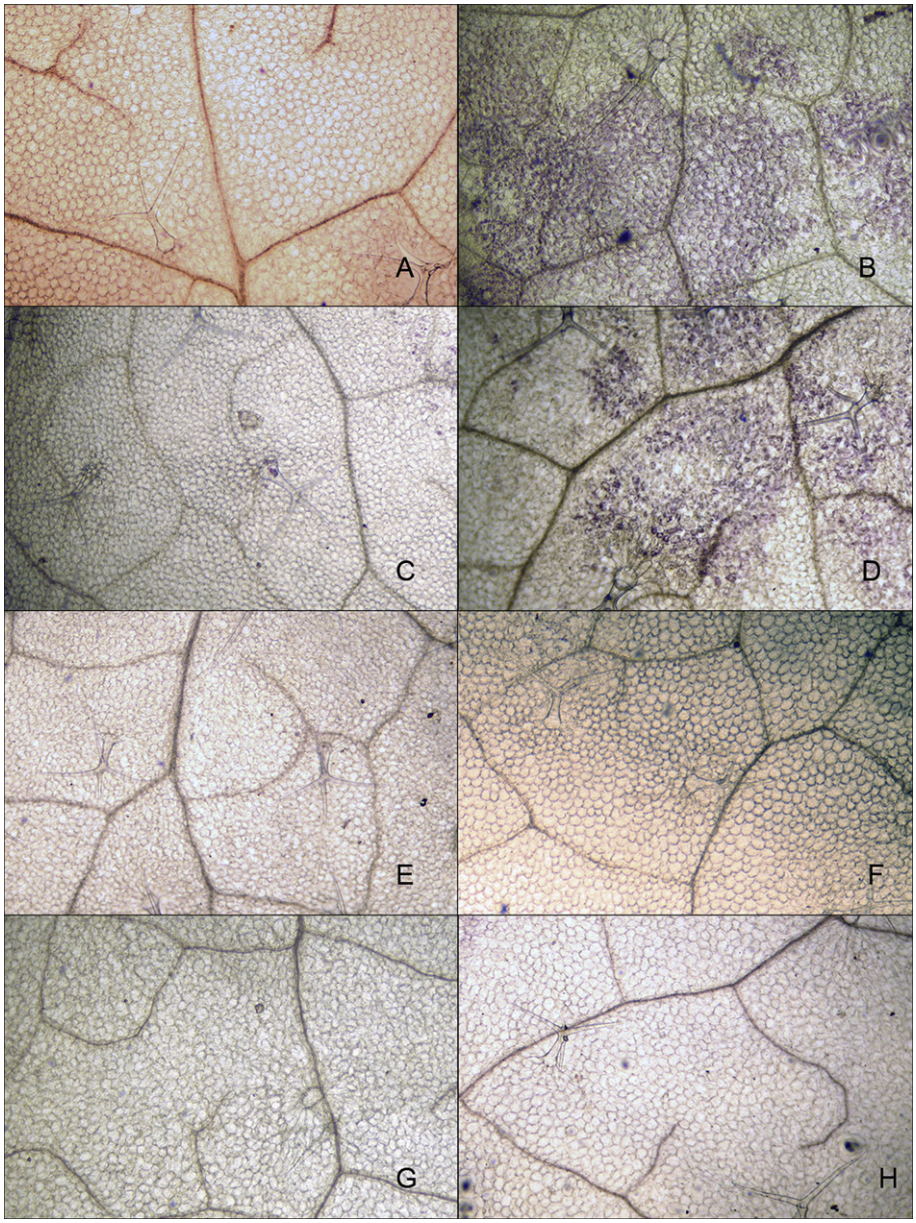

Fig. 3. Superoxide radical generation in Arabidopsis WT tissues under SA action (Histochemical assay with nitroblue tetrazolium). A – control; B – SA infiltration; C – SA + 1-butanol; D – SA + 2-butanol; E – 1-butanol; F – 2-butanol; G – DPI; H – SA + DPI.

guard cells. The final physiological effect of the cascade is stomatal closure [15].

Using stomatal closure as a biotest for a salicylic acid activity in guard cells we studied an effect of different analogs and inhibitors on the signal transduction. It was shown that hydrogen peroxide closed *rbohD* stomata as well as wild-type, and 4-hydroxybenzoic acid (inactive analog of salicylic acid) did not cause any changes in stomatal aperture. Plant leaves pretreatment with DPI blocked SA-induced stomatal movement in wild-type plants. The same stomatal effect in transgenic and DPI-treated plants indicates a key role of D isoform of the NADPH-oxidase in salicylic acid signaling. Stomatal movement inhibition by adding of 1-butanol to incubation buffer suggests about involvement of PLD to SA cascade in guard cells (Fig. 5). As a positive control we used H_2O_2 treatment that caused stomatal closure in both genotypes. Adding of 1-butanol slightly affected on the reaction stimulated by H_2O_2 that is another evidence to upstream localization of PLD *vs*. ROS production in the signaling events. But stomatal aperture is less decreased in H_2O_2 + 1-butanol than in H_2O_2 only. These might suggest about a control loop between H_2O_2 and PLD that would activate NADPH oxidase to produce ROS that could also participate in stomatal closure. On the ground of the obtained results was constructed the schematic model of salicylic acid signaling cascade in guard cells (Fig. 6).

3. Conclusion

Exogenic application of salicylic acid induces in guard cells signaling cascade with an effect of stomatal closure. The intermediate stages of the process are activation of lipid signaling enzymes, especially phospholipase D that results in phosphatidic acid generation and subsequent induction of superoxide production by NADPH oxidases, mainly RbohD. The findings suggest the novel molecular mechanism of salicylic acid signaling network in plant cells including lipid metabolism and ROS formation.

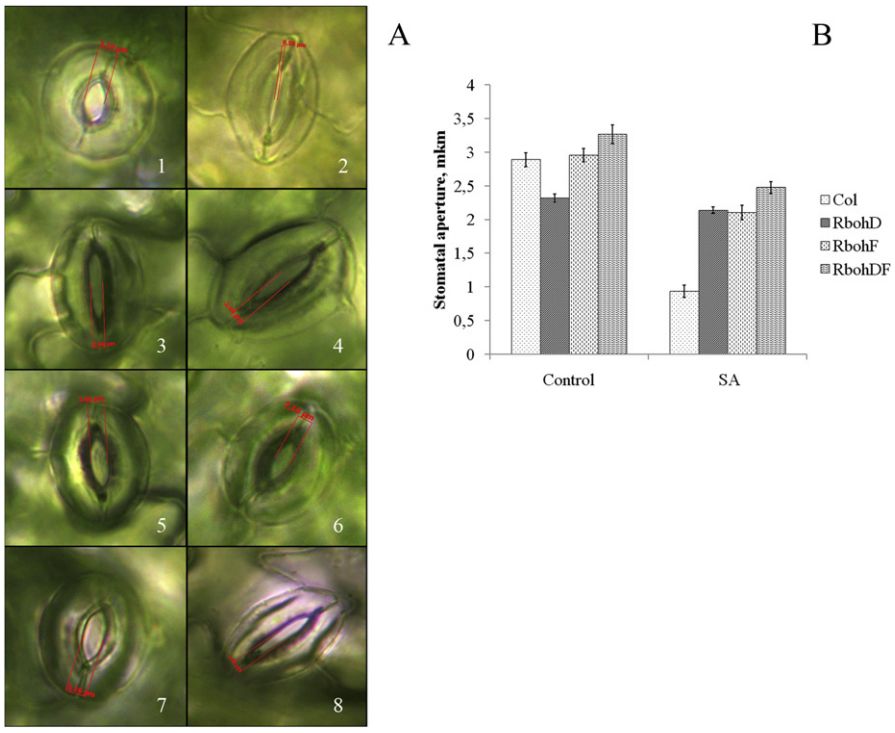

Fig. 4. Effect of SA application on epidermis of WT, *rbohD*, *rbohF* and double mutant *rbohDF* plants (A – guard cells photo, B – stomatal aperture) 1 – Col; 2 – Col + SA; 3 – RbohD; 4 – RbohD + SA; 5 – RbohF; 6 – RbohF + SA; 7 – RbohDF; 8 – RbohDF + SA.

4. Materials and methods

4.1. Plant material and experimental conditions

Arabidopsis seeds ecotype Columbia-1 as wild-type and mutant lines *rbohD*, *rbohF* and *rbohDF* were obtained from The Nottingham Arabidopsis Stock Centre (NASC). Seeds were sown in soil and cold treated at 4 °C overnight. Plants were grown under 14-h light/10-h dark grown in controlled growth chambers at 22 °C with a 16 h photoperiod. For all experiments 4- to 5-week-old plants were used.

4.2. Phospholipid labeling

Full mature leaves of *Arabidopsis thaliana* were labeled by [^{33}P] orthophosphate and then exposed to phytohormonal application. Leaves were incubated for 14 h at 25 °C in the dark in 100 mM Tris–HCl buffer (pH 6.15). Labeling solution contained with 3.7 MBq [^{33}P] orthophosphate per 200 mg of plant tissues. After incubation leaves were washed from non-incorporated [^{33}P] orthophosphate. To study the possible PLD activation we moved leaves to the medium with 0.8% 1-butanol in Tris–HCl buffer (pH 6.15) and incubated for 1 h in the dark prior to SA (1 mM) application. Control samples were incubated with 1-butanol (2-butanol) or SA alone. The experimental samples were fixed in liquid nitrogen after the 15, 30 min of SA treatment [25].

4.3. Phospholipid extraction

Lipids were extracted by adding 3.75 ml $CHCl_3/CH_3OH/HCl$ (50:100:1, v/v) to each sample. Two-phase system was prepared by adding $CHCl_3$ and 0.9% NaCl. Samples were vortexed for 15 s and

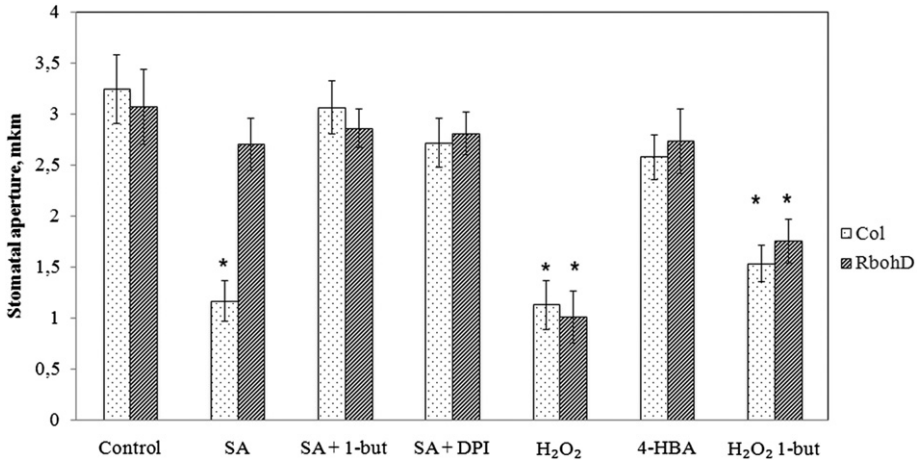

Fig. 5. Effect of 1-butanol, DPI, H_2O_2 and 4-hydroxybenzoic acid on SA-induced stomatal movement regulation of WT and *rbohD* plants.

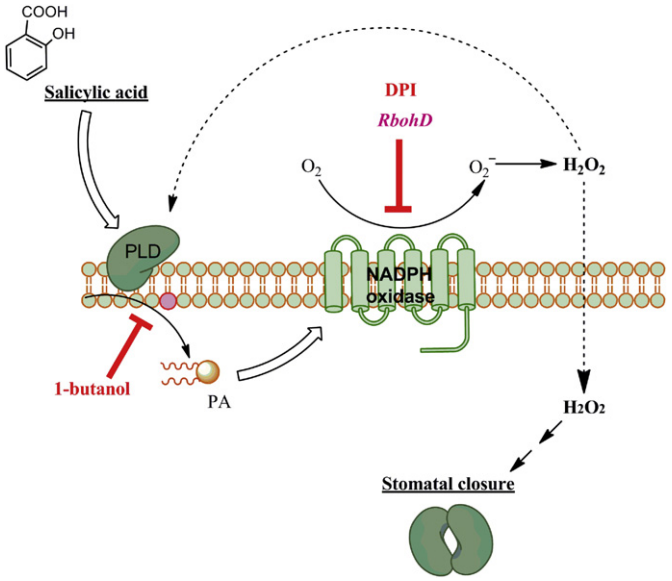

Fig. 6. Salicylic acid signal transduction cascade in guard cells involving phospholipase D and NADPH-oxidase RbohD.

centrifuged for 2 min. The lower organic phase was washed with 3.75 ml $CHCl_3/CH_3OH$/1 M HCl (3:48:47, v/v), dried under nitrogen gas stream and stored at −20 °C until further use.

4.4. Thin-layer chromatography and scintillation counting

Lipids were dissolved in 100 μl $CHCl_3/CH_3OH$ (2:1, v/v). Then samples (5 μl) were analyzed on thin-layer chromatography (TLC) plate (200 × 200 × 0.25 mm, Merck, Germany). To compare different samples, equal amounts of radioactivity were loaded on the same plate. The chromatography was performed using ethyl acetate/iso-octane/acetic acid/H_2O(12:2:3:10, v/v) as an organic upper phase. [^{33}P] phospholipids were visualized on TLC plates by autoradiography on X-ray film Retina XBM (Ukraine-Germany). Spots of equal areas were scrapped off and the radioactivity was quantified by liquid scintillation counting with SL-8 cocktail on Rack Beta 1219 counter (Finland) [26].

4.5. Histochemical assay

Induction of ROS production by NADPH-oxidase under SA infiltration was detected in histochemical assay with nitroblue tetrazolium (NBT). All the reagents in the NBT staining procedure were prepared in a 100 mM Tris–HCl buffer at pH 6.15. A 1-mL syringe with no needle was used to pressure infiltrate a spot area on one leaf half. As an infiltration solution we used 1 mM salicylic acid, 0.8% 1-butanol, 0.8% 2-butanol and 200 μM diphenylene iodide DPI in different combinations. For NBT staining, leaves were detached after infiltration and then were vacuum infiltrated in 0.1% (w/v) NBT in Tris HCl buffer and immersed in the same at room temperature for 30 min. The blue precipitates of reduced NBT were visualized after boiling the leaves in glycerol:ethanol 1:4 (v/v) for 10 min [27].

4.6. Stomatal assay

Stomatal assay was provided according to Melotto et al. with modifications [20]. Fully expanded young leaves were kept in Tris–HCl buffer (pH 6.15) under white light for 2 h to open stomata and then transferred to solution with chemicals for 30 min (1 mM salicylic acid, 1 mM salicylic acid, 0.8% 1-butanol, and 200 μM DPI, 0.15% hydrogen peroxide and 1 mM 4-hydroxybenzoic acid). Then epidermal leaf parts were observed under a microscope (Zeiss Primo Star) and pictures of random regions were taken. The width of the stomatal aperture was measured using the AxioVision 2008 software. Experiments were repeated three times with the same results.

4.7. Data analysis and statistics

Mean values, standard errors of means and the significance of differences between mean values were calculated using Excel and *t*-test statistical programs.

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