PLANT SIGNALING & BEHAVIOR
2021, VOL. 16, NO. 4, e1869415 (8 pages)
https://doi.org/10.1080/15592324.2020.1869415

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RESEARCH PAPER

Participation of calcium ions in induction of respiratory response caused by variation potential in pea seedlings

Andrey Khlopkov, Oksana Sherstneva, Maria Ladeynova, Marina Grinberg, Lyubov Yudina, Vladimir Sukhov, and Vladimir Vodeneev

Department of Biophysics, Lobachevsky State University of Nizhny Novgorod, Nizhny Novgorod, Russia

ABSTRACT

Electrical signals in plants caused by external stimuli are capable of inducing various physiological responses. The mechanisms of transformation of a long-distance electrical signal (ES) into a functional response remain largely unexplored and require additional research. In this work, we investigated the role of calcium ions in the development of ES-induced respiratory response. Gradual heating of the leaf causes the propagation of variation potential (VP) in the pea seedling. The propagation of VP leads to a transient activation of respiration in an unaffected leaf. During the VP generation, a transient increase in the intracellular calcium concentration takes place. A calcium channel blocker inhibits the respiratory response, and a calcium ionophore induces the activation of respiration. Inhibitory analysis has showed that the VP-induced increase in respiration activity is probably associated with calcium-mediated activation of rotenone-insensitive alternative NADPH dehydrogenases in mitochondria.

ARTICLE HISTORY
Received 12 November 2020
Revised 22 December 2020
Accepted 22 December 2020

KEYWORDS
Calcium ions; respiration; variation potential; heat stress

Introduction

Plants face many stress factors throughout their lives and need to adapt to them. An effective adjustment of physiological processes in a plant is ensured by the functioning of a complex of signaling systems. At the same time, rapidly growing stress factors require prompt adjustment, which develops with the participation of fast signals. Among these fast signals, electrical signals (ES) should be especially noted. ES modify the activity of many processes in a plant,[1,2] including photosynthesis and respiration. These processes provide energy exchange, which is one of the key factors in the adaptation of plants to stressful conditions. The development of ES leads to a temporary reduction of the photosynthesis activity,[3–5] as a result of which the ATP content in the cell rises.[6] As a rule, respiration is activated under the ES propagation.[6–9] However, an ES-induced decrease in respiratory activity was registered in a number of cases.[10]

Photosynthesis in plants is the main process for energy gain under conditions of sufficient lighting. At the same time, respiration plays a quite significant role in plant organism. It is the main source of energy in non-photosynthetic tissues, and also supplies ATP for the majority of cell processes, since most of the ATP produced in photosynthesis is used for the synthesis of carbohydrates.[11] In addition, mitochondrial respiration is a site of ascorbate biosynthesis,[12] as well as a source of organic acids, including those necessary for nitrogen assimilation.[13,14] Respiration contributes significantly in metabolic (intermediates of biochemical reactions and cycles) and regulatory pathways related to photosynthesis.[15,16] Such a complex concerted process provides an effective adaptation of the plant to changing environmental conditions.

Being one of the key physiological processes in plant, respiration undergoes regulation at different stages and in different time intervals, from relatively long changes of protein transcription and translation to rapid changes of the activity of enzymes or respiratory complexes.[15,17,18] However, the mechanism of such rapid regulation, in particular, in response to the propagation of stress electrical signals, is still unclear. It can be assumed that the short-term and rapid changes of respiration observed under the ES propagation are associated with a drastic change in ionic concentrations. The role of ionic concentration in the regulation of physiological processes has been well studied in case of photosynthesis.[19] In particular, the H^+ and Ca^{2+} ions can play a key role in triggering changes of the photosynthetic activity. During ES generation, an influx of Ca^{2+} ions into the cell occurs, which causes a temporary inhibition of the H^+-ATPase activity and, therefore, a shift in the intra- and extracellular pH.[19–21] Such pH shifts were shown to modify photosynthesis activity by regulating CO_2 influx into the cell, as well as changes in the activity of the electron transport chain of chloroplasts. Along with the pH shifts that occur during ES generation, the increase in intracellular calcium concentration also appears to have a direct impact on photosynthetic activity.[22] It is supposed that the rise in the cytoplasmic calcium concentration during ES generation stimulates Ca^{2+} influx into chloroplast stroma, which can cause an inactivation of enzymes of Calvin cycle[23,24] and lead to acidification of thylakoid lumen and enhancement of non-photochemical quenching of fluorescence. It is also known that calcium has a significant effect on the activity of a number of enzymes and complexes involved in respiration,[25–29] including alternative NADPH-dehydrogenases[25–28] or complex IV.[29]

CONTACT Vladimir Vodeneev v.vodeneev@mail.ru Department of Biophysics, State University of Nizhny Novgorod, Nizhny Novgorod 603950, Russia.

Considering the fact that the increase in the cytoplasmic concentration of calcium, which takes place during the ES generation, can lead to calcium increase in the mitochondrial matrix,[30,31] it seems very likely that Ca^{2+} can be involved in the respiration response formation.

The aim of this work was to study the mechanism of ES-induced changes in respiratory activity, in particular, the elucidating of the Ca^{2+} ions role in this process.

Materials and methods

Plant material

Studies were carried out on 10–16-d-old pea seedlings (*Pisum sativum L.*) cultivated hydroponically in a Binder KBW-240 plant growth chamber (Binder GmbH, Tuttlingen, Germany) at 24°C under a 16/8-h light/dark photoperiod. Some of the experiments were performed on the model systems: protoplasts isolated from the leaves of pea seedlings, and intact leaves detached from the whole plant.

Electrical measurements

Plant electrical activity was measured by a system for recording surface potentials composed of surface Ag^{+}/AgCl electrodes EVL-1M3.1 (Gomel Plant of Measuring Equipment, Gomel, Belarus), a three-channel high-impedance amplifier IPL-113 (Semico, Novosibirsk, Russia), and a personal computer. Measuring electrodes contacted with a plant by cotton threads wetted with standard solution (1 mM KCl, 0.5 mM $CaCl_2$, 0.1 mM NaCl). The measuring electrodes were arranged on the stem in the internode below the stimulated leaf (E_s) and on the leaflet paired to the leaflet, the respiration activity of which was recorded (E_l), the reference electrode (E_R) was placed in standard solution surrounding the plant root (Figure 1d).

Electrical signal was induced by gradual heating of the second adult leaf immersed in a thermostated cuvette with water. Heating was carried out from an initial temperature of 23°C to 55–60°C with a rate of 7–9°C per minute.

Gas exchange measurements in intact plants

Activity of a plant leaf respiration was recorded using a system that includes an infrared gas analyzer GFS-3000, a measuring head Dual-PAM gas-exchange Cuvette 3010-Dual (Heinz Walz GmbH, Effeltrich, Germany), and a personal computer (the measured area was 1.3 cm^2) (Figure 1).

The plant was placed in the measuring system with roots immersed in a standard solution. A leaflet of the third adult leaf (the first leaf below the stimulated one) was placed in the cuvette of the measuring head. After 20 minutes, the air supply to the cuvette and the recording of the leaf gas exchange parameters were started. The air temperature in the cuvette was 23°C, the CO_2 concentration was 360 µmol mol^{-1}, and the relative humidity was about 60%. The measurements were carried out in the dark. All the plants adapted for 60 minutes before the stimulation. The registration of respiration and electrical activity was performed simultaneously.

Inhibitory analysis of the mechanisms of respiration response

To study the mechanisms of development of the VP-induced respiratory response, we used the following specific inhibitors: rotenone (mitochondrial complex I inhibitor), NaN_3 (complex IV inhibitor), DPI (diphenyleneiodonium chloride, inhibitor of rotenone-insensitive alternative NADPH dehydrogenase), SHAM (salicylhydroxamic acid, alternative oxidase inhibitor), $LaCl_3$ (calcium channel blocker). Before recording, both leaflets from the node under study were incubated for 40 minutes in water (control group) or the corresponding inhibitor solution (0.1 mM rotenone, 20 µM DPI, 0.5 mM NaN_3, 10 mM SHAM, 5 mM $LaCl_3$).

In a separate series of experiments, the detached intact leaf of pea seedling was used as the object of the study. In this case, the leaflet was placed in a gas analyzer cuvette, and the petiole was placed in a cell with a standard solution. The adaptation period before the start of measurements was 90 minutes. 10 minutes after the start of recording, a calcium ionophore A23187 (Sigma-Aldrich, USA) was added to the solution surrounding the petiole cut (final concentration of 1 µM).

Registration of the respiration activity in pea protoplasts

The protoplasts were isolated from the leaves of intact pea seedlings in a medium containing 0.4 M sorbitol, 20 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 30 mM MES-KOH, pH 5.5,[32] with the addition of 1% cellulase, 0.2% pectinase and 0.2% BSA. The resulting suspension was passed through a filter with a pore diameter of 50 µm and centrifuged for 5 minutes (30 g, 4°C), and then the precipitated protoplasts were resuspended in the isolation medium.

The protoplast respiration activity was recorded by the polarographic method using an Oxygraph Plus System (Hansatech Instruments Ltd, Norfolk, United Kingdom). 1 ml of medium and 0.2 ml of protoplast suspension were placed into a cuvette located above the cathode of the oxygen electrode. The measurement was carried out at t = 25°C and in an opaque box to protect the protoplasts from light. A magnetic stirrer integrated into the control unit was used to prevent protoplast sedimentation.

3 minutes after the start of recording, 20 µl of A23187 solution with a final concentration of 0.2 µM (experimental record) or 20 µl of the solvent in which A23187 was dissolved (control record) were added into the cuvette using a microsyringe (Hamilton, USA).

Measurements of intracellular calcium concentration

Ca^{2+}-sensitive fluorescent dye fluo-4, AM (Ex/Em = 494/516 nm, Molecular Probes, USA) was used to study the dynamics of intracellular calcium concentration. A part of the stem of the intact pea seedling between the 2nd and 3rd adult leaves was incubated in 20 µM dye solution (1 mM fluo-4, AM solution in DMSO was diluted to 20 µM using a standard solution) for 17 hours at t = 4°C. Then the plant was washed in standard solution at room temperature for about 2 hours. The dynamics of Ca^{2+} concentration during the propagation of

an electrical signal was registered with an LSM 710 NLO system (Carl Zeiss Microscopy GmbH, Germany). The pea seedling was placed on the microscope stage with roots immersed in a standard solution. Fluorescence was excited at 488 nm and recorded using two channels: the first channel (500–550 nm) corresponded to the fluorescence of the dye; the second channel (620–735 nm) corresponded to the chloroplast autofluorescence. The acquisition time for a separate image in a time series was 7.75 s.

Ratiometric Ca^{2+}-sensitive fluorescent dye fura-2, AM (Ex/Em = 340/505 nm, Molecular Probes, USA) was used for a precise quantitative analysis of the changes in calcium intracellular concentration. The dye was loaded as described above. The concentration of fura-2, AM in the incubation medium was 10 μM. The dye fluorescence was registered using a Shimadzu RF-5301 PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan) equipped with an optical fiber and device for measuring outside the cuvette section ('Lyagushka' device, PCG 'Granat', Russia). Simultaneously with the recording of fluorescence, the recording of electrical activity was carried out. The ratio of the dye fluorescence excited at 380 and 340 nm was used for ratiometric analysis. Fluorescence was registered at 505 nm. The plant adaptation before the stimulation lasted for 60 min; the recording continued for 60 min after the plant stimulation.[4]

Statistics

Each experiment was performed on a separate plant. The number of repetitions in each series of experiments is presented in the Figures' legends. Representative records of individual measurements, averaged curves, mean values and standard errors are presented. Pearson correlation coefficient was used for correlation analysis. Statistical analysis was performed using Student's *t*-test.

Results

Influence of variation potential on respiration activity

The influence of the variation potential (VP) induced by gradual heating on the gas exchange intensity was studied in an unaffected leaf of the pea seedling (Figure 1). Local heating caused the propagation of an electrical signal – a variation potential, with the amplitude of 60 ± 4 mV registered in the stem at a distance of about 3 cm from the stimulation zone. In the leaf at a distance of about 6 cm from the stimulation zone, where the registration of gas exchange was carried out, the amplitude of the electrical signal was 36 ± 4 mV (Figure 1a). An increase in respiratory activity associated with VP propagation was recorded in 94% of cases. The activation of respiration occurred one minute after VP propagation and had amplitude of 0.83 ± 0.07 μmol m^{-2} s^{-1} and duration of about two minutes (Figure 1c). In cases when VP did not propagate into the unstimulated leaf, there was no pronounced change in respiratory activity (Figure 1b). The dependency of the amplitude of respiration changes on the VP amplitude is shown in Figure 2. The Pearson correlation coefficient (r) between the amplitudes of the electrical signal and respiratory response in the studied leaf was 0.7 ($p < .0001$).

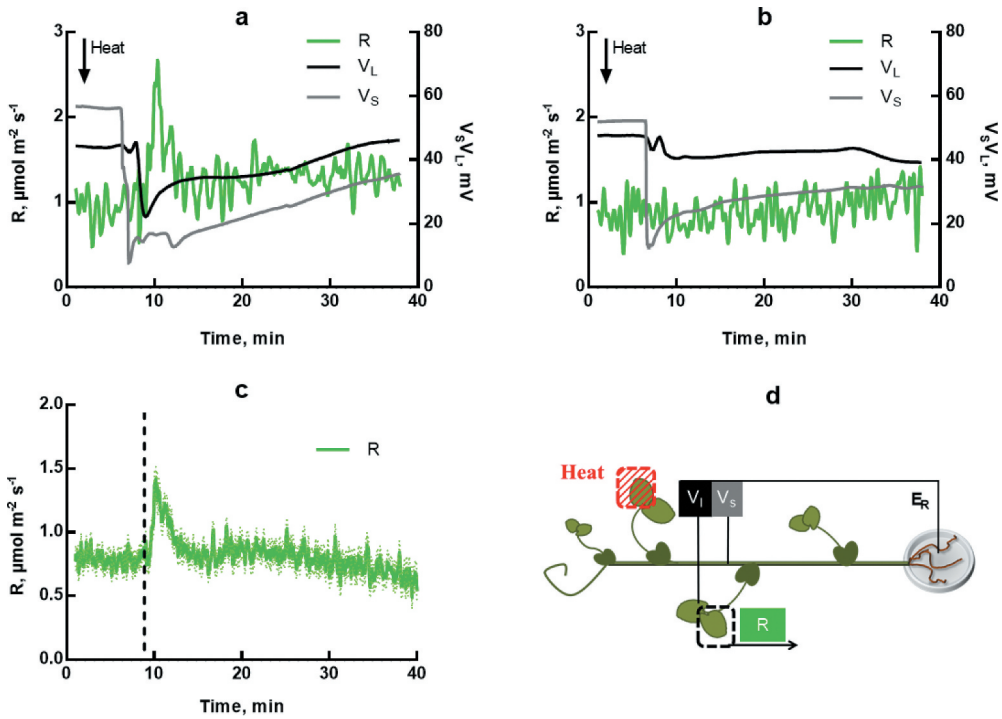

Figure 1. Dynamics of electrical and respiration activity induced by local heating of the leaf of pea seedlings. (a). Electrical signal propagated into the lamina of the unstimulated leaf (typical record). (b). Electrical signal did not propagate into the lamina of the unstimulated leaf (typical record). (c). Dynamics of respiration activity in the unstimulated leaves of pea seedlings induced by local heating ($n = 41$). The dotted line indicates the moment of the VP generation. (d). Scheme of the experimental system for measuring stem (V_S) and leaf (V_l) surface potential and activity of respiration (r). The reference electrode (E_R) was placed in contact with plant roots. The black arrow indicates the start of the local heating.

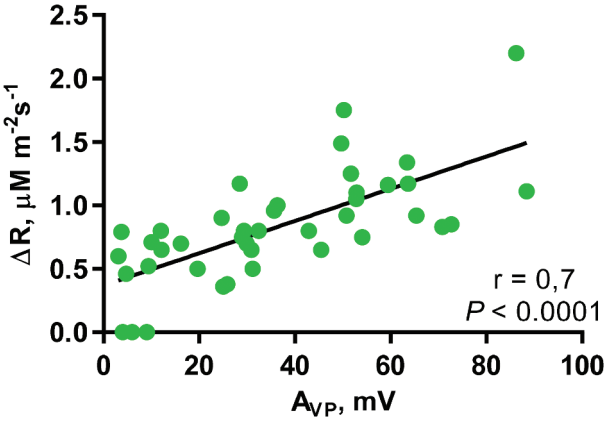

Figure 2. Correlation analysis of the amplitude of the respiration activity changes (ΔR) with the VP amplitude (A_{VP}) in the unstimulated pea leaf induced by the local heating (*n = 41*). Pearson correlation coefficient was used for analysis. *t*-test, $p < 0.0001$.

Dynamics of intracellular Ca^{2+} concentration during the generation of variation potential

Figure 3a shows a fluorescent image of pea seedling cells loaded with fluo-4, AM fluorescent dye, responding to an increase in the Ca^{2+} concentration with fluorescence enhancement. VP generation was shown to be accompanied by a transient rapid Ca^{2+} concentration increase followed by its slow recovery within several minutes (Figure 3a). A quantitative analysis of changes using the fura-2, AM ratiometric fluorescent dye showed that the amplitude of the intracellular Ca^{2+} concentration changes was 110 ± 17 nM (Figure 3b).

Influence of the intracellular Ca^{2+} concentration increase on respiratory activity

The analysis of the direct effect of an intracellular Ca^{2+} rise on the respiration activity was carried out on model systems: protoplasts isolated from the leaves of pea seedlings and a detached pea leaves. The addition of the A23187 ionophore, which induces Ca^{2+} influx into the cell, to the protoplast suspension led to a transient increase in O_2 consumption rate (Figure 4a). The amplitude of the changes was 0.8 ± 0.2 nmol O_2 ml^{-1} min^{-1}. The duration of the respiration activation was about 7 minutes.

In experiments on a detached leaf, ionophore A23187 was added to the solution surrounding the petiole cut. The addition of a calcium ionophore led to a slow increase in respiration activity in the leaf (by 0.36 ± 0.14 μmol m^{-2} s^{-1}), which began after the ionophore addition (Figure 4b).

Along with the experiments on model systems with artificially increased Ca^{2+} concentration, the role of calcium ions in the induction of respiration responses was also investigated in experiments with blocking Ca^{2+} influx into the cell on whole plants. The calcium channel blocker $LaCl_3$ caused a decrease in the amplitude of the respiratory response by 73 ± 14% compared to the control (Figure 5). Along with this, there was a decrease in the VP amplitude in the leaf to 14 ± 4 mV (37 ± 10% of control).

Inhibitory analysis of the mechanisms of respiratory response

The mechanisms of the VP-induced respiratory response development were investigated using specific inhibitors of the mitochondrial electron transport chain (ETC) components: rotenone (complex I), NaN_3 (complex IV), DPI (an alternative rotenone-insensitive NADPH dehydrogenase), SHAM (an alternative oxidase) (Figure 6). The specific inhibitor of complex I, rotenone, caused a tendency to decrease in the respiratory response (but without statistical significance) (Figure 6a). The alternative oxidase inhibitor SHAM also had no effect except a slight insignificant increase in the respiratory response. In the presence of the complex IV inhibitor sodium azide, there was a tendency to a decrease in the amplitude of the respiratory response; however, no statistically significant decrease was found either.

The use of a specific inhibitor of the alternative rotenone-insensitive NADPH dehydrogenase DPI caused a statistically significant decrease in the amplitude of the respiratory response to 0.5 ± 0.06 μmol m^{-2} s^{-1} (Figure 6a). At the same time, despite the downward trend, no statistically significant changes in the VP amplitude were revealed (Figure 6b).

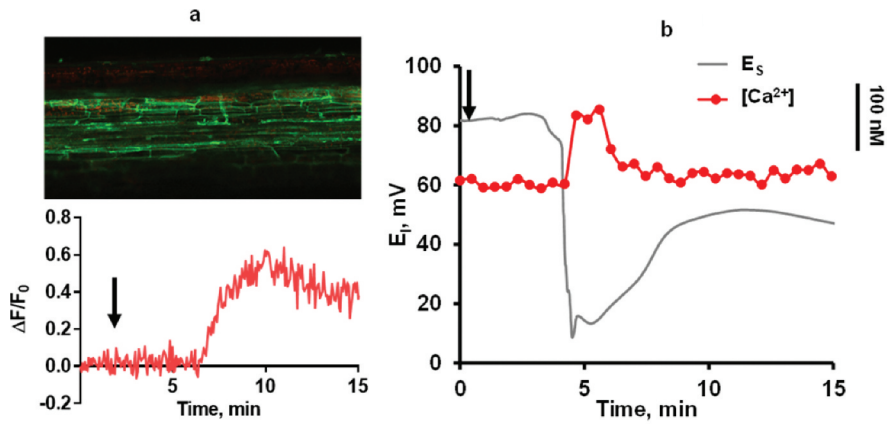

Figure 3. Dynamics of intracellular calcium concentration during the VP generation in pea seedlings. (a). Dynamics of the fluorescence intensity of a Ca^{2+}-sensitive fluorescent dye fluo-4, AM, loaded into stem cells, induced by local heating of the second adult leaf of pea seedling. The inset shows the fluorescence image of pea seedling stem cells loaded with fluo-4, AM fluorescent probe. (b). Changes of the electrical potential (V_S) and intracellular Ca^{2+} concentration (investigated using the fura-2, AM ratiometric fluorescent dye) in pea stem induced by local heating of the second adult leaf. The black arrow indicates the start of the local heating.

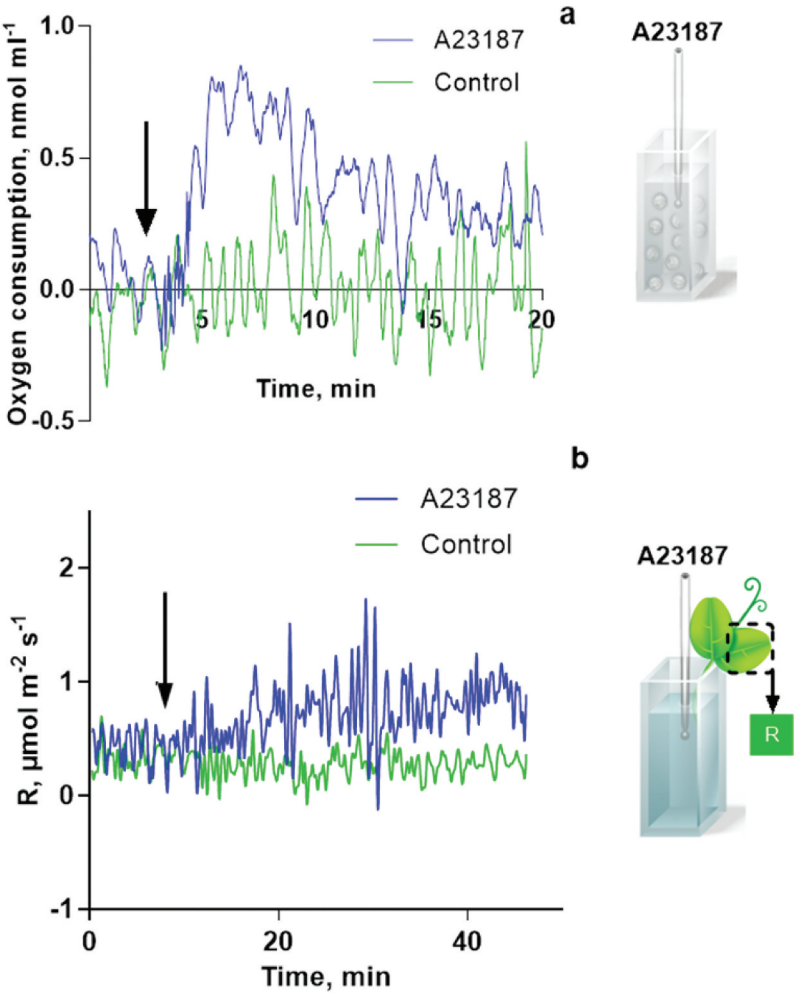

Figure 4. Dynamics of gas exchange parameters induced by the Ca^{2+} ions influx into the cell (using calcium ionophore A23187). (a). Respiration activity (Oxygen consumption) in pea leaf protoplasts after the ionophore addition into the medium. The figure shows the curves averaged over 6 experimental and control measurements. (b). Activity of respiration (r) in pea leaves induced by the ionophore addition into the medium surrounding the leaf petiole (typical record). The arrow indicates the moment of addition of the ionophore (A23187) or equal volume of the solvent (control).

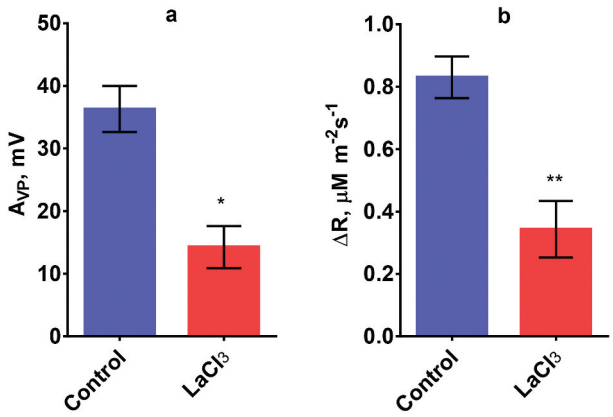

Figure 5. The effect of the calcium channel blocker $LaCl_3$ on the amplitude of VP (A_{VP}) and amplitude of respiration response (ΔR) induced in unstimulated leaf by the heating ($n = 6$). Means ± SE, *t*-test, * – $p < .05$, ** – $p < .01$.

Analysis of the effect of the studied inhibitors on the VP amplitude in the treated leaf (Figure 6b) showed that only sodium azide caused a reduction of the VP amplitude (13 ± 5 mV versus 36 ± 4 mV in the control).

Discussion

The performed study demonstrated that gradual heating of the leaf of pea seedling causes a variation potential, the propagation of which induces a temporary activation of respiration in unstimulated leaves. The presence of a correlation between the VP amplitude and the respiratory response (Figure 2) indicates the role of VP in the induction of a systemic response caused by local heating. This result is in a good agreement with the published data on the key role of ES in the induction of changes in respiration and photosynthesis activity.[2,6,19,33]

It can be assumed, that respiration activation can be resulted from the activation of gas exchange due to an increase in the degree of stomatal openness, or by the regulatory VP influence on intracellular processes. The results obtained in the work[34] show that alteration of respiratory activity due to degree of stomatal openness is unlikely. Thus the generation of ES in the trap of *Dionaea muscipula* was shown to be accompanied by transient activation of respiration in the dark; however, stomatal conductance was not affected. The same conclusion was made in the work,[33] in

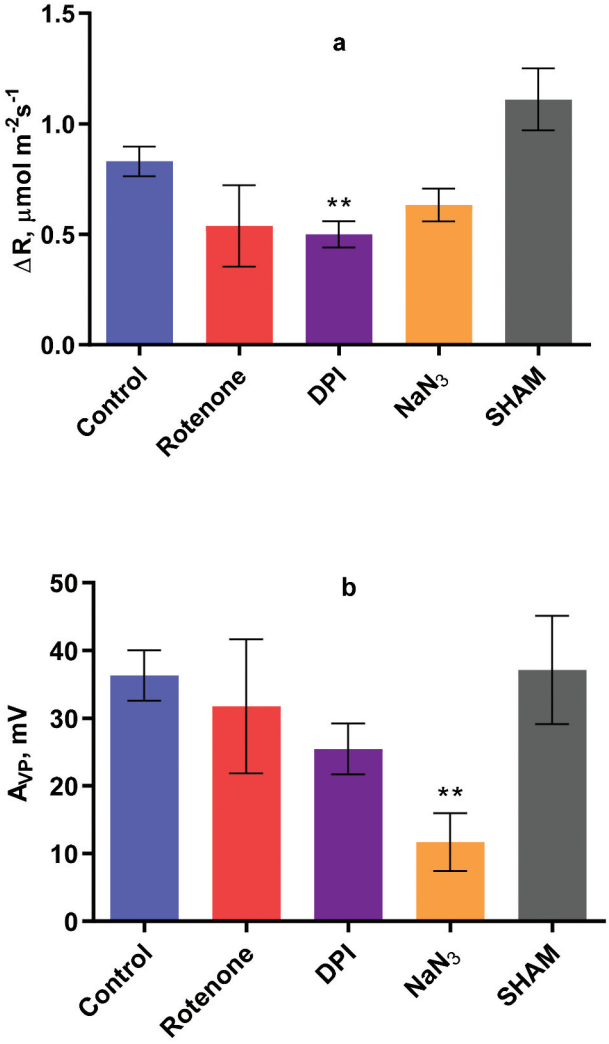

Figure 6. The effect of the mitochondrial ETC complex I inhibitor rotenone ($n = 6$), alternative NADPH dehydrogenase inhibitor DPI ($n = 6$), mitochondrial ETC complex IV inhibitor NaN_3 ($n = 6$), and alternative oxidase inhibitor SHAM ($n = 6$) on (a) the amplitude of respiration (ΔR) response and (b) the amplitude of VP (A_{VP}) induced in unstimulated leaf by the local heating. Means ± SE, *t*-test, ** – $p < .01$.

which a small contribution of this mechanism to the change in respiration caused by an electrical signal in *Conocephalum conicum* was noted.

Another probable mechanism of the VP-induced respiratory response is the regulation of mitochondrial respiration activity. It is believed that one of the key regulatory factors is the ratio of ATP/ADP in the cell. This mechanism was considered in work of Pavlovic and co-workers,[8] in which it was suggested that the activation of respiration observed during the ES generation in *Dionaea muscipula* is associated with a drastic consumption of ATP (≈29% of the total pool) during ES-induced trap closure, which led to an elevated ADP content and activation of the respiratory enzymes. However, it seems likely that the decrease in ATP content associated with ES propagation is typical of plants capable of rapid movement. For example, no significant changes in the ATP content were found in maize plants after the propagation of ES, induced by both cold and damage.[35] In addition, even an increase of ATP content 5 minutes after VP propagation was observed in pea plants.[6] These results make it unlikely that a decrease in ATP content causes the respiration activation in peas, in contrast to plants capable of rapid movement.

A change in the intracellular ionic concentrations, in particular, the most important intracellular messenger Ca^{2+}, can be considered as a probable mechanism for the induction of the respiratory response caused by VP. It should be noted that long-distance stress signals in plants have a complex nature and may integrate ROS, calcium and electrical potential waves.[36,37] The calcium wave, which represents a transient increase in the intracellular Ca^{2+} concentration, develops along with the wave of electrical potential during VP propagation (Figure 3). The possible role of calcium ions in the activation of respiration is supported by the data obtained in our work: Ca^{2+} influx suppression leads to inhibition of the respiratory response (Figure 5), and an artificial increase in Ca^{2+} using an ionophore is sufficient to activate respiration (Figure 4). The published data on the sensitivity of a number of ETC enzyme complexes in plant mitochondria to Ca^{2+} concentration[25–29] allow us to consider them as potential targets. The performed inhibitory analysis showed that the VP-induced activation of respiration is not associated with the activation of alternative oxidase. The significant inhibition of the respiratory response in the presence of a specific inhibitor of alternative mitochondrial NADPH dehydrogenases may indicate the primary role of this enzyme in VP-induced respiratory activation. At the same time, the observed tendency to a decrease of the VP-induced respiratory response amplitude under the action of rotenone and NaN_3 may indicate the involvement of complexes I and IV in this process.

Based on the data obtained, we hypothesized the following mechanism of induction of the respiration response caused by an electrical signal (Figure 7). Local stimulation causes the propagation of the variation potential, the generation of which is associated with the activation of ligand-dependent calcium channels[38] and the calcium influx into the cell, causing an increase in its concentration in the cytosol and, probably, in mitochondria. An increase in calcium concentration leads to the activation of rotenone-insensitive alternative NADPH dehydrogenases. Activation of these enzyme complexes of ETC in plant mitochondria, directly or indirectly, increases the rate of oxidation of NADPH and NADH in the ETC, which, in turn, accelerates the work of the tricarboxylic acid cycle and, as a result, enhances the CO_2 release observed in the experiment. It should be noted also, that the possible role of other components of the complex stress signal, which represents interacting calcium, ROS and electrical potential waves,[36] in the development of the respiratory response cannot be neglected. In particular, an increase in ROS content can contribute to this process.[39,40]

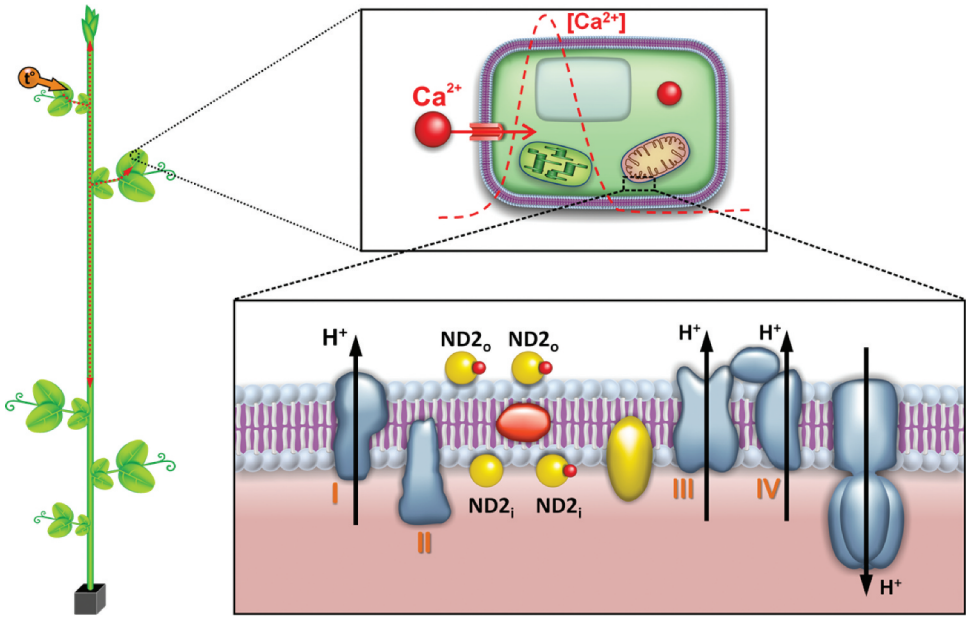

Figure 7. A scheme of the possible mechanism of respiration activity changes under ES generation. Local leaf heating causes an electrical signal generation and propagation through the plant. The development of ES in intact parts of the plant is accompanied by a transient increase in the intracellular concentration of Ca^{2+} ions. Such an increase can cause changes in the functioning of various components of the mitochondrial respiratory chain, primarily the rotenone-insensitive alternative NADPH dehydrogenases, the activation of which leads to an acceleration of cellular respiration reactions.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Ministry of Science and Higher Education of the Russian Federation for large scientific projects in priority areas of scientific and technological development under grant number [075-15-2020-774].

ORCID

Oksana Sherstneva http://orcid.org/0000-0001-8497-7676
Lyubov Yudina http://orcid.org/0000-0002-6702-2445
Vladimir Sukhov http://orcid.org/0000-0002-8712-9127
Vladimir Vodeneev http://orcid.org/0000-0002-3726-5577

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