1   **Cover Page**

2   **Title: Long-Distance Electrical and Calcium Signals Evoked by Hydrogen Peroxide in**
3   **Physcomitrella**

4   **Short title:** Hydrogen peroxide-evoked signals in Physcomitrella

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60 **Abstract**

61 Electrical and calcium signals in plants are one of the basic carriers of information transmitted

62 over a long distance. Together with reactive oxygen species (ROS) waves, electrical and

63 calcium signals can participate in cell-to-cell signaling, conveying information about different

64 stimuli, e.g. abiotic stress, pathogen infection, or mechanical injury. There is no information

65 on the ability of ROS to evoke systemic electrical or calcium signals in the model moss

66 Physcomitrella and on the relationships between these responses. Here, we show that external

67 application of hydrogen peroxide evokes electrical signals in the form of long-distance

68 changes in the membrane potential, which transmit through the plant instantly after

69 stimulation. The responses were calcium dependent, since their generation was inhibited by

70 lanthanum, a calcium channel inhibitor (2 mM) or EDTA, a calcium chelator (0.5 mM). The

71 electrical signals were partially dependent on glutamate receptor ion channels (GLR), since

72 the knockout of GLR genes only slightly reduced the amplitude of the responses. The basal

73 part of the gametophyte, which is rich in protonema cells, was the most sensitive to hydrogen

74 peroxide. The measurements carried out on the protonema expressing fluorescent calcium

75 biosensor GCaMP3 proved that. We also demonstrate upregulation of a stress-related gene

76 which appears in a distant section of the moss 8 minutes after H_2O_2 treatment. The results

77 help to understand the importance of both types of signals in the transmission of information

78 about the appearance of ROS in the plant cell apoplast.

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Introduction

As an early terrestrial plant, the moss Physcomitrella (new botanical name *Physcomitrium patens*), from the beginning of the adaptation to land conditions, had to cope with exposure to UV radiation, ozone, wounding, and many other abiotic and biotic stressors that enhance the synthesis of reactive oxygen species (ROS) (Rensing et al., 2020, 2008; Tucker et al., 2005). Physcomitrella is an emerging model plant with a fully sequenced genome (Rensing et al., 2008; Lang et al., 2018). It is widely used to study defense mechanisms against environmental stress factors (Frank et al., 2005; Hoernstein et al., 2023; Koselski et al., 2019; Saidi et al., 2009). It was previously demonstrated in our lab that Physcomitrella is capable of generating action potentials (APs) after illumination with light of sufficient (over-threshold) intensity, cooling, glutamate (Glu) treatment, etc. (Koselski et al., 2020, 2019, 2008). Electrophysiological experiments with the application of ion channel inhibitors and manipulation of ion gradients across the plasma membrane indicated that Ca^{2+} fluxes from external and internal stores were involved in the generation of APs in Physcomitrella. They interacted with K^+ and, to a lesser extent, with Cl^- fluxes. Our recent study on GCaMP mutants allowing monitoring of changes in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) demonstrated that local Glu application caused an increase in $[Ca^{2+}]_{cyt}$ confined to the site of the Glu treatment, whereas AP was transmitted to distant cells (Koselski et al., 2020). This was rather an unexpected result, because in other plant species examined before, action potentials or variation potentials (VPs, known also as Slow Wave Potentials, SWPs) spread together with Ca^{2+} waves and seem mutually dependent (Choi et al., 2017, 2014; Gilroy et al., 2016). In the model vascular plant *Arabidopsis thaliana* two glutamate receptors *At*GLR3.3 and *At*GLR3.6 were identified as key players in wound-induced SWP generation and transmission (Farmer et al., 2020; Mousavi et al., 2013). Both genes are predominantly expressed in vascular tissues: phloem and xylem contact cells, respectively. Glutamate (and other amino acids) released from wounded tissues binds to the Ligand Binding Domain (LBD) subunit of the glutamate receptor-like channels facilitating opening of the channel pore and in consequence initiating a Ca^{2+} wave and other downstream responses, like jasmonate (JA) key genes and other stress-related gene expressions (Grenzi et al., 2022).

According to the recent findings, in vascular plants, in addition to the ion fluxes facilitating regenerative $[Ca^{2+}]_{cyt}$ wave formation and transmission, the ROS based component supplements the long-distance signaling system (Baxter et al., 2014; Gilroy et al., 2016; Kurusu et al., 2015; Marcec et al., 2019). NADPH oxidases (respiratory burst oxidase

116 homologs, RBOHs) localized in the plasma membrane seem to be good candidates to fit this

117 scheme. RBOHs cause reduction of oxygen to superoxide anion (O_2^{-}), which is quickly

118 converted to H_2O_2. They possess EF-hand motives which activate them upon binding of Ca^{2+}

119 to enhance ROS production (Marcec et al., 2019; Wojtaszek, 1997). Before the machinery of

120 ROS scavenging reduces the ROS level back to normal, its temporary excess affects many

121 metabolic and signaling pathways and their components. H_2O_2 produced in the apoplast can

122 be quickly transported to the cytosol via aquaporins (Tian et al., 2016). Recently, a specific

123 apoplastic H_2O_2 receptor was discovered, Hydrogen-Peroxide-Induced Ca^{2+} Increases 1

124 (HPCA1) (Wu et al. 2020). It is a membrane-spanning protein composed of an extracellular

125 H_2O_2 sensor and a cytoplasmic kinase component, which is postulated to activate Ca^{2+}-

126 permeable channels after H_2O_2 binding to the sensor (Foyer, 2020; Wu et al., 2020). Thus, ion

127 channels can be affected from both sides of the plasma membrane. It was previously

128 demonstrated that ROS can activate ion channels in different plant species classified in

129 different branches of the phylogenetic tree of characean algae (Demidchik et al., 1997) to

130 dicots and monocots (Wu et al., 2015).

131 In vascular plants, hydrogen peroxide and hydroxyl radicals affect Ca^{2+}-permeable channels

132 in the plasma membrane and in the internal store – the vacuole. In *Arabidopsis thaliana*, two

133 classes of non-selective calcium-permeable channels are postulated to be involved: GLR

134 channels activated by glutamate and cyclic nucleotide-gated channels CNGCs (Finka et al.,

135 2012; Mousavi et al., 2013). Additionally, annexin1 has been reported to be involved in ROS-

136 induced $[Ca^{2+}]_{cyt}$ elevation (Richards et al., 2014). It has been demonstrated that homologs of

137 GLU and CNGC channels were involved in signaling processes in Physcomitrella (Finka and

138 Goloubinoff, 2014; Koselski et al., 2020; Ortiz-Ramírez et al., 2017). In addition to calcium-

139 permeable channels, K^{+} channels in the plasma membrane have been found to respond to an

140 increase in the ROS level (Demidchik et al., 2010). Massive K^{+} efflux in ROS-treated plants

141 was one of the first observations of plant response to this type of stress factors (Demidchik et

142 al., 2010; Nassery, 1979). In Arabidopsis, GORK (Guard Cell Outward Rectifying K^{+}) and

143 SKOR (Stellar Potassium Outward Rectifier) channels have been identified to be responsible

144 for that effect (Demidchik, 2018). These channels are also regarded as good candidates to

145 pass an outward current during the AP repolarization phase in excitable plants (Cuin et al.,

146 2018).

147 In the present study, we examined the effects of H_2O_2 treatment of intact Physcomitrella.

148 Electrical potential changes and $[Ca^{2+}]_{cyt}$ transients were measured in wild type plants (WT)

149 and $glr1^{KO}$ mutants treated with H_2O_2. We demonstrated that in the WT plants, H_2O_2 evoked

150 APs recorded both in the gametophyte leaves and protonema. The susceptibility of the

151 protonema to the treatment was much higher than in the leaf cells (0.5 mM versus 5 mM).

152 These signals were blocked by La^{3+} - a calcium channel inhibitor and by EDTA – a Ca^{2+}

153 chelator. In the $glr1^{KO}$ mutants, the electrical signals were reduced but not totally blocked,

154 which reveals that GLR channels are not crucial in H_2O_2-induced membrane potential

155 changes and Ca^{2+} fluxes in Physcomitrella. Involvement of other factors affecting $[Ca^{2+}]_{cyt}$ is

156 discussed. Looking for downstream effects of the signals, we demonstrate an enhanced

157 expression of stress-related genes.

Results

159 *Electrical signals in leaf cells*

160 The microelectrode measurements carried out on leaf (phylloid) cells from the Physcomitrella

161 gametophyte proved that the application of hydrogen peroxide evoked systemic electrical

162 signals in the form of membrane-potential changes transmitted along the plant. The long-

163 distance responses transmitted from the basal part of the plant (from the rhizoid side) and

164 recorded in apical leaf cells are presented in Fig. 1A. Experiments with different H_2O_2

165 concentrations indicate an influence of the concentration on amplitude and shape of

166 membrane potential changes recorded during the response. Stimulation of the basal part of the

167 plant with 0.05 mM and 0.1 mM H_2O_2 evoked irregular membrane potential changes with

168 similar amplitude, duration, and rate of depolarization (Fig. 1A, Tab 1). After an increase of

169 H_2O_2 concentration to 0.5 mM, significant changes in basic parameters describing the

170 membrane potential changes were obtained (Fig. 1A, Tab 1). The cell membrane depolarized

171 over twice as fast than in the lower concentrations (14.5±1.6 mV/s, n=18), and reached more

172 positive values. The amplitude of the depolarization amounted to 68±3 mV (n=19) and was

173 about 30 mV higher than after treatment with 0.05 and 0.1 mM, H_2O_2, respectively. Another

174 difference occurred in the shape of the responses, where after the peak of depolarization

175 evoked by 0.5 mM H_2O_2, a several-minute plateau of the membrane potential was recorded.

176 Hydrogen peroxide in the concentration of 0.5 mM was used to compare transmission ability

177 of electrical signals in the opposite direction - from the apical to the basal part of the plant

178 (Fig. 1B). The electrical signals transmitted from the apical part of the plant characterized in a

179 lower rate of depolarization (2.3±0.4 mV/s, n=8), amplitude (22±5 mV, n=9) and more

180 negative values of maximum depolarization (-142±8 mV, n=9). The additional feature was an

181 absence of a characteristic plateau of the membrane potential. The shape of the membrane
182 potential changes resembled the responses obtained after direct stimulation of a cell in the
183 apical part of the plant (Fig. 1C) or removing of the basal part (Fig. 1D), where a 10-fold
184 increase in the H_2O_2 concentration (to 5 mM) evoked responses similar to these transmitted
185 from the basal part after 0.5 mM H_2O_2 administration. These results indicate that the highest
186 susceptibility to hydrogen peroxide occurs in the basal part of the plant having protonema
187 cells and rhizoids, which act as an initial place for the generation of long-distance electrical
188 signals propagated toward the plant apex. The results also showed that an increase of the
189 H_2O_2 concentration facilitates generation of fully developed responses resembling action
190 potentials (APs).

191 Action potentials belong to the basic long-distance electrical signals recorded in plant cells
192 whose generation is dependent on an influx of calcium ions into the cytoplasm. We decided to
193 study the dependence of the recorded responses on the presence of an inhibitor of calcium
194 channels (2 mM lanthanum) or a calcium chelator (0.5 mM EDTA), respectively (Fig. 2 A,
195 B). Apart from effects of lanthanum and EDTA, the possibility to reverse the evoked effects
196 was also examined. Each plant was stimulated twice - after initial immersion for 3-4 hours in
197 lanthanum or EDTA, and then after the exchange of the solution back to the standard solution.
198 Immersion of the plants in lanthanum caused a total blockage of the response in 10 of the 19
199 tested cells. The responses in the other plants had a significantly reduced amplitude (to 9±2
200 mV, n=9). Lanthanum also shifted the resting potential to positive values (to -130±3 ± mV,
201 n=19). The depolarization after the lanthanum application was slower (the rate amounted to
202 0.5±0.2 ±mV/s, n=8) and reached more negative values (-128±5, n=9). After a washout of
203 lanthanum from the measuring chamber, the amplitude of the responses increased to 27±4 mV
204 (n=4), which indicated that inhibition of the responses by lanthanum is partially reversible.
205 EDTA totally blocked the responses in 9 of the 18 tested cells. Similar to lanthanum, EDTA
206 evoked reduction of the amplitude (to 13±4 mV, n=9), shift of the resting potential to more
207 positive values (to -122±5-± mV, n=18) and reduction of the rate of depolarization (to
208 1.4±0.7±mV/s, n=7). The shift of the resting potential after EDTA was partially reversible,
209 since a washout resulted in a restoration of the resting potential close to the values measured
210 in standard solution (-144±4 mV, n=18). The comparison of all these parameters of the
211 membrane potential changes is compiled in Table 2.

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214   *Electrical signals in protonema cells*

215   The results of the measurements carried out on the leaf cells indicated that it is hard to evoke

216   electrical signals in such cells, and the responses can start mainly from the protonema cells

217   and/or rhizoids - probably the target for the action of hydrogen peroxide. In order to study this

218   hypothesis, we decided to examine the effect of H_2O_2 on electrical membrane potential

219   changes in protonema cells. In these measurements, stimulation was carried out by

220   microinjection of H_2O_2 in three regions: initially into a chain of protonema cells adjacent to

221   the tested cell with the inserted microelectrode, then directly into the tested cell, and at the

222   end into the gametophyte base.

223   The results confirmed that, although electrical signals in the chain of protonema cells are

224   transmitted from cell to cell, the responses recorded in the same cell differed depending on the

225   region of stimulation (Fig. 4, Tab. 3, Supplementary Video S1). The stimulation of the

226   gametophyte base was the most effective, as it evoked electrical signals in each tested cell

227   immediately upon stimulation. In comparison to the electrical signals recorded in the leaf

228   cells, the signals from the protonema reached a higher amplitude (81 ± 4 mV, n=8) and

229   duration (486 ± 100 s, n=8). Surprisingly, weaker effects were achieved by the direct

230   stimulation of the tested cell or the stimulation of adjacent cells located close to the tested

231   cell. This method of stimulation evoked electrical signals with a smaller amplitude (66 ± 6 mV,

232   n=8) than when H_2O_2 was applied in the gametophyte base. The responses recorded in the

233   protonema after the direct or indirect stimulation also exhibited a low depolarization rate

234   (1.6 ± 0.6 mV/s, n=8), which was lower than the responses to the stimulation of the basal part

235   of the gametophyte (9.3 ± 2.5 mV/s, n=8) or than those recorded in the leaf cells. Cell-to-cell

236   transmission of the electrical signal evoked by stimulation of the single cells from the chain of

237   protonema cells was rarely recorded and occurred only in 2 out of 18 tested plants

238   (Supplementary Fig. 1 and Video 2).

239   One of the candidates responsible for cell-to-cell communication in plants is the GLR

240   receptor, which can act as a non-selective calcium-permeable channel. The participation of

241   the GLR receptor in the transmission of electrical signals recorded in our experiments was

242   tested with the use of a *glr1* mutant of Physcomitrella (Pp*glr*^{KO}). In the Pp*glr1*^{KO} mutants, as

243   in the wild type (WT), electrical signals propagated from the base of the gametophyte to the

244   leaf and protonema cells (Fig. 3, 4, Supplementary Video S3). In comparison to WT,

245   electrical signals recorded in the leaf cells of Pp*glr1*^{KO} had a reduced amplitude (to 46±3 mV,

246   n=26) and a lower depolarization rate (9.6±1.2 mV/s, n=24). The amplitude and rate of

247   depolarization of responses in the protonema cells from Pp*glr1*^{KO} recorded after the

248   stimulation of the basal gametophyte part reached similar values to those recorded in the WT

249   protonema cells (78±3 mV, n=10 and 8.9±3 mV/s, n=10, respectively). As in the WT

250   protonema cells, the direct or indirect stimulation of the protonema cells from Pp*glr1*^{KO}

251   evoked a smaller amplitude (63±4 mV, n=10) and a lower depolarization rate (1.1±0.3 mV/s,

252   n=10) than after the stimulation of the basal part of the Pp*glr1*^{KO} gametophyte. All these

253   changes in the parameters of changes in membrane potential in the protonema cells are

254   presented in Table 3.

255   *Calcium signals*

256   Calcium signals were recorded in plants expressing GCaMP3, i.e. a fluorescent calcium

257   indicator. Fluorescence measurements of calcium signals recorded after the stimulation of the

258   basal part of the plant indicated that the application of 0.5 mM H_2O_2 evoked calcium waves

259   which propagated from cell to cell in thread-like protonema cells. In contrast to electrical

260   signals, the calcium signals were slower and appeared a few minutes after the application of

261   the stimulus (Fig. 5, Supplementary Video S4). In some plants, the calcium signals did not

262   start at the stimulation site but appeared at some distance from the stimulation, acting as a

263   new source for calcium waves. In such a situation, calcium wave propagation was observed in

264   both directions - into and from the stimulation site. The rate of calcium signals propagation

265   along the protonema towards the site of the stimulation was similar to that in the opposite

266   direction, reaching 5.5±0.7 µm/s (n=4) and 5.2±0.3 µm/s (n=7), respectively. A characteristic

267   trait of some recorded calcium signals was its decrement with distance (Supplementary Fig. 2,

268   Supplementary Video S5). As in the case of electrical signals, the observation of calcium

269   signals in the stimulated leaves was possible after the increase of the hydrogen peroxide

270   concentration to 5 mM (Supplementary Video S6). In turn, in contrast to electrical signals,

271   calcium signals in leaf cells after application of 0.5 mM H_2O_2 to the basal part of the plant

272   were observed occasionally only in several cells from single leaves, but never (in none of 30

273   tested plants) in all leaves (Supplementary Video S7).

274   *Expression of genes in distant regions of the moss*

275   To analyze whether the electrical signal which was triggered upon local treatment would be

276   accompanied by changes in gene expressions in distant tissues, we selected candidate genes

277 and performed quantitative real-time PCR (qPCR). The candidates were selected based on
278 homology to Arabidopsis genes differentially expressed after local stress stimulus (Zandalinas
279 et al., 2019). In that study, a high light stimulus was locally imposed on selected Arabidopsis
280 rosette leaves and changes in gene expression in the locally treated and the distant, non-
281 treated leaves (local and systemic response, respectively) were investigated by RNAseq
282 analysis. From this published list, we selected candidate genes which were upregulated in the
283 systemic response if their expression was also inducible via H_2O_2 treatment and depended on
284 the function of the respiratory burst oxidase homolog D (RBOHD). Here, the gene encoding a
285 galacturonosyltransferase-like 10 protein (AT3G28340) was 5-fold upregulated 5 minutes
286 after the light stress treatment (Zandalinas et al., 2019). In Physcomitrella, we identified four
287 homologous proteins (Pp3c5_28420V3.1, Pp3c25_14930V3.1, Pp3c16_25090V3.1,
288 Pp3c2_18670V3.1) via *BlastP* search (Altschul et al., 1997) against the Physcomitrella
289 proteome (Lang et al., 2018). In analogy to Zandalinas et al. (2019) we tested their
290 responsiveness to H_2O_2 treatment and submerged entire gametophores in 0.5 mM H_2O_2 for 8
291 minutes. Here, we employed hydroponic gametophore cultures and analyzed the expression of
292 the candidate genes with qPCR. Of the four Physcomitrella homologues, only one gene
293 (Supplementary Fig S3, Pp3c16_25090V3.1) exhibited a significant increase in gene
294 expression (p = 0.0305) and hence, was selected for further analysis. For Pp3c5_28420V3.1.
295 an increase in gene expression was detectable (Supplementary Fig S3) but the difference was
296 not significant. Nevertheless, this gene was included for further experiments.

297 For these two selected candidates we tested whether both genes are regulated in gametophore
298 sections distant from a local treatment with H_2O_2. Apices of gametophores were harvested
299 after treatment with 0.5 mM H_2O_2 at the base for 8 min (Supplementary Fig S4) as well as
300 corresponding untreated apices. A significant upregulation of Pp3c16_25090V3.1 (p =
301 0.00184) was observed (Fig. 6) whereas an upregulation of Pp3c5_28420V3.1 was indicated,
302 but was not statistically significant (p = 0.06075). Thus, a local stress stimulus (here: H_2O_2 at
303 the gametophore base) triggers alteration of gene expression within 8 minutes in distant
304 sections (here: apex) in Physcomitrella.

Discussion

306 Plant cell signaling is based mainly on generation and transmission of different types of
307 signals including electrical, calcium, and reactive oxygen species (ROS). Investigations of the
308 relationship between these signals allow unraveling signaling pathways triggered in response

309   to biotic and abiotic stresses. In this study, we tried to answer the question whether external

310   application of ROS (hydrogen peroxide) evokes systemic response in the form of electrical

311   and calcium signals, to analyze the interdependence of these signals and their impact on gene

312   expression in distant regions.

313   The importance of ROS in the plant defense system has been evidenced by many experiments

314   (Huang et al., 2019). One of the first studies focused on the involvement of ROS in cell-to-

315   cell communication in *Arabidopsis* (Miller et al., 2009) proved that local stimuli produced

316   ROS waves propagating through the plant at a rate of 8.4 cm/min and the response was

317   dependent on a gene RBOHD (Respiratory Burst Oxidase Homolog D) encoding plant

318   NADPH oxidase involved in the production of ROS. The rate of propagation of such

319   RBOHD-related ROS signals recorded in different tissues ranged from ~400 to 1400 µm/sec

320   and was dependent on the type of stress (Choi et al., 2017). Together with propagation of the

321   signal, the accumulation of extracellular ROS was observed along the path of the signal

322   (Miller et al., 2009), indicating that each cell along the path is able to activate RBOHD and

323   release ROS, which in turn trigger adjacent cells to carry out the same process. In such an

324   autopropagation process, named ROS-induced ROS-release (RIRR), hydrogen peroxide

325   generated by RBOHD can be regarded as a long-distance signal. However, in the study by

326   Miller et al. (2009), external application of H_2O_2 did not evoke a ROS wave indicating

327   involvement of some other signal molecules in the propagation of the ROS wave.

328   Calcium is a candidate for such a signal molecule and together with ROS can cooperate in

329   long-distance transmission of information about stimuli. For example, calcium-dependent

330   protein kinase CPK5 playing a role in plant immunity is important for cell-to-cell

331   communication based on ROS waves (Dubiella et al., 2013). There are also other ways of

332   activation of RBOH proteins by calcium, including direct binding of this ion to the EF-hand

333   motif on the RBOH protein (Kimura et al., 2012) or binding of phosphatidic acid to the same

334   protein whose accumulation in the cell can be induced by calcium (Zhang et al., 2009). In

335   addition to the calcium-induced ROS release process, ROS-induced calcium release is

336   possible. For example, it has been shown that ROS can activate different calcium permeable

337   channels, e.g. hyperpolarization-activated Ca^{2+} channels in root cells (Demidchik et al., 2007),

338   Ca^{2+} influx channels in guard cells (Pei et al., 2000), or Ca^{2+} permeable channels regulated by

339   annexin1 (Richards et al., 2014). Recent studies carried out on Arabidopsis allowed the

340   discovery of a plasma-membrane leucine-rich-repeat receptor kinase, i.e. hydrogen-peroxide

341   induced Ca^{2+} increase 1 (HPCA1), which links the perception of apoplastic H_2O_2 with Ca^{2+}

342   signaling (Wu et al., 2020). One of the most probable mechanisms of the cooperation between

343   calcium and ROS in long-distance transmission of signals is that an increase in cytoplasmic

344   calcium evoked by a local stimulus can induce production of ROS via Ca^{2+}-RBOH

345   interactions and accumulation of ROS in the apoplast. The transport of ROS from the apoplast

346   to the cell, probably carried out by aquaporins or other channels, would evoke ROS-induced

347   calcium release, which in turn could induce ROS production (Gilroy et al., 2014).

348   As shown by the results of our study, H_2O_2-evoked calcium signals are not systemic

349   responses, such as those observed earlier in *P. patens* under osmotic stress and salt

350   stimulation (Storti et al., 2018). Calcium waves with a velocity of about 400 µm/s in response

351   to local salt stress were also measured in Arabidopsis (Choi et al., 2014), where blockage of

352   calcium channels by lanthanum inhibited not only the calcium waves but also the ROS-

353   regulated transcriptional marker (ZAT12), indicating that calcium and ROS waves are closely

354   linked. Assuming the interconnection of calcium signals and ROS waves, it is unlikely that

355   the extracellular application of H_2O_2 in our experiments evoked the ROS wave, since the

356   H_2O_2-evoked calcium signals admittedly appeared at some distance from the stimulation site

357   but propagated with a decrement (Fig. 3). The other feature of the H_2O_2-evoked calcium

358   signals in Physcomitrella, i.e. the slower rate of propagation than in the case of the ROS wave

359   (above 5 µm/s), also indicates a low probability of appearance of a calcium-associated ROS

360   wave. The ability to evoke local calcium signals but not self-propagating calcium waves by

361   the extracellular H_2O_2 application implies engagement of some other factors taking part in the

362   transmission of information about such stimuli. It seems that, in *P. patens*, electrical signals,

363   which appear at the moment of stimulation and propagate along the whole plant, are a proper

364   carrier of the information about H_2O_2 enhancement. There still remains the question of the

365   relationships between electrical, calcium, and ROS signals.

366   The relationship between ROS and electrical signals was previously confirmed in Arabidopsis

367   mutants lacking RBOHD, where propagating electrical signals after heat stress or high light

368   had a significantly reduced amplitude or were totally blocked in comparison to the wild type

369   (Suzuki et al., 2013). Taking into account the ability of extracellular ROS (mainly hydrogen

370   peroxide) to activate different ion channels (Demidchik, 2018), including calcium-permeable

371   channels which can participate in long-distance electrical signals, it seems that ROS can

372   promote electrical signals along the ROS wave path. This hypothesis can be also supported by

373   the similar velocity of different types of plant self-propagating electrical signals to the

374   velocity of the ROS wave [like action potentials (20-400 cm/min) and system potentials (5-10

375   cm/min); (Zimmermann et al., 2009)]. In Physcomitrella, the H_2O_2-evoked electrical signals
376   propagated immediately from the basal part along the whole plant (Fig, 1 and 2), indicating
377   that the signals appeared before the slowly propagating calcium waves. In cooperation with
378   ROS and calcium waves, electrical signals, which are probably the fastest carriers of
379   information, can initiate the whole array of plant defense responses. It is also probable that, by
380   reaching cells distant from the stimulation site, electrical signals initiate a calcium-wave
381   response only in some cells, in which the threshold of calcium signal generation is lower than
382   in other cells. The relevance of this assumption is proved by the features of some calcium
383   waves observed in the protonema cells, where at a distance from the stimulation site in the
384   basal part of the plant, only a few protonema cells in the thread-like chain generated calcium
385   signals propagating in two directions - into and out of the site of stimulation.

386   One of the candidates of ion channels participating in long-distance electrical signals in plants
387   are glutamate receptor-like channels (GLR), which was confirmed in experiments carried out
388   on Arabidopsis (Mousavi et al., 2013; Salvador-Recatalà, 2016; Salvador-Recatalà et al.,
389   2014). Wound-induced electrical signals recorded in this species were dependent on clade III
390   GLRs (GLR 3.3 and GLR 3.6) and played a crucial role in the distal production of jasmonates
391   taking part in plant defense responses (Mousavi et al., 2013). It is a matter of discussion if
392   those channels are directly responsible for Ca^{2+} fluxes since they are predominantly expressed
393   in endomembranes (Farmer et al., 2020; Nguyen et al., 2018).

394   In Physcomitrella, only two *GLR* genes have been identified (Verret et al., 2010). Both genes
395   (*PpGLR1* and *PpGLR2*) are paralogs to *GLR* clade III from Arabidopsis (De Bortoli et al.,
396   2016) and encode channels participating in chemotaxis and reproduction of Physcomitrella
397   (Ortiz-Ramírez et al., 2017). A patch-clamp study indicated that GLR1 is a calcium-
398   permeable ion channel localized in the cell membrane and partially inhibited by glutamate
399   receptor antagonists (Ortiz-Ramírez et al., 2017). These data indicate that GLR1 in
400   Physcomitrella, similar to GLR 3.3 and GLR 3.6 from Arabidopsis, can be important in the
401   transmission of long-distance electrical signals. This hypothesis was not fully confirmed in
402   our study, since H_2O_2 evoked long-distance electrical signals in the Physcomitrella $glr1^{KO}$
403   mutants; however, the response differed in the amplitude (Tab. 1). Such a "weak" effect of the
404   GLR1 receptor knockout suggests that, in addition to GLR channels, other channels must be
405   engaged in the long-distance propagation of ROS-induced electrical signals. The most
406   important channels taking part in the generation of electrical signals in Physcomitrella are
407   probably calcium-permeable, given the blockage of H_2O_2-evoked responses by the calcium

408 channel inhibitor (lanthanum) or the calcium chelator (EDTA) (Fig. 1D). It is also probable
409 that, in addition to ion channels in the plasma membrane, a significant role in the transmission
410 of electrical signals is assigned to intracellular calcium channels. One of the best-known
411 intracellular channels permeable e.g. to calcium is the two-pore channel 1 (TPC1) located in
412 the vacuolar membrane, the tonoplast (Dadacz-Narloch et al., 2011). A patch-clamp study
413 carried out in our laboratory demonstrated that TPC channels in Physcomitrella vacuoles
414 conduct Ca^{2+} currents (Koselski et al., 2013). Involvement of the channel in the transmission
415 of long-distance calcium waves has been demonstrated (Choi et al., 2014), but there is no
416 information about the role of TPC1 in the transmission of electrical signals. As calcium-
417 permeable channels, TPC1 may be involved in the release of calcium from intracellular
418 compartments (Qudeimat et al., 2008) leading to calcium-induced calcium release (CICR), a
419 desired phenomenon for long-distance signal transmission, but this assumption arouses
420 controversy (Pottosin et al., 1997; Ward et al., 1994). The role of H_2O_2 in CICR is
421 questionable since MIFE (Microelectrode Ion Flux Estimation) and patch-clamp studies
422 carried out on vacuoles from *Beta vulgaris* demonstrated that H_2O_2 suppressed the Ca^{2+} efflux
423 from the vacuole and slow vacuolar (SV) currents carried by TPC1 (Pottosin et al., 2009).

424 Effectively, the treatment with 0.5 mM H_2O_2 at the base of gametophores was sufficient to
425 trigger an increase in gene expression in the apex of a component of the homogalacturonan
426 biosynthesis (Fig. 6, Pp3c16_25090V3.1). Here, the homologous candidate
427 Pp3c5_28420V3.1 was not significantly upregulated although an increasing trend was
428 detectable. This agrees with publicly available data indicating that both genes are not co-
429 expressed. Galacturonosyltransferase-like proteins such as Pp3c16_25090V3.1 act in the
430 pectin assembly (homogalacturonan biosynthesis) of the primary cell wall (reviewed in Loix
431 et al., 2017). Under oxidative stress, cell-wall pectins also represent a source for the
432 biosynthesis of ascorbic acid (García-Caparrós et al., 2021; Valpuesta and Botella, 2004),
433 which in turn is used to detoxify ROS such as H_2O_2. Both selected candidates are homologues
434 of an Arabidopsis isoform (AT3G28340) whose gene expression is regulated via ROS waves
435 (Zandalinas et al., 2019). However, it should be noted that Galacturonosyltransferase-like
436 proteins comprise a large gene family with at least 21 members in Arabidopsis and 17 genes
437 in Physcomitrella (Van Bel et al., 2022) and clear ortholog relations are not yet resolved. The
438 expression of the Arabidopsis homolog (AT3G28340) was 5-fold increased only in systemic
439 leaves distant from a local high light stress impulse (Zandalinas et al., 2019) and those data
440 further indicate that the increase of expression was a response to a ROS wave. In contrast, the

441   two selected homologues in Physcomitrella were not regulated by high light stress

442   (Supplementary Fig S5) but their expression increased after heat stress. Heat stress in plants is

443   accompanied by the elevated production of reactive oxygen species (ROS) such as H_2O_2

444   (reviewed in Mittler et al., 2022). In summary, these data show that the gene expression of at

445   least one of the two Physcomitrella candidates (Pp3c16_25090V3.1) is responsive to ROS.

446   Consequently, the increase of expression in the untreated apex (Fig. 6) was likely based on a

447   propagating ROS-wave.

448   Taken together, our study demonstrates differences in the generation and propagation of

449   H_2O_2-evoked electrical and calcium signals in the model moss Physcomitrella. Many of the

450   applied variants of measurements indicated that the basal part of the gametophyte is the most

451   excitable region, probably because a large number of protonema cells are juvenile cells from

452   an early stage of new gametophyte development. In comparison to the leaf cells, the responses

453   in the protonema exhibited higher amplitudes and lasted longer, which may indicate higher

454   susceptibility of such cells to ROS (Table 2 and 3, Fig. 1 and 4). The main difference between

455   the electrical and calcium signals was the velocity of propagation, which was higher in the

456   electrical signals. The other difference was the ability to propagate without a decrement; in

457   contrast to the electrical signals, calcium signals diminished with distance, even if they

458   appeared in some distance from stimuli (Fig. 5, Supplementary Video S4 and S5). Given

459   these differences, we propose that H_2O_2-evoked long-distance electrical signals are the first to

460   reach distant regions of the plant and activate calcium signals, but not in every cell. The

461   protonema cells were the most susceptible to calcium signals, indicating that the signals play

462   a key role in young and developing cells. A similar observation was reported in our previous

463   work focused on glutamate-evoked calcium signals (Koselski et al., 2020). Electrical and

464   calcium signals were not the only effect of H_2O_2 application. Gene expression analysis proved

465   that apart from the signals, an increase of stress-related genes expression is observed in not

466   stimulated distant regions of plants (Fig. 6). The increase in the gene expression appeared

467   after 8 minutes - the time close to duration of electrical signals. Similar time scale of electrical

468   signals and genes expression, raises the question about interdependence of the both

469   phenomena.

470   **Materials and methods**

471   Cultivation of plant material used for electrophysiological and calcium imaging analysis

472 Physcomitrella gametophytes were grown on KNOP solid agar medium (Reski and Abel,
473 1985) in 160 mm diameter Petri dishes. The wild-type plants (WT) and knockout *glr1*
474 mutants (Pp*glr1*[KO]) were used for the analysis of membrane potential changes in leaf and
475 protonema cells with use of the microelectrode technique. A Physcomitrella mutant
476 expressing GCaMP3 was used for the fluorescence imaging of changes in the calcium
477 concentration. The plants were grown in a growing chamber (Conviron Adaptis A1000,
478 Conviron, Winnipeg, Canada) at a photoperiod of 16/8 light/dark, with light intensity 50
479 μmol/m^2 s, and at temperature set to 23°C.

480 Cultivation of plant material used for gene expression analysis

481 Physcomitrella WT protonema (new species name: *Physcomitrium patens* (Hedw.) Mitt.
482 (Medina et al., 2019); ecotype "Gransden 2004" was cultivated in Knop medium with
483 microelements. Knop medium (pH 5.8) containing 250 mg/L KH_2PO_4, 250 mg/L KCl, 250
484 mg/L $MgSO_4$ x 7 H_2O, 1,000 mg/L $Ca(NO_3)_2$ x 4 H_2O and 12.5 mg/L $FeSO_4$ x 7 H_2O was
485 prepared as described (Reski and Abel, 1985) and 10 mL per litre of a microelement (ME)
486 stock solution (309 mg/L H_3BO_3, 845 mg/L $MnSO_4$ x 1 H_2O, 431 mg/L $ZnSO_4$ x 7 H_2O, 41.5
487 mg/L KI, 12.1 mg/L Na_2MoO_4 x 2 H_2O, 1.25 mg/L $CoSO_4$ x 5 H_2O, 1.46 mg/L $Co(NO_3)_2$ x 6
488 H_2O) as described (Egener et al., 2002; Schween et al., 2003). The suspension culture was
489 dispersed weekly with an ULTRA-TURRAX (IKA) at 18,000 rpm for 90 s.

490 Hydroponic cultures of Physcomitrella gametophores were cultivated as described in
491 Hoernstein et al. (2023). Glass rings covered with mesh (PP, 250 m mesh, 215 m thread, Zitt
492 Thoma GmbH, Freiburg, Germany) were prepared as described in Erxleben et al. (2012).
493 Protonema suspension was adjusted to a final density of 440 mg/L (dry weight per volume) as
494 described (Decker et al., 2017) and evenly distributed in equal volumes on the mesh surface.
495 Glass rings covered with protonema were placed in Magenta®Vessels (Sigma-Aldrich, St.
496 Louis, USA). KnopME medium was supplemented until it touched the bottom of the mesh.
497 The medium was changed every 4 weeks.

498 Suspension cultures and hydroponic cultures were cultivated under standard light conditions
499 (55 μmol photons/m^{2}s) at 22°C in a 16h/8h light/dark cycle.

500 Measurements of membrane potential in leaf cells

501 The method of membrane-potential measurements with microelectrodes was similar to that
502 employed in Koselski et al. (2020). Plastic Petri dishes used in the microelectrode

503   measurements were divided into two chambers with a barrier. The barrier had a small (1 mm

504   width) gap sealed with Vaseline. Before the experiments the plants were incubated for 3-6

505   hours at light intensity of 50 µmol/m^2 in a bath solution containing (in mM) 1 KCl, 1 CaCl$_2$,

506   50 sorbitol, and 2 HEPES, pH 7.5 (buffered by Tris). The stimuli were introduced by

507   application of 500 µL of a bath solution supplemented with 0.5 mM hydrogen peroxide. The

508   lanthanum chloride or EDTA influence on the H$_2$O$_2$-evoked responses described in this study

509   was assessed by application of one of these substances into one of the Petri dish

510   compartments containing the basal part of the plant. Borosilicate glass capillaries (1B150F-6,

511   World Precision Instruments, Sarasota, USA) were used to make micropipettes with the use

512   of a P-30 micropipette puller (Shutter Instrument Co., Novato, USA), filled with 100 mM

513   KCl, and connected to the FD223 electrometer (World Precision Instruments, Sarasota, USA).

514   A Sensapex SMX (Sensapex, Oulu, Finland) electronic micromanipulator was used for

515   positioning and insertion of the microelectrode. The reference electrode was composed of an

516   Ag/AgCl$_2$ wire inside a plastic tube filled with 100 mM KCl and ending with a porous tip.

517   The measured data were acquired by a Lab-Trax-4 device (World Precision Instruments,

518   Sarasota, USA) working with LabScribe2 software, which also allowed analyses of the data.

519   The analysis of statistical differences was performed in SigmaStat 4.0 (Systat Software Inc.,

520   California, USA). Recordings of changes in the membrane potential were recorded with 2 Hz

521   data collection frequency. Figures were prepared with the use of Sigma Plot 9.0 (Systat

522   Software Inc., California, USA) and CorelDraw12 (Corel Corporation, Ottawa, Canada)

523   software.

524   Measurements of membrane changes in protonema cells

525   The plants were prepared in the same way as for the measurements carried out on the leaves.

526   The microelectrode was positioned and inserted using a PatchStar (Scientifica, East Sussex,

527   UK) micromanipulator and observed under an Olympus IX71 (Tokyo, Japan) microscope

528   with a camera (Artcam-500MI, Tokyo, Japan) working with QuickPhoto Camera software

529   (version 2.3, Promicra, Prague, Czech Republic). A CellTram Vario microinjector

530   (Eppendorf, Hamburg, Germany) with a borosilicate glass micropipette (with a diameter of

531   about 3 µm) was used for the application of H$_2$O$_2$ onto the cell surface. Likewise, in our

532   previous paper (Koselski et al. 2020), 1 mM methyl blue was used for staining of the

533   stimulating solution, which allows observation of its dispersion. Live recording of membrane

534   potentials visible in LabScribe3 software and microscope camera images were recorded by

535   OBS software (ver. 23.2.1; Open Broadcaster Software, Massachusetts, USA).

536   Fluorescence calcium imaging

537   Films and images of calcium concentration changes in the GCaMP3 *P. patens* mutants were

538   recorded with the use of NIS-Elements AR software (ver.5.20.00 Nikon, Tokyo, Japan)

539   working with a Nikon Eclipse Ti fluorescence microscope equipped with a Nikon Plan UW

540   2X WD:7,5 objective and a Nikon DS-Ri2 camera (Nikon, Tokyo, Japan). The excitation of

541   fluorescence was provided by a Prior lumen 200 metal arc lamp (Prior Scientific Instruments

542   Ltd., Cambridge, England) and a 495-nm dichroic mirror with a standard GFP excitation filter

543   470 ± 20 nm. Images of fluorescence emission were recorded with 1-s exposure and a

544   standard GFP fluorescence emission barrier filter 525 ± 50 nm (Nikon, Tokyo, Japan).

545   Stimulation of the plants was carried out by microinjection using a CellTram Vario

546   microinjector (Eppendorf, Hamburg, Germany). The standard solution supplemented with

547   H_2O_2 was visible due to tinting by 0.025 µM fluorescein.

548   Treatment of plants used for gene expression analysis

549   Gametophores from hydroponic cultures were used for the treatment with H_2O_2. Half of the

550   gametophores from one glass ring were cut approximately in the middle and only the upper

551   half of the gametophores (~100 mg) was used as control sample. Cut gametophore apices

552   were gently dried by dabbing with filter paper. The glass ring with the remaining uncut

553   gametophores was transferred into a new Magenta®Vessel containing KnopME with 0.5 mM

554   H_2O_2. After eight minutes, the upper half of the remaining uncut gametophores (treated

555   sample) was harvested as described before. In total, gametophores from four independent

556   hydroponic cultures (four biological replicates) were sampled.

557   RNA extraction and quantitative real-time PCR (qPCR)

558   Extraction of RNA was done with the innuPREP Plant RNA Kit (Analytic Jena, Jena,

559   Germany) using the extraction buffer "PL". 5 µg total RNA were treated with DNAseI

560   (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) at 37°C for 1 hour and integrity of

561   the RNA was checked on Agarose gels. 2 µg DNAseI digested RNA were used for reverse

562   transcription using the TaqMan[TM] Reverse Transcription kit (N8080234, Thermo Fisher

563   Scientific) with random hexamer primers. Reverse transcription was performed at 42°C for 1

564   hour. A non-transcribed control without the addition of MultiScribe[TM]RT enzyme was

565   included. Primers for the qPCR were designed using Primer3Plus software

566   (https://www.primer3plus.com/, Untergasser et al., 2012) with qPCR settings and an

567 efficiency of 2 was confirmed using a 1:2 dilution series of cDNA. Melting curve analysis

568 was performed to exclude the presence of off-targets. qPCR was performed in 96-well plates

569 using the SensiFast^{TM} SYBR No-ROX Kit (Bioline) in technical triplicates for each biological

570 replicate. 50 ng cDNA were used for each technical triplicate and the PCR reaction was

571 performed in a LightCycler$^{®}$ 480 (Roche, Basel, Switzerland). -RT and water controls for

572 each primer pair were included. The PCR reaction was performed in 45 cycles with a melting

573 temperature of 60°C. Expression analysis of the genes Pp3c5_28420V3 and

574 Pp3c16_25090V3 (genes of interest, GOI) was performed as described (Bohlender et al.,

575 2020) in relation to the housekeeping (reference) genes L21 (Pp3c13_2360V3, Beike et al.,

576 2015) and LWD (Pp3c22_18860V3, Schuessele et al., 2016). All primers are listed in

577 Supplementary Table 1. The expression levels were calculated relative to the reference genes

578 according to Livak and Schmittgen (2001) using the software for the LightCycler® 480

579 (V1.5.0, Roche). Relative expression is represented as $2^{(-\Delta CT)}$ with $\Delta CT = CT_{[GOI]} -$

580 $CT_{[reference]}$. Figure 6 was created and statistics were calculated in R (R Core Team, 2022).

581 Statistical significance was tested via one-way Anova with subsequent post-hoc test.

582 Significance was accepted at $p < 0.05$.

Data Availability

584 The data underlying this article are available in the article and in its online supplementary

585 material.

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Author contribution

597   M.K. conceived and designed the manuscript, prepared the main part of experiments,

598   analyzed and interpreted the data, and drafted the main part of the work. P.W. participated in

599   preparation of the membrane potential measurements. K.T. participated in writing and revised

600   the manuscript. S. N. W. H and R. R. designed qPCR experiments, analyzed data and helped

601   writing the manuscript.

602   **Disclosures**

603   Conflicts of interest: No conflicts of interest declared

604

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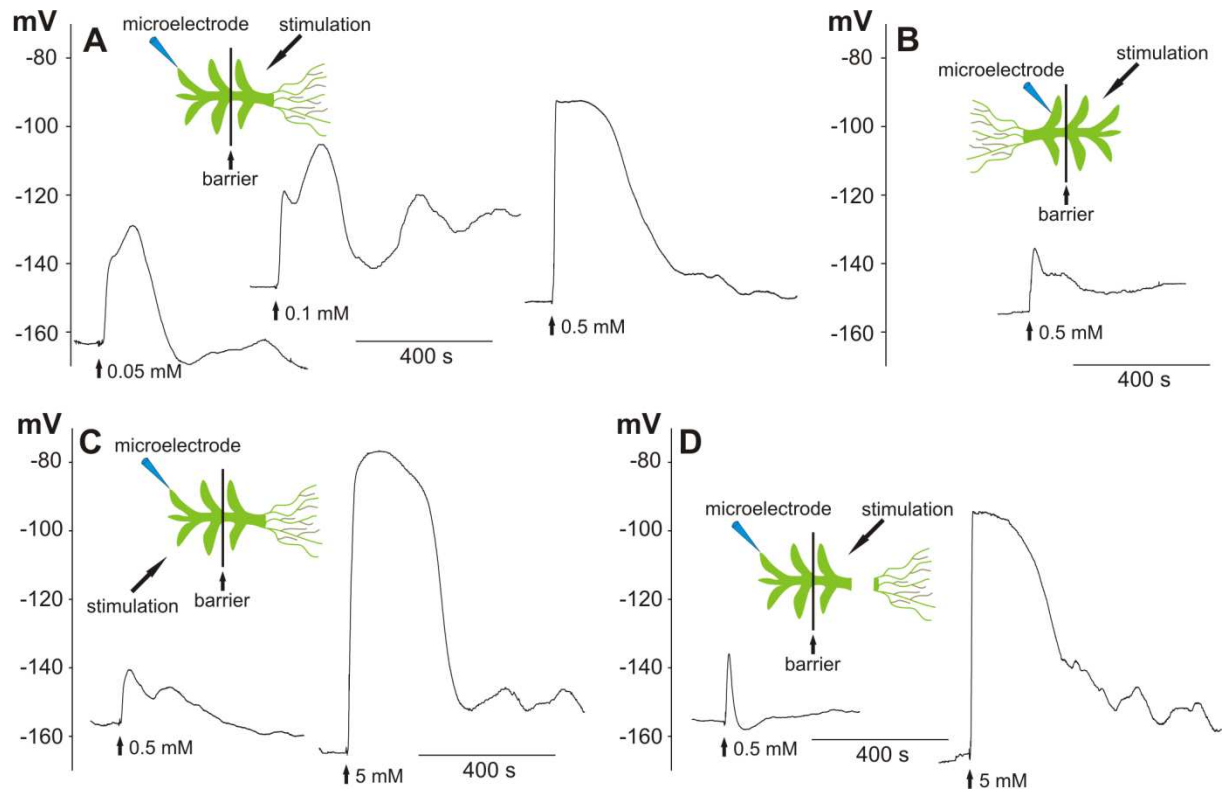
Fig. 1. Hydrogen peroxide-evoked long-distance electrical signals recorded in a Physcomitrella leaf (phylloid) cell. The recordings were carried out in bipartite chambers with a barrier separating two parts of the gametophyte. Schemes presenting the site of stimulation and insertion of the microelectrode are in placed on the top of the recordings. A – long-distance electrical signals in the form of membrane potential changes recorded after the stimulation of the basal part of the gametophyte with different H_2O_2 concentrations. B - electrical signals recorded after stimulation of the apical part of the gametophyte. C and D - reduction of the sensitivity of the leaf cells to H_2O_2 recorded after direct stimulation of the tested cell located in the apical part of the gametophyte and in the gametophyte with a cut-off basal part, respectively. Vertical axes present the values of the membrane potential (in mV).

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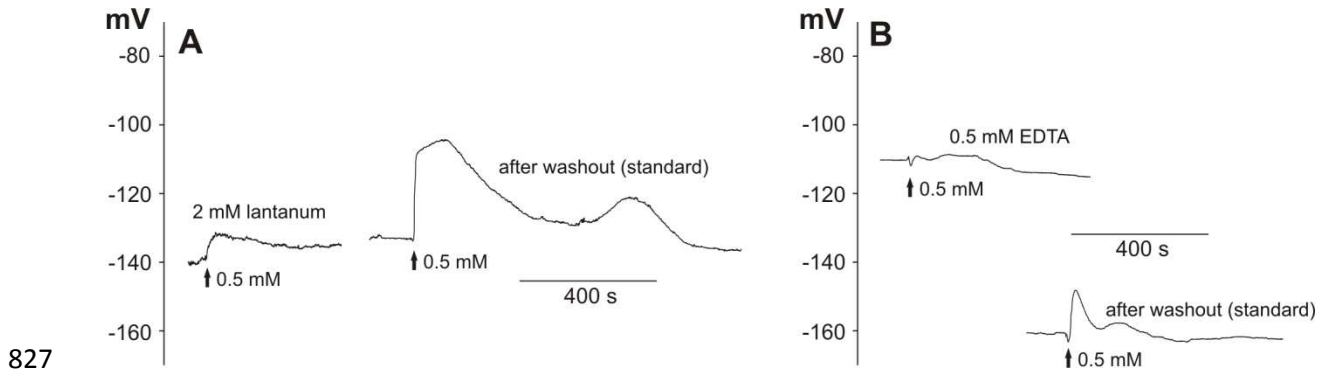
Fig. 2. Blockage of H_2O_2-evoked long-distance electrical signals by 2 mM lanthanum (A) and 0.5 mM EDTA (B). The method of stimulation was the same as in Fig. 1A. The representative membrane potential changes show the effects of preincubation in lanthanum (A) or EDTA (B), respectively, and then washout of the drugs carried out on the same plant.
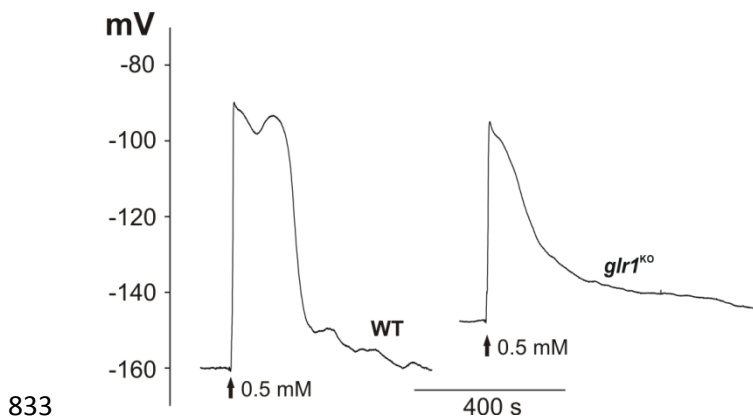
Fig. 3. Comparison of H_2O_2-evoked long-distance electrical signals recorded in wild type and Pp$glr1^{KO}$ mutant. The method of stimulation was the same as in Fig. 1A.
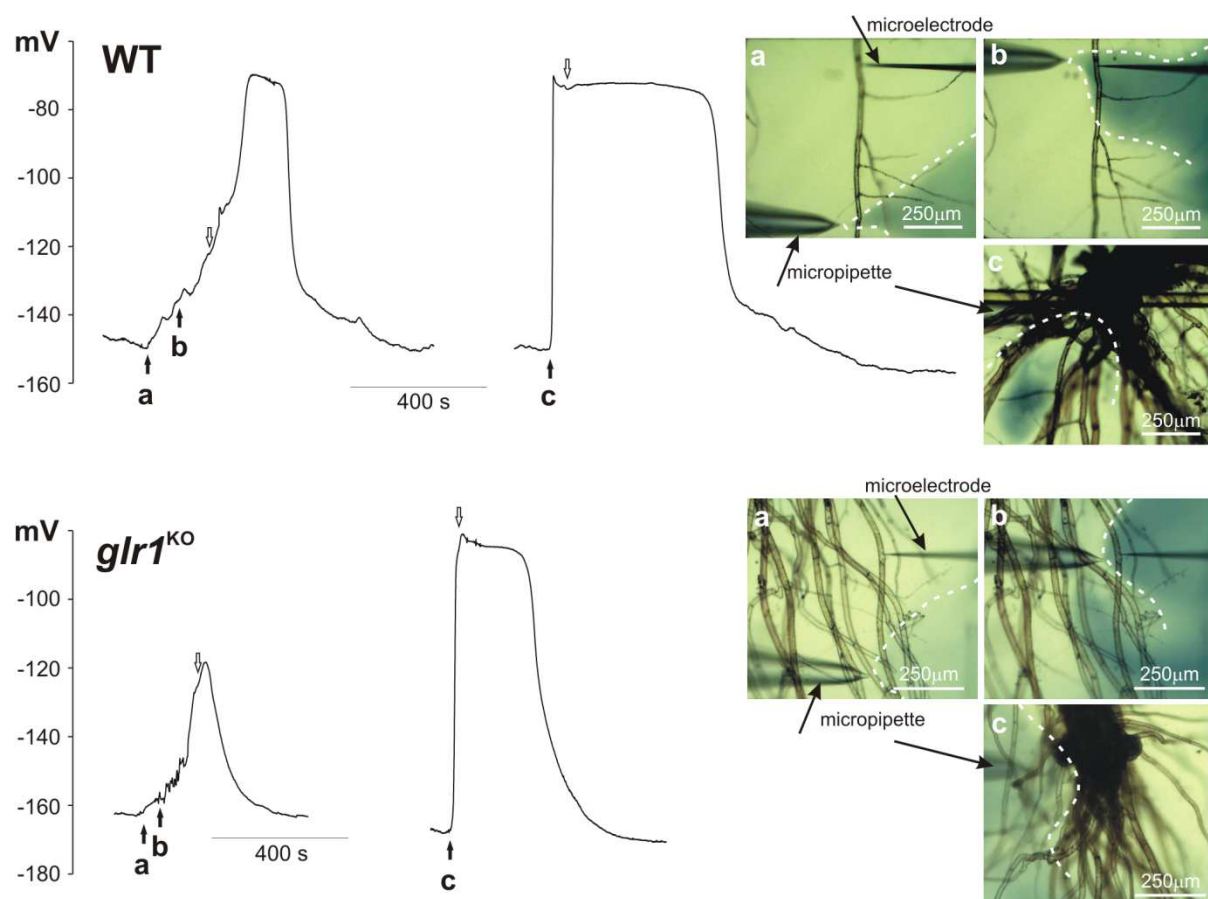
Fig. 4. Hydrogen peroxide-evoked membrane potential changes recorded in protonema cells from wild type and Pp$glr1^{KO}$ mutant. Hydrogen peroxide (0.5 mM) was injected into the selected site by the micropipette. In the pictures placed on the right side of presented traces, three different measurement variants are presented: a - stimulation of the cells adjacent to the tested cell (site of the microelectrode insertion), b - direct stimulation of the tested cell, and c - stimulation of the basal part of the gametophyte. The pictures show dispersion of H_2O_2 stained with 1 mM aniline blue (marked by dashed white lines) recorded 5 seconds after time points a, b, and c marked on the trace by black arrows. The end of stimulation (removal of the micropipette from the measuring chamber) was marked by white arrows.

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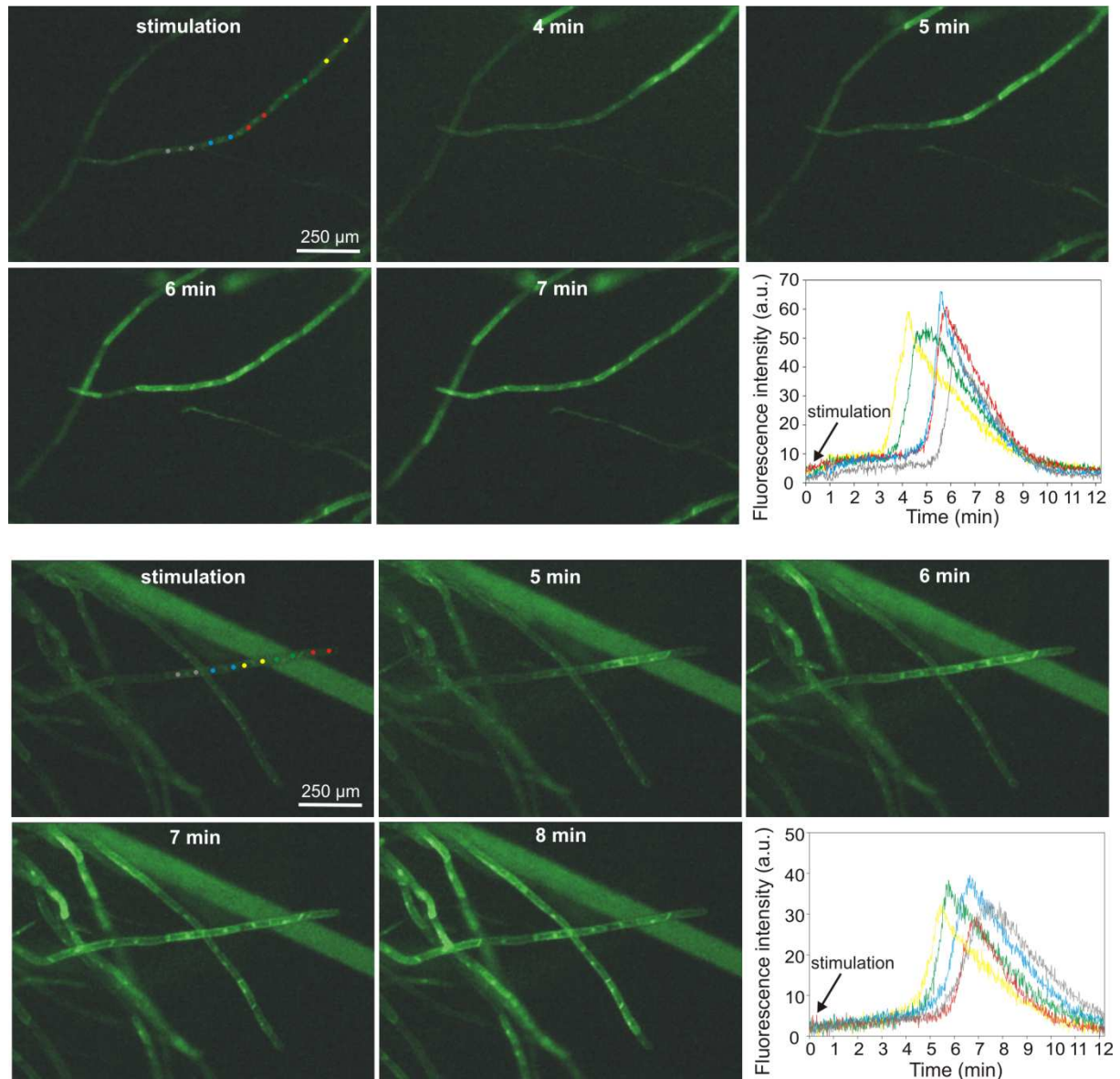
Fig. 5. Hydrogen peroxide-evoked calcium signals recorded in protonema cells expressing fluorescent calcium biosensor GCaMP3. For each cell two circular ROIs (Region of Interest) located in cytoplasmic region from both sides of the nucleus were analyzed. The two panels of pictures show two different types of calcium signal propagation - at the upper the signal propagate in one direction starting from the site of stimulation (basal part of the gametophyte) and at lower panel, the signal propagation starts from one cell (marked by yellow ROIs) and propagate in two opposite directions. Recorded during the experiment changes in fluorescence intensity in different ROIs are presented in the bottom right corner of each picture panels.

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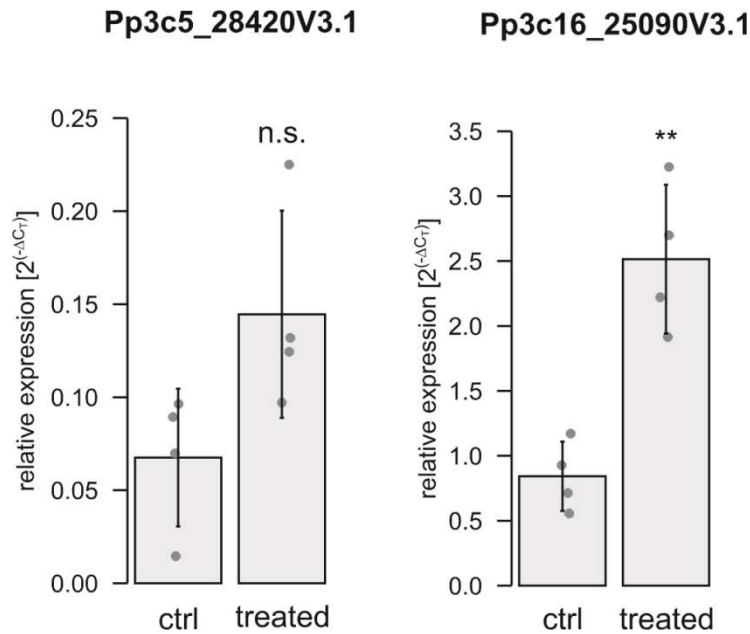
Fig. 6. Gene expression analysis by qPCR of selected gene candidates. Apices were used from untreated gametophores and gametophores treated at the base with 0.5 mM H_2O_2 for 8 min. Dots represent biological replicates (mean values from three technical replicates). Bars are mean values from the four biological replicates with standard deviation. Relative expression ($2^{(-\Delta CT)}$) of Pp3c5_28420V3.1 and Pp3c16_25090V3.1 is calculated against the reference genes L21 (Pp3c13_2360V3.1) and LWD (Pp3c22_18860V3.1) according to Livak & Schmittgen (2001). Significance levels are based on a one-way Anova with subsequent post-hoc test (**p < 0.01); n.s. = not significant.

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873 **Table 1. Values of electrical signal parameters obtained in leaf cells after the application**

874 **of hydrogen peroxide in different concentrations and different variants of experiments.**

	V_0 (mV)	V_{max} (mV)	A (mV)	$t_{1/2}$ (s)	R_{dep} (mV/s)
basal stimulation 0.5 mM	-154±3 (n=19)	-86±3[a, b, c, d, e] (n=19)	68±3[a, b, c] (n=19)	236±44[a] (n=17)	14.5±1.6[a, b, c, d, e] (n=18)
basal stimulation 0.1 mM	-161±4 (n=14)	-123±6[d, i] (n=14)	38±6[g] (n=14)	169±33 (n=13)	6±1.4[e] (n=13)
basal stimulation 0.05 mM	-158±2 (n=12)	-121±7[e, j] (n=12)	37±6[h] (n=12)	140±15 (n=11)	4.4±1[d] (n=11)
apical stimulation 0.5 mM	-165±5 (n=13)	-142±8[a, f, k] (n=9)	22±5[b, e, j] (n=9)	85±20[c, f] (n=8)	2.3±0.4[b] (n=8)
direct apical stimulation 0.5 mM	-153±4 (n=9)	-136±7[b, g, l] (n=9)	17±7[a, d, i] (n=9)	89±28[d, g] (n=8)	2.9±1.6[a] (n=6)
direct apical stimulation 5 mM	-153±5 (n=11)	-75±5[f, g, h, i, j] (n=11)	78±4[d, e, f, g, h] (n=11)	316±52[e, f, g] (n=11)	11.6±3.5 (n=9)
basal stimulation of plant without rhyzoids 0.5 mM	-156±3 (n=15)	-130±7[c, h, m] (n=12)	24±5[c, f, k] (n=12)	51±12[a, b, e] (n=12)	3.3±0.8[c] (n=12)
basal stimulation of plant without rhyzoids 5 mM	-153±3 (n=10)	-86±6[k, l, m] (n=10)	67±6[i, j, k] (n=10)	389±79[b, c, d] (n=9)	10.5±2.1 (n=10)

875 Table Footnote: Basal stimulation, apical stimulation, direct apical stimulation and basal

876 stimulation of plant without rhyzoids represent the variants of experiments presented in Fig.

877 1A, B, C and D, respectively. The values denote mean ± standard error. Dunn's test was

878 performed for all pairwise comparisons (P<0.05). V_0 - value of the membrane potential before

879 stimulation, V_{max} - maximum value of the membrane potential recorded during the response,

880 A - amplitude of the response, R_{dep} - the rate of depolarization measured in half of the

881 amplitude on a section equal to the half of the amplitude, $t_{1/2}$ - duration of the response

882 measured in half of the amplitude, n-number of tested plants.

883 **Table 2. Values of electrical signal parameters obtained in leaf cells after the application**

884 **of 0.5 mM hydrogen peroxide in the basal part of the gametophyte.**

	V_0 (mV)	V_{max} (mV)	A (mV)	$t_{1/2}$ (s)	R_{dep} (mV/s)
standard (WT)	-154±3[a, b, c] (n=19)	-86±3[a, b, c] (n=19)	68±3[a, b, c, d] (n=19)	236±44 (n=17)	14.5±1.6[a, b, c, d] (n=18)
2 mM La^{3+}	-130±3[b] (n=19)	-128±5[a, d] (n=9)	9±2[a, e] (n=9)	205±25 (n=3)	0.5±0.2[a, e] (n=8)
after washout of 2 mM La^{3+}	-130±4[c] (n=19)	-104±5 (n=5)	27±4[d] (n=4)	224±34 (n=15)	3.6±0.8[c] (n=14)
0.5 mM EDTA	-122±5[a, d, e] (n=18)	-115±7[c] (n=9)	13±4[b] (n=9)	126±32 (n=6)	1.4±0.7[b, f] (n=7)
after washout of 0.5 mM	-144±4[e] (n=18)	-121±7[b] (n=8)	24±6[c] (n=8)	63±14 (n=7)	2.5±0.7[d] (n=7)
standard (Pp*glr1*^{KO})	-146±3[d] (n=26)	-100±4[d] (n=26)	46±3[e] (n=26)	194±21 (n=25)	9.6±1.2[e, f] (n=24)

885 Table Footnote: The responses recorded in the wild type (WT) were obtained in the standard

886 solution or the standard solution supplemented with 2 mM LaCl$_3$ or 0.5 mM EDTA. The *P.*

887 *patens* knockout mutants (Pp*glr1*^{KO}) were tested in the standard solution. Explanations - as in

888 Table 1.

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896 **Table 3. Values of parameters of electrical signals in protonema cells after the**

897 **application of 0.5 mM hydrogen peroxide directly/indirectly to the cell or in the basal**

898 **part of the gametophyte.**

	V_0 (mV)	V_{max} (mV)	A (mV)	$t_{1/2}$ (s)	R_{dep} (mV/s)
direct/indirect stimulation (WT)	-155±8 (n=8)	-89±9 (n=8)	66±6 (n=8)	403±105 (n=7)	1.6±0.6[a, d] (n=8)
basal stimulation (WT)	-155±7[d, h] (n=8)	-74±4 (n=8)	81±4[a] (n=8)	486±100 (n=8)	9.3±2.5[a, b] (n=8)
direct/indirect stimulation (Ppglr^{KO})	-146±4 (n=10)	-84±5 (n=10)	63±4[a] (n=10)	300±70 (n=10)	1.1±0.3[b, c] (n=10)
basal stimulation (Pp$glr1^{KO}$)	-155±2 (n=10)	-77±3 (n=10)	78±3 (n=10)	294±25 (n=9)	8.9±3[c, d] (n=10)

899 Table Footnotes: Direct and indirect stimulation correspond to the measuring variants

900 presented at Fig. 4. Explanations - as in Table 1.

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A

mV

microelectrode   stimulation

barrier

↑ 0.05 mM

↑ 0.1 mM

400 s

↑ 0.5 mM

B

mV

microelectrode   stimulation

barrier

↑ 0.5 mM

400 s

C

mV

microelectrode

stimulation   barrier

↑ 0.5 mM

↑ 5 mM   400 s

D

mV

microelectrode   stimulation

barrier

↑ 0.5 mM   400 s

↑ 5 mM

A

mV

-80

-100

-120 2 mM lantanum

-140

↑ 0.5 mM

after washout (standard)

↑ 0.5 mM

400 s

B

mV

-80

-100 0.5 mM EDTA

-120 ↑ 0.5 mM

-140

400 s

-160 after washout (standard)

↑ 0.5 mM

WT

- mV
- −80
- −100
- −120
- −140
- −160
- a
- b
- c
- 400 s

microelectrode

a — 250μm

b — 250μm

micropipette

c — 250μm

glr1^KO

- mV
- −100
- −120
- −140
- −160
- −180
- a
- b
- c
- 400 s

microelectrode

a — 250μm

b — 250μm

micropipette

c — 250μm